Macrophage Clearance of Cell Death During Mouse Embryogenesis

VICTORIA LOUISE CAMP

A Thesis Submitted for the Degree of Doctor of Philosophy at the University of London 1998

Department of Anatomy and Developmental Biology, University College London.
ABSTRACT

Macrophages are haematopoietically derived cells. In adult tissues they have been shown to be motile and phagocytic. This investigation examines their function as phagocytes during mouse embryonic development, in particular focusing on their role in the clearance of apoptotic cells in the remodelling footplate and during organogenesis of the metanephric kidney.

Using the macrophage specific monoclonal antibody, F4/80, macrophages are revealed in the developing limb from embryonic day (E) 11.5 and in the metanephric kidney from E12.5. In these organ systems, macrophage appearance is tightly correlated with that of programmed cell death, suggesting that the majority, if not all, of the dead cells are engulfed and cleared away by macrophages.

By injecting F4/80 into the interdigital space of a living mouse embryo’s footplate at E13.5, macrophages can be “tagged” and their movements followed. 24 hours after labelling, tracking experiments reveal that macrophages move out of the interdigit, proximally, as far as the base of the limb.

To further examine the role of macrophages in development, two strategies for macrophage depletion have been studied. Macrophages within cultured E11.5 kidney rudiments were killed by exposure to toxic-liposomes. This treatment results in organ cultures that were smaller, appear less differentiated and have fewer tubules. The PU.1 null mouse provides a further opportunity to investigate development in the absence of macrophages. These mice have no macrophages and yet their limbs appear superficially to develop in an identical fashion to their wild-type litter mates. Transmission electron microscopy of PU.1 null footplates reveals that mesenchymal fibroblasts are able to compensate and can engulf dying cells in the absence of macrophages; this is one of the first examples of cell (as opposed to gene) redundancy revealed by transgenic mouse strategies, and phagocytic clearance of dying cells in the embryo cannot be the prime role of macrophages.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................................2

TABLE OF CONTENTS .............................................................................................................3

TABLE OF FIGURES ..................................................................................................................7

ABBREVIATIONS .....................................................................................................................9

ACKNOWLEDGMENTS ............................................................................................................12

CHAPTER ONE .........................................................................................................................13

GENERAL INTRODUCTION

Phagocytic cells exist in a variety of developing systems: worms, flies and chicks ......................................................... 14

The genetics of the death programme are unfolding from studies in C.elegans and Drosophila ........................................... 31

Programmed cell death in the limb ......................................................................................................................... 51

Lim b specification and induction ......................................................................................................................... 48

Lim b patterning .................................................................................................................................................. 49

Programmed cell death in metanephric development ................................................................................................. 45

Kidney Development ........................................................................................................................................... 38

Overview ......................................................................................................................................................... 38

Experimental support for reciprocal interactions .................................................................................................. 44

Programmed cell death serves many purposes in development ............................................................................... 37

Antibodies to Complement Receptor, Type 3 (CR3) .................................................................................................. 23

Sialoadhesin ........................................................................................................................................................ 23

Macrophage location in adult tissues ..................................................................................................................... 24

Macrophages are key players in the immune/inflammatory response ........................................................................ 25

Chemotaxis and the migratory machinery ............................................................................................................... 26

Where are macrophages found in the embryo? ....................................................................................................... 27

Overview ......................................................................................................................................................... 13

Programmed Cell Death ........................................................................................................................................ 29

Overview ......................................................................................................................................................... 29

A ntero-posterior patterning ..................................................................................................................................... 50

Proximo-distal patterning ......................................................................................................................................... 49

Anter o-posterior patterning .................................................................................................................................... 50

Dorso-ventral patterning .......................................................................................................................................... 51

Experimental support for reciprocal interactions .................................................................................................. 44

Programmed cell death in metanephric development ................................................................................................. 45

Lim b Development ............................................................................................................................................. 46
CHAPTER TWO ..............................................................................................................55

GENERAL MATERIALS AND METHODS

Mice ...............................................................................................................................55
Husbandry ...................................................................................................................55
Embryo culture .........................................................................................................56
Open uterus surgery ..................................................................................................57
Kidney organ culture ...............................................................................................58
Resin histology and electron microscopy ..................................................................60
Resin histology .........................................................................................................60
Transmission electron microscopy ..........................................................................60
Scanning electron microscopy ..................................................................................61
F4/80 immunohistochemistry ....................................................................................61
Sections .....................................................................................................................61
Wholmounts .............................................................................................................63
Cell death markers ...................................................................................................63
The nuclear dye 7-amino actinomycin D (7-AAD) ......................................................63
Apoptag in situ apoptosis detection kit - fluorescein (Oncor) S7110-KIT .....................64
Tracking techniques ..................................................................................................66
Manipulations of macrophage numbers ....................................................................67
Toxic liposomes ........................................................................................................67
The transgenic mouse PU.1 ....................................................................................67

CHAPTER THREE .....................................................................................................69

THE ROLE OF MACROPHAGES IN THE CLEARANCE OF PROGRAMMED CELL DEATH IN THE DEVELOPING KIDNEY

Introduction ..............................................................................................................69
Materials and Methods ...........................................................................................73
Histology and electron microscopy ..........................................................................73
Resin histology .........................................................................................................74
Transmission electron microscopy ..........................................................................74
Scanning electron microscopy ..................................................................................74
F4/80 immunohistochemistry ....................................................................................75
Double labelling of sections for apoptosis and macrophages ....................................76
Apoptag labelling of dead cells ...............................................................................77
Wholmount preparations .........................................................................................78
Results ......................................................................................................................79
Location and shape of the mesonephros and metanephros .......................................79
Programmed cell death spreads as a wave throughout the regressing mesonephros .................................................80
In the metanephric kidney cell death was localised to the mesenchyme and mesenchymally-derived tissues ..........80
Confirmation of cell death by labelling the cut ends of the DNA ..................................81
In both the developing mesonephros and metanephros, wherever there was programmed cell death there were also macrophages ..........................................................................................81
By E14.5 swollen macrophages containing apoptotic bodies were more commonly found in the metanephric kidney ........................................................................................................83
Double labelling experiments show definitively that macrophages engulf apoptotic cells in the metanephric kidney ........................................................................................................83
Discussion ...............................................................................................................98

CHAPTER FOUR .......................................................................................................102

IN VITRO EXAMINATION OF THE METANEPHRIC KIDNEY

Introduction ..............................................................................................................102
Materials and Methods ...........................................................................................105
Organ Cultures ..........................................................................................................105
Resin Histology .........................................................................................................109
F4/80 immunohistochemistry ....................................................................................110
Double labelling of organ cultures for apoptosis and macrophages .........................110
Liposomes ................................................................................................................111
Results ......................................................................................................................112
Normal development of E11 kidney organ cultures ................................................112
Effect of liposome treatment ....................................................................................113
Discussion ...............................................................................................................134
CHAPTER FIVE ..........................................................................................................................136
INVESTIGATION OF MACROPHAGES IN NORMAL LIMB DEVELOPMENT

Introduction..........................................................................................................................136
Material and Methods.......................................................................................................140
  Histology and electron microscopy..............................................................................140
  Resin histology...............................................................................................................141
  Transmission Electron Microscopy..............................................................................141
  Scanning Electron Microscopy.....................................................................................142
  F4/80 immunohistochemistry.......................................................................................142
  Sections............................................................................................................................142
  Wholemounts................................................................................................................143
  Double labelling of limbs for apoptosis and macrophages..........................................144
  Labelling interdigital macrophages for subsequent “live tracking”............................144
  Embryo culture of E13.5...............................................................................................145
  Open uterus surgery......................................................................................................145
Results................................................................................................................................150
  Extensive programmed cell death occurs between E13.5 and E14.5.........................150
  Macrophages enter the limb at E11.5...........................................................................150
  Double-labelling of E13.5 limbs for macrophages and apoptotic cells........................151
  Macrophages can be tracked in vivo...........................................................................151
Discussion..........................................................................................................................164

CHAPTER SIX ......................................................................................................................166
LIMBS IN A MACROPHAGE-LESS MOUSE EMBRYO

Introduction.......................................................................................................................166
Materials and Methods....................................................................................................171
  The transgenic mouse Pu.1.............................................................................................171
  Harvesting of embryos..................................................................................................172
  Details of the litters........................................................................................................172
  Histology and electron microscopy............................................................................173
   Resin histology.............................................................................................................174
   Transmission electron microscopy............................................................................174
   Scanning electron microscopy..................................................................................174
   Analysis of interdigit regression................................................................................175
Results................................................................................................................................178
  Sculpting of the footplate occurs at the normal rate in Pu.1 null embryos..................178
  Footplate development...............................................................................................178
  Fibroblasts can engulf dead cells in the absence of macrophages.............................179
Discussion..........................................................................................................................191

CHAPTER SEVEN ...............................................................................................................193
GENERAL DISCUSSION

Summary.............................................................................................................................193
Do macrophages have further functions in development?.............................................194
What molecular machinery allows a macrophage to recognise and engulf a dead cell, and which parts of this machinery might be upregulated by non-macrophage phagocytes?..195
What more will studies of cell death clearance in worms and flies tell us?..................198
Evolution of macrophages - what is their primary role in organisms?.........................199

REFERENCES .....................................................................................................................202

APPENDIX ..........................................................................................................................230
1/2 Strength Karnovsky - Karnovsky (1965).................................................................230
Araldite resin....................................................................................................................230
Apoptag in situ apoptosis detection kit - fluorescein (Oncor: S7110-KIT).................231
Leibovitz’s L-15 Medium with L-Glutamine (Gibco)................................................232
Dulbecco's Modified Eagle Medium/Nutrient Mix F12 (1:1) with L-Glutamine, 15mM HEPES (Gibco) .................................................................................................................................................. 233
Preparation of multilamellar liposomes composed of phosphatidylcholine and cholesterol, with the drug dichloromethylene diphosphonate (C12MDP) - Van Rooijen (1989) ............................................................................................................. 234
Explant Saline ............................................................................................................................................................ 236
Culture Saline ............................................................................................................................................................ 236
Hartmann’s Solution - Compound Sodium Lactate (Fresenius Ltd) ........................................................................ 237
Lysis Buffer ............................................................................................................................................................ 237

PUBLICATIONS................................................................................................................................................ INSIDE BACK COVER

| Figure 1. 1 | The haematopoietic lineage | 17 |
| Figure 1. 2 | Genes involved in programmed cell death in *C.elegans* | 32 |
| Figure 1. 3 | E11.5 mouse embryo showing the relative positions of the three vertebrate kidneys | 39 |
| Figure 1. 4 | Overview of genes involved in limb development | 47 |
| Figure 3. 1 | Scanning electron micrographs of the mesonephros and metanephros in the developing mouse embryo | 85 |
| Figure 3. 2 | Resin histology and transmission electron microscopy of the mesonephros at E11.5 | 87 |
| Figure 3. 3 | Resin histology of the developing metanephros | 89 |
| Figure 3. 4 | Nuclear staining of apoptotic cells in tails of developing nephrons | 91 |
| Figure 3. 5 | Resin histology and transmission electron microscopy of the metanephric kidney | 93 |
| Figure 3. 6 | F4/80 immunohistochemistry in the mesonephros and early metanephros | 95 |
| Figure 3. 7 | F4/80 immunohistochemistry and 7-AAD labelling of macrophages and dead cells in the metanephros | 97 |
| Figure 4. 1 | Diagram of how E11.5 kidney rudiments were dissected out of the embryo in preparation for organ culture | 108 |
| Figure 4. 2 | Light microscopy of the E11.5 kidney rudiment as it develops in culture over a period of five days | 116 |
| Figure 4. 3 | Graphs of normal kidney rudiment growth and tubule development over the culture period | 118 |
| Figure 4. 4 | Resin histology of E11.5 kidney rudiments after 96 hours in culture | 120 |
| Figure 4. 5 | Wholemount F4/80 immunohistochemistry of an E11.5 kidney rudiment at the start and at the end of the culture period | 122 |
| Figure 4. 6 | Light microscopy of the kidney rudiments treated with liposomes at 24 hours | 124 |
| Figure 4. 7 A | Graph of kidney rudiment growth of organ cultures treated with liposomes at 24 hours | 127 |
Figure 4.7 B: Graph of tubules that formed in organ cultures treated with liposomes at 24 hours

Figure 4.8: Resin histology of organ cultures treated with PBS-liposomes or toxic-liposomes

Figure 4.9: F4/80 immunohistochemistry of liposome treated organ cultures at 96 hours

Figure 5.1: Diagram of the female mouse prepared for open uterus surgery

Figure 5.2: Scanning electron micrographs of the sculpting events that occur during footplate remodelling

Figure 5.3: Light and electron microscopy of interdigital tissue in the E13.5 mouse footplate

Figure 5.4: Wholemount F4/80 immunohistochemistry of developing mouse limbs at E11.5 and E13.5

Figure 5.5: Attempts to DiI and dextran label macrophages in the interdigital region of the E13.5 footplate

Figure 5.6: Wholemount immunohistochemistry to reveal F4/80 labelled cells in hindlimbs just after interdigit injection and up to 24 hours

Figure 6.1: A haematopoietic lineage diagram to indicate where PU.1 may be acting

Figure 6.2: Diagram of the interdigit angle measurements of E13.5 footplate

Figure 6.3: Scanning electron micrographs of E13.5 and E14.5 hindlimbs from wild-type and PU.1 null embryos

Figure 6.4: Graph of the angles of the first and second interdigits in the hindlimbs of wild-type and heterozygote embryos, contrasted with PU.1 nulls

Figure 6.5: Resin histology of wild-type and PU.1 null hindlimbs at E13.5

Figure 6.6: Resin histology of wild-type and PU.1 null hindlimbs at E14.5

Figure 6.7: Transmission electron microscopy of E13.5 hindlimbs in wild-type and PU.1 null embryos
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>AER</td>
<td>apical ectodermal ridge</td>
</tr>
<tr>
<td>AGM</td>
<td>aorta, gonads and mesonephros</td>
</tr>
<tr>
<td>ANZ</td>
<td>anterior necrotic zone</td>
</tr>
<tr>
<td>A-P</td>
<td>antero-posterior</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bcl-2</td>
<td>B cell lymphoma/leukemia-2</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>caspase</td>
<td>&quot;c&quot; refers to the cysteine protease activity, while the &quot;aspase&quot; refers to the cleavage after aspartic acid</td>
</tr>
<tr>
<td>C.elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>ceds</td>
<td>C.elegans death genes</td>
</tr>
<tr>
<td>ces</td>
<td>cell-death specification</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>colony-forming unit - granulocyte-macrophage</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscope</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CR₃</td>
<td>complement receptor, type 3</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>dcp-1</td>
<td>Drosophila caspase-1</td>
</tr>
<tr>
<td>Dil</td>
<td>indocarbocyanine</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s Modified Eagle Medium/Nutrient Mix F12 (1:1) with L-Glutamine, 15mM HEPES</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>dnBMPR-1B</td>
<td>dominant negative type I BMP receptor</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>D-V</td>
<td>dorso-ventral</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>egl-1</td>
<td>egg laying</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>En-1</td>
<td><em>Engrailed</em>-1</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>Fig</td>
<td>figure</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>gcm</td>
<td>glial cell missing</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell line derived neurotrophic factor</td>
</tr>
<tr>
<td>GF</td>
<td>growth factor</td>
</tr>
<tr>
<td>glide</td>
<td>glial cell deficient/</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HB</td>
<td>heparin-binding</td>
</tr>
<tr>
<td>HET</td>
<td>heterozygote</td>
</tr>
<tr>
<td>hid</td>
<td>head involution defective</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin-1β-converting enzyme</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>KGF</td>
<td>keratinocyte growth factor</td>
</tr>
<tr>
<td>L-15</td>
<td>Leibovitz's L-15 Medium</td>
</tr>
<tr>
<td>LS</td>
<td>longitudinal section</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MDP-1</td>
<td>Macrophage-Derived Proteoglycan-1</td>
</tr>
<tr>
<td>MSR</td>
<td>murine scavenger receptor</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>nuc-1</td>
<td>nuclease-1</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>P-D</td>
<td>proximo-distal</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PMNs</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PNZ</td>
<td>posterior necrotic zone</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
</tbody>
</table>
PZ  progress zone
R-fng  Radical fringe
rpm  revolutions per minute
rpr  reaper
RT-PCR  reverse transcription-polymerase chain reaction
SEM  scanning electron microscopy
SER  sialoadhesin
SR-A  murine scavenger receptor class -A
SREBP  sterol regulatory element binding proteins
Ssh  Sonic hedgehog
TdT  terminal deoxynucleotidyl transferase
TEM  transmission electron microscopy
TGF-α  transforming growth factor-alpha
TGF-β  transforming growth factor-beta
TNF-α  tumour necrosis factor-alpha
TRITC  tetramethylrhodamine isothiocyanate
WT  wild-type
WT-1  Wilms’ tumour associated gene
ZPA  zone of polarising activity

3H  tritiated
7-AAD  7-amino actinomycin D
±  and
+/+  homozygous wild-type
+-  heterozygous
-/-  homozygous null

**Units**

°  degrees  c  centi
°C  degrees Celsius  μ  micro
kDa  kiloDaltons  m  milli
g  gram
L  litre
m  metre
M  molar
ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr Paul Martin, for his encouragement, help and advice. Prof. Siamon Gordon and Dr Andrew McKnight, Pathology Oxford, kindly provided the F4/80 antibody used throughout this project. Members of the Department of Anatomy and Developmental Biology, UCL who were very patient with me, were Mark Turmaine and Suhel Miah for electron microscopy, and Kate Whitley for confocal microscopy. Dr Adrian Woolf, Institute of Child Health, London, taught me how to culture E11.5 kidney rudiments, and Dr Richard Lang and Dr Graciana Diez-Roux, Skirball Institute, New York, kindly made the liposomes used for depletion studies. Prof. Richard Maki and Dr Scott McKercher, Burnham Institute, La Jolla, California, permitted the use of PU.1 transgenic mouse embryos.

Thank you to past and present members of the lab who assisted in a number of different ways. Many thanks to Aileen, George and Katie who generously gave of their time, particularly during the final stages of my thesis.

Finally, I gratefully acknowledge the support of the Medical Research Council in funding this project.
CHAPTER ONE

General Introduction

Macrophages

Overview

Metchnikoff (1892) first used the term "macrophage" meaning "big eater" to describe phagocytic cells belonging to the mononuclear phagocyte system which were capable of engulfing and degrading large particles (van Furth, 1970). Macrophages are multifunctional cells. In the adult, macrophages and their lineage relatives populate almost all tissues of the body where it is presumed they play a "housekeeping" role, phagocytosing dying cells as tissues turn over. It is clear that they are also crucial in acute tissue damage situations where an inflammatory response must be raised for efficient tissue repair (Haslett and Henson, 1996; Riches, 1996). Macrophages first appear in the embryo at early limb bud stages. Since inflammatory responses and tissue repair are not normal requirements for embryonic development and the embryo is in a sterile environment, consideration needs to be given as to the function of macrophages during the course of embryogenesis.
Phagocytic cells exist in a variety of developing systems: worms, flies and chicks

Cells with phagocytic capacity are not restricted to vertebrate organisms. In the worm Caenorhabditis elegans (C.elegans), where the genetics of programmed cell death have been dissected, neighbouring cells are seen to engulf each dying cell at the time of, or soon after, death (Sulston and Horvitz, 1977; Robertson and Thomson, 1982; Ellis et al., 1991a). In C.elegans it seems that no specific cell population is responsible for phagocytosis, but specific genes exist which are responsible for directing the engulfment and removal of dead cells. These are the so called C.elegans death genes (cds): ced-1, ced-2, ced-5, ced-6, ced-7, ced-8 and ced-10 (Hedgecock et al., 1983; Ellis et al., 1991a). It is thought that these genes encode proteins for recognition, binding, and finally, engulfment of dying cells. Genetic studies have classified them into two complementary groups representing two parallel phagocytosis pathways, one involving ced-2, ced-5 and ced-10, and the other using ced-1, ced-6, ced-7, and ced-8 (Ellis et al., 1991a). If there is a mutation in any one of these genes, then relatively few dead cells remain unengulfed in the developing worm (Ellis et al., 1991a). However, if there are two mutations, one in each group, then the engulfment process is more severely disrupted and many corpses remain (Ellis et al., 1991a; White, 1993). Downstream of the engulfment process the DNA (deoxyribose nucleic acid) of the dead cell appears to be degraded by a nuclease encoded by the gene nuc-1 (nuclease-1; Hedgecock et al., 1983). From extensive mutation studies, Hedgecock and colleagues (1983) have shown that generally cell death is genetically independent of the engulfment process. However, engulfment can cause cell death in a few cases (Sulston and White, 1980).

In Drosophila there is a population of phagocytic cells called hemocytes, which have the potential to differentiate into macrophages (Tepass et al., 1994). Using the enhancer-trap line E8-2-18 (Bier et al., 1989) and a peroxidasin antibody (Nelson et al., 1994) as markers, Tepass and colleagues (1994) have mapped out the movements of Drosophila hemocytes from early stages of development. These hemocytes, or multi-purpose blood
cells, are first seen within the head mesoderm in the embryo at stage 10, about two hours after gastrulation, illustrating that macrophage-like cells are present from a very early stage in development (Tepass et al., 1994). These cells then extend long processes and migrate via the hemolymph throughout the whole embryo. Programmed cell death is first seen in *Drosophila* embryos a short while later, at stage 11, and hemocytes are seen to contain apoptotic bodies. Tepass and colleagues (1994) now describe the cells as "macrophages". The phagocytic activity of hemocytes is dependent on their location within the developing embryo and on what structures are undergoing organogenesis. The hemocyte to macrophage conversion appears dependent on the amount of cell death in their neighbourhood. In mutants where there is an increase in the amount of cell death, a greater percentage of hemocytes become macrophages (Weigel et al., 1989; Tepass and Knust, 1993). It is interesting to note that cell death still occurs in mutant embryos which are lacking in macrophages, suggesting that, in flies at least, cell death is not triggered by the presence of phagocytes (Tepass et al., 1994).

In vertebrate embryos, the chief phagocytic cell, the macrophage, is haematopoietically derived. The lineage history of the macrophage was elegantly demonstrated in avian embryos by Cuadros and colleagues (1992) by constructing chick embryo-quail yolk sac chimeras. Using quail antibodies specific for cells of the blood and endothelial lineage, the earliest phagocytic cells in the embryo were shown to originate from the yolk sac. Double labelling using acid phosphatase histochemistry confirmed that these cells were active and analogous to macrophages found in mammals (Cuadros et al., 1992). Markers to track the location and movements of macrophages in chicks include the antibody CVI-CHNL-68.1 (Jeurissen et al., 1988) and, more recently, a whole battery of antibodies isolated by immunising rabbits with tissue from late stage chick hind limbs (Rotello et al., 1994). One of these six new "anti-engulfens" antibodies recognises an epitope specific to the macrophage plasma membrane and the others bind macrophage specific cytoplasmic structures (Rotello et al., 1994).
The monocyte lineage is first present in the yolk sac

In murine embryos the lineage history of macrophage has been carefully analysed. Haematopoiesis results in the formation of eight distinct cell lineages from a single pluripotent stem cell (Crocker and Milon, 1992), and is a dynamic, self-renewing system, able to maintain a balance between cell proliferation and differentiation (Till and McCulloch, 1980). The different cell types that are produced (Fig. 1.1) all have separate functions important for maintaining homeostasis within the body (Gordon et al., 1992a).

In the adult organism, the route from a stem cell in the bone marrow to a mature macrophage begins with a precursor cell or colony-forming unit, granulocyte-macrophage (CFU-GM), which becomes committed to one of two alternative pathways, ultimately giving rise to cells that will differentiate into either granulocytes or monocytes (Auger and Ross, 1992). Precursors committed to the monocyte lineage are called monoblasts; they are extremely immature and contain only small amounts of non-specific esterase enzyme. The monoblast divides to yield two promonocytes (Auger and Ross, 1992). The promonocyte is a highly mitotic cell stage, and after about twenty hours each promonocyte will divide to produce two daughter monocytes. Each monocyte moves into the blood stream, where it circulates without division under normal conditions (van Furth, 1970). Using tritiated (3H) thymidine, monocytes can be labelled prior to their “release” into the circulation, and consequently, the amount of time that they spend migrating can be calculated. The average transit time in the blood for a monocyte before it enters a tissue and terminally differentiates into a mature macrophage is around thirty two hours (van Furth and Cohn, 1968). Resident macrophages then adopt particular characteristics dependent on their location. For example macrophages of the skin, Langerhans cells, extend many long, thin plasma membrane processes beneath or within the epithelia: and, in the liver, the resident macrophages located in the sinuses and called Kupffer cells, contain more cytoplasm and only have a few shorter processes (Gordon et al., 1988a).
Figure 1.1: The haematopoietic lineage

(Designed with the advice of A. Grigoriadis, UMDS, London)
Macrophages are relatively long-living cells, surviving for a matter of months as opposed to days (van Furth, 1970; Gordon et al., 1992a). Local proliferation in adult tissues is unusual, the well known exception being alveolar macrophages in the lung (Tarling et al., 1987). The turnover of macrophages is largely dependent on bone marrow production (Gordon et al., 1992a). Once they have carried out the highly active role of phagocytosis, they are either engulfed by other macrophages, or trapped in lymph nodes; it is believed that they are not re-circulated (Gordon, 1986).

In mammalian embryos, the earliest organ of haematopoiesis is the yolk sac (Moore and Metcalf, 1970; Huang and Auerbach, 1993). The first macrophage precursors can be detected in the mouse yolk sac as early as embryonic day 5 (E5), and mature macrophages can be identified using the F4/80 monoclonal antibody in the yolk sac from E9 (Gordon, 1986). As embryogenesis proceeds, a blood vessel network is established and blood islands are formed (Gordon et al., 1992a). Macrophages circulate freely in the blood stream and can rapidly leave blood vessels, such that, by E10 macrophages are found in the liver (Morris et al., 1991). Haematopoiesis starts in the liver at E11. It then moves to the spleen around E15, and a couple of days later to the bone marrow (Morris et al., 1991); these latter two organs continue haematopoietic activity into adulthood.

It now seems that haematopoiesis is not as simple as previously thought, with both embryonic and adult haematopoietic stem cells arising from the ventral yolk sac or extra-embryonic tissue. Perhaps there are two sources of haematopoiesis; a “primitive” (embryonic) one, and a “definitive” (adult) one, both of which are derived early in embryonic development (Zon, 1995). The stem cells from the “primitive” site all die during development, while those of the “definitive” one persist into adulthood (Zon, 1995). Secondary haematopoietic activity, as seen in the liver, spleen and bone marrow, does not appear, as previously thought, to be seeded from the yolk sac (Robb, 1997); rather it may derive from the intra-embryonic tissue which is dorsally derived (Zon, 1995). The paraaortic splanchnopleura, which consists of the caudal splanchnic
mesoderm, endoderm of the developing gut and endothelium of arteries, has been identified by several groups as a pre-liver intra-embryonic site of haematopoiesis in the E7 - E8 mouse (reviewed in Dzierzak and Medvinsky, 1995). Another area, which comprises the aorta, gonads and mesonephros (AGM), has also been shown to contain CFU (colony-forming-unit; Godin et al., 1995). A recent in vitro study performed by Cumano and colleagues (1996) showed that the paraaortic splanchnopleura contained lymphoid progenitors, as well as erythroid and myeloid precursors, while the yolk sac contained only erythroid and myeloid progenitors. In another experiment, Medvinsky and Dzierzak (1996) examined the yolk sac and the AGM, and found that CFU and long term repopulating-haematopoietic stem cells were present in the AGM at E10. However, these results do not, as yet, definitively show that the yolk sac does not seed haematopoietic activity in the liver and subsequent organs of haematopoiesis (Robb, 1997).

Chick-quail and quail-chick chimeras have clearly illustrated that there is an intra-embryonic source of haematopoietic stem cells from around the dorsal aorta (Dieterlen-Lievre et al., 1993), the yolk sac only being responsible for a primitive short term haematopoiesis. This has also been found to be the situation in Xenopus, where experiments have shown that the haematopoiesis which occurs in the adult, arises from intra-embryonic tissue, rather than from the ventral blood islands or yolk sac (Kau and Turpen, 1983).

Regulators of haematopoietic development

Haematopoiesis is a process which requires coordinated expression of cell-specific genes to give rise to the different blood cell phenotypes (Shivdasani and Orkin, 1996). To date, a number of genes that may be key in regulating various steps of haematopoietic
lineage pathways have been identified. SCL/tal-1 is an oncoprotein containing a 50 - 60 motif with similarity to that found in myc genes (Kallianpur et al., 1994). It is a member of the basic-helix-loop-helix family of transcription factors, which have been shown to be important in controlling the development of different lineages (Olson and Klein, 1994; Lee et al., 1995). Immunohistochemistry reveals that SCL/tal-1 protein is first present from E7.5 in embryonic and extra-embryonic tissues. It is found in the yolk sac at the time of haematopoiesis, and later in the liver and spleen (Kallianpur et al., 1994). It seems that SCL/tal-1 is critical in the lineage decision to form or maintain haematopoietic stem cells progenitors, since, in the transgenic knockout mice null for SCL/tal-1, all cells of the haematopoietic lineages are lost (Porcher et al., 1996; Shvidasani and Orkin, 1996).

PU.1 is a transcriptional regulator and a member of the ets family expressed exclusively by cells of the haematopoietic lineage (Scott et al., 1994; McKercher et al., 1996). Disruption of the PU.1 gene in transgenic knockout mice results in the failure of formation of macrophages, mature B lymphocytes and neutrophils (McKercher et al., 1996). PU.1 does not appear to affect commitment of haematopoietic stem cells to the myeloerythroid and lymphoid lineages, since erythrocytes, megakaryocytes, precursor T and B lymphocytes, and immature neutrophils can be found in the null mice; rather the block is one of differentiation downstream of the initial commitment (McKercher et al., 1996). Osteoclasts, bone-resorbing cells, are also absent in PU.1 mutant mice, and consequently these mice develop osteopetrosis, which results in an increase in skeletal mass (Tondravi et al., 1997).

Downstream of SCL/tal-1 and PU.1, further transcription factors regulate later lineage decisions. c-fos is a member of a multigene family and a component of the AP-1 transcription factor complex (Angel and Karin, 1991). This proto-oncogene is important in determining the osteoclast-macrophage lineage split, as shown by c-fos knockout mice.
which, like PU.1 nulls, go on to develop osteopetrosis because cells that should differentiate as osteoclasts differentiate as macrophages instead (Grigoriadis et al., 1994).

There are numerous immunological markers of macrophages

Macrophages have previously been identified using morphological criteria such as a ruffled plasma membrane, presence of endocytic vesicles, adherence and phagocytic activity (Austyn and Gordon, 1981). M1/70 was the first antibody to be described in the mouse that labelled phagocytic cells (Springer et al., 1979). This antibody binds to the Mac-1 antigen, a member of the beta 2 (β2) subfamily of integrins, on macrophages and polymorphonuclear leukocytes (PMNs; Springer, 1990; Gordon et al., 1992b), so is not totally specific or reliable as a macrophage marker. Another approach used to detect macrophages was to develop antibodies against Fc receptors which identify the Fc domain of IgG found on macrophages (Unkeless et al., 1979). This technique of identification was again complicated by the fact that lymphocytes and PMNs also express Fc receptors.

- **F4/80**

To date the most efficient and effective antibody for definitively labelling mature mouse macrophages in a variety of organ systems is the F4/80 monoclonal antibody (Austyn and Gordon, 1981). This antibody is specific for mouse blood monocytes and macrophages derived from bone marrow precursors. It does not label PMNs, lymphocytes or fibroblasts. The F4/80 antigen has now been cloned (McKnight et al., 1996). It is a 160 kilodaltons (kDa) molecular weight glycoprotein, consisting of an amino-terminal containing seven epidermal growth factor (EGF) - like domains and a carboxyl-terminal with homology to the seven transmembrane-spanning family of hormone receptors. The seven tandem EGF-like domains of the amino-terminal each
have three disulphide bonds, which are thought to contribute to the tightly folded structure. Five of these domains also show motifs important in calcium ion binding. The carboxyl-terminal contains numerous hydrophobic residues, confirming that the molecule spans the membrane many times. This information suggests that the F4/80 antigen could act via two separate ligand-binding interactions, one provided for by the amino-terminal and the other the carboxyl terminal. Potentially, these domains might reflect the F4/80 antigen’s ability to interact both with a diffusible peptide ligand, and by cell adhesion to fixed substratal molecules, thereby allowing macrophages to respond differently to various microenvironments. The existence of further cell-restricted membrane antigens has led to the production of other monoclonal antibodies, and to a fuller picture of macrophage distribution (Gordon et al., 1988b).

- **FA.11**

Smith and Koch (1987) isolated the antibody FA.11 which recognizes macrosialin, an intracellular membrane glycoprotein of molecular weight 85 - 90 kDa. It appears to be present on resident and activated macrophages, and is a definitive marker of phagocytosis (Rabinowitz and Gordon, 1991). Whilst this antigen is more widely expressed than F4/80, extensive N and O glycosylations lead to variable staining patterns (Gordon et al., 1992b). A study by Holness and colleagues (1993) showed that macrosialin is a murine homologue of CD68 and a member of the lysosome-associated membrane glycoproteins (Lamps), with an unusual mucin-like extracellular domain. Glycosylation sites on macrophages are affected in a variety of ways, dependent upon the nature of the inflammatory stimuli. By having numerous sites the macrophages can be more sensitive to changes in their environment, and hence more efficient (da Silva et al., 1996).

- **2F8**

The murine scavenger receptor (MSR) is a homotrimeric transmembrane glycoprotein with an unusual collagenous domain (da Silva et al., 1996). The antibody 2F8
recognises type I and type II scavenger receptors from class A (SR-A), but not all macrophage populations are reactive to 2F8 (Hughes et al., 1995). Activated macrophages express MSR, implicating it as a main "player" in the inflammatory and immune response (da Silva et al., 1996). Platt and colleagues (1996) have proposed a role for SR-A in the clearance of apoptotic thymocytes in vitro, since thymic macrophages from mice with a deficient SR-A gene have reduced phagocytosis.

- **Antibodies to Complement Receptor, Type 3 (CR₃)**

The antigen CR₃ is important for cell adhesion and in the inflammatory response (Rosen and Gordon, 1987). It has variable expression on tissue macrophages but is constant in culture, most of the labelling being restricted to the plasma membrane (Gordon et al., 1992b). PMNs, circulating monocytes and natural killer (NK) cells, derived from the same stem cell population as mature macrophages, all strongly express this antigen (Springer et al., 1979; Beller et al., 1982). Antibodies to CR₃ include M1/70 which reacts with the β2 leukocyte integrin family (Springer, 1990), and 5C6 which recognizes a pronase-stable epitope (Rosen and Gordon, 1987).

- **Sialoadhesin**

Sialoadhesin (previously termed Sheep Erythrocyte Receptor; SER) is a divalent, cation-independent, lectin-like haemagglutinin (Morris et al., 1988) expressed on the plasma membrane of stromal macrophages, where it is important for binding, but not engulfment, of cells. It is concentrated at points of contact between stromal macrophages and erythroid and myeloid cells in the bone marrow (Crocker and Gordon, 1989). Antibodies against SER recognise sub-populations of specialised macrophages found in the spleen, lymph nodes and liver (Morris et al., 1992).
Macrophage location in adult tissues

As described previously, the F4/80 monoclonal antibody is a sensitive marker for labelling monocytes/macrophages in mouse tissue, and has enabled a careful documentation of this cell lineage in adult organs and tissues. Numerous macrophages are routinely found in the bone marrow (Hume et al., 1984b), spleen, liver, lymph nodes (Hume et al., 1983b), gut and entrances to the body (Hume et al., 1984c) - sites that might be expected given the origin of macrophages and what is known about the tasks they perform. However, F4/80 positive cells are also found in the central nervous system (Perry et al., 1985), kidney (Hume and Gordon, 1983), adrenal glands and the pituitary gland (Hume et al., 1984a). It seems that, once a macrophage has completed its function, it is either removed from the system by fellow macrophages, or trapped in lymph nodes, so that it is no longer free to circulate in the blood stream and establish elsewhere (Gordon et al., 1986). In the central nervous system, it appears that macrophages infiltrate primarily with the purpose of removing old neurones and axons which have undergone apoptosis, and, having engulfed this neural debris, they then mature into microglia (Hume et al., 1983a; Perry et al., 1985), which are acknowledged to be the resident phagocytes in the central nervous system throughout adult life.

A comprehensive study of macrophage distribution has been carried out in the adult kidney using the F4/80 monoclonal antibody (Hume and Gordon, 1983). This study shows the highest numbers of macrophages in the medullary interstitium. Since the macrophages are so well integrated with the other cells of this area, they were not initially thought to be a different cell type, until the F4/80 antibody revealed many cells in this location to be of the monocyte lineage. Another area of the adult kidney where macrophages abound is the macula densa and the glomerular capillary bed, forming a physical component of the juxtaglomerular apparatus. Macrophages are also found to a lesser extent in close contact with the cortical proximal and distal convoluted tubules and the Bowman's capsule.
Macrophages are key players in the immune/inflammatory response

Macrophages are recruited to any site of tissue damage by a range of chemotactic factors. Reverse transcription-polymerase chain reaction (RT-PCR) studies have shown that activated macrophages at such a site of inflammation express a number of growth factors and cytokines (Rappolee et al., 1988). Macrophage and granulocyte-macrophage colony stimulating factor (M-CSF & GM-CSF) are both secreted by macrophages, and have an effect on their growth and differentiation (Bartocci et al., 1987). The cytokine interleukin-1 (IL-1) has a positive loop effect on macrophages causing production of more IL-1. Tumour necrosis factor-alpha (TNF-α) can also induce the release of IL-1 from macrophages and vice versa (Nathan, 1987), and they both also activate the release of CSF (Philip and Epstein, 1986; Dinarello et al., 1987). Transforming growth factor-beta (TGF-β), is secreted by macrophages, as well as other cells involved in the repair process. It affects a wide range of cell types and elicits a number of cellular responses (Roberts and Sporn, 1996), including a widespread release of other growth factors (Wahl et al., 1987). Platelet-derived growth factor (PDGF) is a secreted mitogen consisting of a disulphide-bonded dimer of two polypeptide chains (Heldin and Westermark, 1996). PDGF has been implicated as a chemotactic factor in the process of wound healing (Ross et al., 1986). There are at least nine members of the Fibroblast Growth Factor (FGF) family (reviewed in Abraham and Klagsbrun, 1996). These polypeptides have a diverse range of functions in a variety of cells and tissues. They are produced by a number of cell types, including macrophages, in response to injury, and are able to stimulate several aspects of the repair mechanism (Abraham and Klagsbrun, 1996). Keratinocyte Growth Factor (KGF, also known as FGF-7) is a member of the FGF family normally expressed by the skin, and its expression is largely increased as a result of injury (Werner et al., 1992). The EGF family is continually acquiring new members, as molecules with EGF-like regions are identified (Nanney and King, 1996). These molecules, which include TGF-α (Derynck et al., 1986) and heparin-binding (HB) EGF-like (Higashiyama et al., 1991), are largely produced by macrophages and
have a role in the repair machinery. Insulin-like Growth Factor-1 (IGF-1) is another factor found to be secreted by macrophages at a wound site (Rappolee et al., 1988).

**Chemotaxis and the migratory machinery**

Cell migration is crucial for many aspects of embryonic development. The coordinated movement of cells contributes to many important morphogenetic processes in embryogenesis (Lauffenburger, 1996). Locomotion of individual cells is dependent upon many integrated functions, and, in particular, requires coordinated control of cell adhesion and organization of the actin cytoskeleton (Downey, 1994), both of which are regulated by members of the Rho family of molecular switches. The actin cytoskeleton allows eukaryotic cells to exhibit a wide range of properties dependent upon its constitution and environment. Three small GTP (guanosine triphosphate) - binding proteins Rho, Rac and Cdc42 have been implicated in the reorganization of the actin cytoskeleton (Hall, 1994). Rho is fundamental for the formation of actin stress fibres, as well as being needed for the establishment and maintenance of focal adhesions (Ridley and Hall, 1992). Rac is necessary for formation of broad flat sheet-like extensions called lamellipodia, and for membrane ruffling (Ridley et al., 1992). Cdc42 is important for the formation of thin cylindrical needle-like projections called filopodia (Nobes and Hall, 1995). As well as regulating actin membrane extensions, Rac and Cdc42 are also involved in regulating the assembly of “focal complexes” which are associated with lamellipodia and filopodia. Lamellipodia and filopodia appear to be the key motile apparatus of various crawling cell types, such as fibroblasts and the growth cones of neurons, and, since macrophages are highly locomotory, patrolling tissues and migrating towards a chemoattractant, it is likely that Rho, Rac and Cdc42 could be playing a similar role in this lineage also. In a macrophage cell line, Allen and colleagues (1997) have shown that, just as in fibroblasts, Rac is required for lamellipodia formation and
membrane ruffling, Cdc42 is necessary for filopodial extension, and Rho is needed for actin stress fibre assembly and cell contraction. Rac and Cdc42 are necessary for the establishment of “focal complexes”, whereby the macrophage adheres to the extracellular matrix substratum. Rac is also important for another function of macrophages, that of phagocytosis (Ridley, 1995). The precise mechanisms by which these molecular switches act have not yet been fully elucidated, but it is clear that a number of chemotactic factors are able to activate one or other of them. Macrophages move in a directed way along a chemical gradient towards a source of chemotactic factor (Auger and Ross, 1992; Downey, 1994). Depending on the environment, macrophages can upregulate or downregulate their receptors to the chemotactic factors and thereby generate an appropriate response.

Where are macrophages found in the embryo?

Using the F4/80 monoclonal antibody, macrophages were detected in many developing organ systems of the mouse embryo (Morris et al., 1991). As described previously, they are first found in the haematopoietic organs, the yolk sac, and, subsequently, in the liver, spleen, thymus and bone marrow. Macrophages are also seen in non-haematopoietic systems, the connective tissue surrounding the neuroectoderm (E12), the lungs (E12), the kidneys (E14), and the gut (E15), and they are found in increasing numbers as organogenesis proceeds (Morris et al., 1991). The precise location of macrophages within these tissues has not been fully examined; neither has their function been determined.

De Felici and colleagues (1986) investigated the existence of macrophages in the developing urogenital ridges of E11.5 mouse embryos. Their culture experiments revealed a population of round granular cells, which were both phagocytic and motile, in
their explant cultures. These cells labelled positive with the F4/80 monoclonal antibody, which indicates that they were macrophages. This strongly implicates a role for macrophages in clearing away the programmed cell death which results from the degenerating mesonephros, as well as their likely involvement in the “removal” of the Mullerian duct in the male and the Wolffian duct in the female. Hopkinson-Woolley and colleagues (1994) showed that macrophages are recruited to the developing limb bud while it is being remodelled. There is an influx of macrophages at the time of digit separation, when there is a massive amount of programmed cell death occurring, suggesting that apoptotic cells are being cleared away by the macrophages. This study discredited the idea that apoptotic cells in the developing limb were cleared away by neighbouring parenchymal cells (Ballard and Holt, 1968; Garcia-Martinez et al., 1993).

In the developing mouse eye, significant remodelling events occur in the first three weeks after birth. Lang and Bishop (1993) disrupted a subpopulation of macrophages found specifically in the eye by running the promoter region of the gene off the A chain of the diphtheria toxin in transgenic mice. This resulted in ocular structures which are normally lost during development, the hyaloid vasculature and the pupillary membrane, persisting. Macrophages therefore, in this case at least, are instrumental in regression of tissues; their presence being essential for development to proceed normally.

The body of this thesis describes a series of experiments investigating the role that macrophages might play in clearing programmed cell death from the embryo by focusing on two developing organ systems, the limb and the kidney. To understand the purpose and outcome of these experiments, a thorough background in development of both these organ systems and what is known about programmed cell death generally is necessary.
Programmed Cell Death

Overview

Cell death can be classified according to morphological, biochemical or circumstantial criteria (Wyllie et al., 1980). Programmed cell death, or apoptosis, has been shown in recent years to play a key role in morphogenesis during the development of many organ systems, where it is used by the embryo as a tool to eliminate unwanted cells. The products of programmed cell death, apoptotic bodies, are rapidly phagocytosed and cleared, so that few dead cells are found in the system at any one time (Raff, 1992). It is perhaps because of this rapid clearance that the extent and importance of developmental cell death has been largely overlooked until recent years.

The extent and time-course of programmed cell death that occurs during development differs between each organ system. The apoptosis found in the developing limb during the remodelling of the footplate giving distinct separate digits is "castastrophic", occurring over a relatively short period of time, and resulting in a dramatic change of shape. The programmed cell death that occurs during the development of the kidney is a more gradual process, described as "trickle-like", resulting in no gross morphological change in kidney shape.

It is still unclear why certain cells die and how the programme of cell death is regulated. There are many different signalling pathways, intracellular and extracellular, which ultimately activate a common cell death programme. However, it is agreed that all nucleated cells constitutively express the protein components required for cell death. A proteolytic cascade is involved in the execution of this pathway. A family of specific intracellular regulatory proteins, caspases (the “c” refers to the cysteine protease activity, while the “aspase” refers to the cleavage after aspartic acid; Alnemri et al., 1996), are
involved in its execution, and during development it may also be controlled at the transcriptional level (Jacobson et al., 1997). The cell death pathway has been found to be similar between tissues and across species barriers, and is even conserved between invertebrates and vertebrates. This has enabled researchers to extrapolate from the genetic “cell death” studies in the worm *C. elegans*, and discover genes dedicated to programmed cell death in vertebrates by virtue of their homology to the *ced* genes.

*Apoptosis differs from necrosis in a number of ways*

Apoptosis is an active “programmed” process of gene-directed cellular destruction serving a biologically significant homeostatic function. Necrosis is a degenerative form of death which is usually the result of some environmental occurrence which is accidental. Generally the two death types can be separated according to the following criteria shown below (Thompson et al., 1992).

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>chromatin condensation</td>
<td>chromatin degeneration</td>
</tr>
<tr>
<td>cell shrinkage</td>
<td>cellular swelling</td>
</tr>
<tr>
<td>plasma membrane &amp; organelles intact</td>
<td>plasma &amp; organelle membranes rupture</td>
</tr>
<tr>
<td>under control of physiological stimuli</td>
<td>result of injury (toxins or ischemia)</td>
</tr>
<tr>
<td>affects single cells</td>
<td>affects group of cells</td>
</tr>
<tr>
<td>product rapidly engulfed, no inflammation</td>
<td>inflammatory response evoked</td>
</tr>
</tbody>
</table>
The first morphological sign of apoptosis is compaction of the nuclear chromatin and the condensation of the cytoplasm. This condensation is accompanied by convolution of the nuclear and cellular membranes and the nucleus breaks into smaller fragments. Membrane-bound spherical or ovoid apoptotic bodies are produced. The internucleosomal DNA is cleaved by endonucleases into multiple fragments of 185-200 base pairs, which gives the classic DNA laddering effect if run on a gel (Arends et al., 1990).

The genetics of the death programme are unfolding from studies in *C. elegans* and *Drosophila*

Programmed cell death has been extensively studied in the worm *C. elegans*, giving a clear model for understanding the basic genetic principles of developmental apoptosis. During the development of *C. elegans*, 1090 cells are formed, of which 131 undergo cell death (Sulston and Horvitz, 1977; Sulston et al., 1983). These 131 cells die at a particular time, in a precise manner, in every worm (Ellis et al., 1991b). The dying cells show the characteristic signs of apoptosis; the cell as a whole rounds up, the nuclear chromatin condenses and the cytoplasm contracts (Ellis et al., 1991b). These dead cells are rapidly engulfed and degraded by neighbouring cells (Ellis et al., 1991a). The deaths occur even in the absence of neighbouring cells, indicating that they are pre-programmed to die and not killed; the death is “suicide”, rather than “murder” (Miura and Yuan, 1996).

Genetically four steps of programmed cell death can be identified; suppression, execution, engulfment and degradation. *Ceds* and more recently cell-death specification (*ces*) genes have been identified in *C. elegans* as key players in somatic cell deaths (Fig. 1.2; Hedgecock et al., 1983; Ellis and Horvitz, 1991).
Figure 1.2: Genes involved in programmed cell death in *C. elegans*

Decision to die ➔ Execution of death ➔ Engulfment ➔ Degradation

 COMMON CELL DEATH PATHWAY

\[ \text{ces-2} \rightarrow \text{ces-1} \rightarrow \text{egl-1} \rightarrow \text{ced-9} \rightarrow \text{ced-3} \rightarrow \text{ced-1} \rightarrow \text{ced-2} \rightarrow \text{nuc-1} \]

\[ \text{ced-4} \rightarrow \text{ced-6} \rightarrow \text{ced-5} \rightarrow \text{ced-7} \rightarrow \text{ced-10} \]

Another gene involved in the decision making process is egg laying (egl-1; Trent, 1983). An endodeoxyribonuclease (nuc-1) has also been identified, which is necessary for the digestion of the DNA of the dead cell (Sulston, 1976; Hedgecock et al., 1983).

*Ced-3* and *ced-4* are required for cell death to occur, and if either of these genes is mutated in such a way as to make it a total null, then cells which normally undergo apoptosis during development will now survive, although the resulting worm does not perform as well as a wild-type *C. elegans* (Ellis and Horvitz, 1986). *Ced-9* is a gene which protects cells against cell death, and, if this gene is inactivated, widespread cell death occurs (Hengartner et al., 1992). Conversely, if this gene is activated, then cell deaths that are normally expected to occur do not (Hengartner et al., 1992).

Mutation in either *ced-3* or *ced-4* will prevent the massive cell death found in a worm with inactivated *ced-9* (Hengartner et al., 1992) - if either *ced-3* and *ced-9* or *ced-4* and *ced-9* are inactivated in a double mutant then no cell deaths occur (Hengartner et al., 1992). It has now become clear that *ced-4* acts as a molecular switch by interacting with and promoting the activity of *ced-3*, which is thought to be the "executioner" (Chinnaiyan et al., 1997). Recent studies have shown that *ced-9* is a binding protein for *ced-4* (Wu et al., 1997). *Ced-9* is localised in the membranes and *ced-4* is found in the cytosol of the cell. Wu and colleagues (1997) propose that *ced-9* regulates cell death by mopping up *ced-4* and localising it to the membranes.

The *ces* genes are candidates for regulators of the cell death programme, controlling the fate of individual cells (Ellis and Horvitz, 1991). *Ces-2* causes cells to die, functioning as a transcriptional repressor of a gene required for cell survival, for example *ces-1* (Ellis and Horvitz, 1991). This is confirmed in *ces-2* loss of function mutants, in which programmed cell death does not occur (Metzstein et al., 1996).
The engulfment genes have been classified into two complementary groups; one involving ced-2, ced-5 and ced-10, and the other using ced-1, ced-6, ced-7, and ced-8 (Ellis et al., 1991a). Mutations in one of these genes alone does not dramatically effect the engulfment and degradation of dead cells by their neighbours, few remain uneaten (Hedgecock et al., 1983; Ellis et al., 1991a); but if there are two mutations, one gene from each group, then the engulfment process is more severely disrupted and many corpses remain (Ellis et al., 1991a; White, 1993).

Nuc-1 digests the DNA of the engulfed dead cell and so a mutation in this gene prevents this final step in the cell death pathway (Sulston, 1976; Hedgecock et al., 1983). From extensive mutation studies, Hedgecock and colleagues (1983) have shown that generally the cell death pathway is genetically independent of the engulfment process, but there are a small number of instances where engulfment appears to trigger cell death (Sulston and White, 1980).

The components of this programmed cell death pathway have been highly conserved throughout evolution.

_Drosophila_ is another good model for investigating the genetics of programmed cell death. The wide diversity of mutants available give us a better understanding of the role played by each gene. In _Drosophila_ three genes have been identified which are involved in the convergence of the different signals to result in the activation of a common cell death pathway. _Reaper (rpr), head involution defective (hid)_ and _grim_ appear to be the mediators between various intracellular and extracellular signals which induce or inhibit the cell death programme. These genes are expressed in cells that are destined to die, about two hours before morphological signs of apoptosis are seen. A depletion mutant in either _rpr, hid_ or _grim_ leads to suppression of cell death, implicating them all as activators of the cell death pathway in _Drosophila_ (White et al., 1994; Grether et al., 1995; Chen et al., 1996). These cell death activators require caspase activity in order to function.
Drosophila caspase-1 (dcp-1) is structurally and biochemically similar to ced-3 (Song et al., 1997). A dcp-1 loss of function mutant, unlike a ced-3 worm mutant, is lethal because of tumourous growth (Song et al., 1997). It appears that Drosophila cannot cope with the resulting extra cells in the same way as C.elegans.

What is known about the death programme in vertebrates

A number of the genes identified in C.elegans have homologues in the mammalian system. The first homologue to be identified was that of ced-3, the gene required for all cell deaths in C.elegans. The vertebrate homologue is interleukin-1β-converting enzyme (ICE; Yuan et al., 1993). There are now several members of the ced-3/ICE family of proteases, and they all induce apoptosis when overexpressed. They all cleave their substrate after aspartate residues, and so they have been renamed caspases (cysteine aspases) in order to simplify the nomenclature (Alnemri et al., 1996). The caspases are all synthesized as pro-enzymes and mediate cell death in a number of ways (Jacobson et al., 1997). Intracellular proteins of the nucleus, nuclear lamina, cytoskeleton, endoplasmic reticulum and cytosol are cleaved by caspases, as they regulate the cell death pathway (Chinnaiyan and Dixit, 1996). Some caspases are also able to activate themselves and each other (Nagata, 1997).

zVAD-fmk, a cell-permeable, irreversible tripeptide inhibitor of some caspases, was found to inhibit programmed cell death, when used in the explanting medium of mouse forelimb organ cultures (Jacobson et al., 1996). The regression of the interdigital tissue in the treated limbs did not occur as normal, indicating that this substance might be important for analysing the role of cell apoptosis in embryogenesis (Jacobson et al., 1996). However, mice lacking ICE do not show any alteration in the normal pattern of cell death, presumably due to genetic redundancy in the caspase family (Li et al., 1995).
It has now become apparent that CPP32/caspase-3 is the family member that has most homology with ced-3 (Nicholson et al., 1995). Mice deficient for CPP32 die either in utero or postnatally, with a significant reduction in programmed cell death in the brain (Kuida et al., 1996).

There are a number of substrates of the caspases that may be key in the death programme. Caspase-3 cleaves poly(ADP-ribose) polymerase (PARP), an enzyme which generates specific size apoptotic fragments (Tewari et al., 1995). However, transgenic knockout mice deficient in PARP develop normally, although they are more susceptible to spontaneous skin disease in adulthood (Wang et al., 1995).

Gelsolin, a protein that is normally involved in the modulation of actin in the cell (Kwiatkowski et al., 1986), has recently been shown to be a substrate for caspase-3. Caspase-3-cleaved-gelsolin injected into cells will directly activate the characteristic morphogenetic changes of apoptosis, suggesting that gelsolin and the actin cytoskeleton are a crucial component of the mammalian programmed cell death pathway (Kothakota et al., 1997). However, gelsolin knockouts, as in PARP knockout mice, are able to survive into adulthood without obvious effects on levels of programmed cell death (Witke et al., 1995).

There are also vertebrate homologues of the worm ced-9. Bcl-2 (B cell lymphoma/leukemia-2), first identified as a site of translocation in many B cell lymphomas, is the closest vertebrate homologue of ced-9, which inhibits apoptosis (Hengartner and Horvitz, 1994). If transfected into sensory neurones starved of neurological growth factors it will protect them from cell death. The bcl-2 family of genes includes both inhibitors (bcl-2, bcl-x) and promoters (bax and bak) of cell death. These family members can both homo-dimerise and hetero-dimerise, and it is the ratio of homodimers that controls whether programmed cell death occurs (Barinaga, 1994; Farrow and Brown, 1996). Bcl-2 deficient mice die shortly after birth due to a
combination of excessive cell death in the kidney, resulting in polycystic kidneys, and an upset in lymphocyte development (Veis et al., 1993). Mice deficient in bcl-x die in utero at E13, due to massive cell death in the nervous system and death of the haematopoietic cells in the liver (Motoyama et al., 1995).

To date, only one vertebrate homologue of the C. elegans genes responsible for engulfment has been identified. Ced-7 has been shown to have structural homology to the mammalian ATP binding cassette (ABC) transporter, ABC1 (Wilson et al., 1994; Luciani and Chimini, 1996). Macrophages in the mouse express this ABC1 transporter (Luciani et al., 1994) and when it is disrupted phagocytosis of dead cells does not occur (Luciani and Chimini, 1996). This illustrates that the ABC transporter molecules are conserved across the evolutionary boundaries.

Programmed cell death serves many purposes in development

In C. elegans development it is thought that cell death is only required as a fine-tuning process (Ellis and Horvitz, 1986). Indeed, gene disruptions that lead to failure of normal cell deaths do not significantly hinder the adult worm. However, in higher organisms it is believed that cell death may play a number of major roles; remodelling of structures, balancing the cell types and removal of abnormal or harmful cells.

In mouse embryos, dead cells are first observed in the inner cell mass and the trophoblast cells shortly after their differentiation (El-Shershaby and Hinchliffe, 1974). Apoptosis is found along the edges of the closing neural tube and the fusing palatal shelves (Glücksmann, 1951). Perhaps the best studied system in which programmed cell death is believed to play a key role is the developing nervous system, where up to half of all cells born will eventually die (Barres et al., 1992). Apoptosis is believed to balance cell
types and numbers, so that the correct nerves reach the appropriate targets (Barres et al., 1992).

The kidney and the limb are two organ systems in which programmed cell death occurs in dramatically different ways. In the kidney the death is “trickle-like” with odd cells dying over a relatively long period of time, while in the limb the death is “catastrophic”, the majority of cells die within a twenty four hour time-frame.

**Kidney Development**

*Overview*

Three distinct phases of kidney development are seen during mammalian embryogenesis, recapitulating the evolutionary history of the organ. All three derive from the two rods of intermediate or nephrogenic mesoderm which “sit” either side of the embryo between the somitic and lateral plate mesenchyme. These organs develop in a cranio-caudal direction and are temporally arranged such that they develop one after the other (Fig. 1.3). The first two organ systems are transitory and die by “catastrophic” programmed cell death; the final organ system is extensively remodelled by programmed cell death during the period of organogenesis.
Figure 1.3: E11.5 mouse embryo, showing the relative positions of the three vertebrate kidneys.
**Pronephros**

The pronephros is the first kidney to form, and it develops from the most cranial portion of the intermediate mesoderm. Also derived from this region of intermediate mesoderm is the nephric duct which migrates caudally and links the pronephros to the cloaca (Vize *et al.*, 1997). In the mammalian system the pronephros is transient and non-functional. The pronephros undergoes a wave of "catastrophic" cell death, leaving only the nephric duct remaining. However, this is not the situation found in lower vertebrates. In the larval stages of fish and amphibia the pronephros acts briefly as a functional kidney and in the adult organism serves as the chief haematopoietic tissue (Frank, 1988; Temmink and Bayne, 1987).

**Mesonephros**

The mesonephros develops more caudally as the pronephros is regressing, and is thought to be induced to differentiate by signals from the migratory nephric duct (Saxén, 1970). The mesonephros extends through the thoracic and lumbar regions of the embryo, beginning to form in the mouse at approximately E9.5 and reaching greatest prominence at E11.5. This organ differentiates to form tubules and immature glomeruli which are believed to act transiently as a crude filtration system and drain via the nephric duct into the cloaca. By E12 in the mouse embryo "catastrophic" cell death is observed in the mesonephros and this regression continues until E13 when only the parts utilised by the permanent kidney and the genital system remain. In the fish and amphibian the mesonephros does not regress and instead remains the major kidney in the adult organism (Torrey, 1965; Saxén, 1987).
Metanephros

The precursor of the permanent adult kidney in the mammalian system is the metanephros. The metanephric kidney develops as a result of complex reciprocal interactions between two tissues, the epithelium of the ureteric bud and the most caudal part of the intermediate mesoderm called the metanephric mesenchyme. The ureteric bud develops as a sprout from the nephric or Wolffian duct which grows towards the condensing metanephric mesenchyme.

- Epithelial-mesenchymal interactions

In the mouse the ureteric bud begins to sprout from the Wolffian duct at E10.5, as a result of chemotactic signals produced by the condensing metanephric blastema. The Wilms' tumour associated gene (WT-1), a transcription factor with four zinc finger domains, is expressed initially by the uninduced mesenchyme when it is condensing to form the blastema. Its expression continues and increases during the inductive phase and is expressed by the mesenchymally-derived epithelium as the nephric tubules are formed (Pritchard-Jones et al., 1990; Armstrong et al., 1993). In mice lacking the WT-1 gene the ureteric bud fails to grow out from the nephric or Wolffian duct and this leads to programmed cell death of the metanephric blastema (Kreidberg et al., 1993).

Wnt-11 is expressed along the nephric duct as it elongates and then becomes localised to a region opposite the metanephric mesenchyme (Kispert et al., 1996). Later it is restricted to the tips of the branching ureteric bud which need to make contact with the metanephric mesenchyme in order to maintain its expression.

Pax-2, a transcription factor of the paired-box-containing gene family, is expressed by the nephric duct, the ureteric bud, the metanephric mesenchyme and the aggregating condensates. It is also found in "comma-shaped" bodies, but there is a noticeable
decrease in expression as these evolve into “S-shaped” bodies (Dressler et al., 1990). It is thought to play an important role in the induction of epithelial structures. Mice which are homozygous mutants for Pax-2 have no kidneys and this is thought to be due to a defect in the development of the nephric duct which degenerates before the ureteric bud sprout from it (Torres et al., 1995).

Another gene involved at this branching stage is c-ret, a tyrosine kinase receptor, expressed by all of the nephric duct epithelium and subsequently by the ureteric bud derivatives (Pachnis et al., 1993). Transgenic knockout mice null for c-ret often fail to form ureteric buds. However, when they do form branching is poor and the morphology of the resulting kidneys is abnormal (Schuchardt et al., 1994). It has been suggested that the ligand for ret is glial cell line derived neurotrophic factor (GDNF), which is expressed by the metanephric mesenchyme from E11.5, in a manner complementary to ret. This ligand/receptor signalling partnership initially appears to play a role in triggering the branching of the ureteric bud and later becomes restricted to the periphery where new nephrons are forming (Pichel et al., 1996). Mice lacking GDNF have no kidneys because the ureteric buds are not induced to sprout from the nephric duct, and although the metanephric mesenchyme begins to condense it soon undergoes programmed cell death (Sanchez et al., 1996).

Bone Morphogenetic Protein (BMP-7) is expressed by metanephric condensates and later in the ureteric bud-derived collecting ducts (Lyons et al., 1995). Mice lacking BMP-7 have kidneys but they are arrested in development. There are a few “comma-shaped” and “S-shaped” bodies, suggesting that the aggregation and differentiation processes are initiated, but that tissue is unable to continue differentiating (Dudley et al., 1995). The metanephric mesenchyme in BMP-7 null mice then undergoes programmed cell death indicating that this growth factor might be acting as a survival factor (Luo et al., 1995).
BF-2, a member of the winged helix family of transcription factors, is expressed by cells which surround the mesenchymal condensates, in particular by those cells which are destined to become stromal cells (Lai et al., 1993). In the BF-2 knockout mouse kidney development is abnormal due to defective ureteric bud growth and lack of mesenchymal-epithelial transition (Hatini et al., 1996). Mesenchymal condensates form but they are unable to fully epithelialise, indicating that the BF-2 is not needed for stromal cell development but rather is acting as an inducer for epithelial formation (Hatini et al., 1996).

- **Mesenchymal-mesenchymal interactions**

After inductive signals have transferred from ureteric bud-derived branch tips to adjacent metanephric mesenchyme aggregation begins, and the aggregations of mesenchyme express Pax-2 and uvomorulin (Thiery et al., 1982; Dressler and Douglass, 1992). The cells establish a polarity and become immature epithelial cells. A renal vesicle is formed which then differentiates into a "comma-shaped" and then subsequently an "S-shaped" body, which precedes final morphogenesis into a mature nephron (Jokelainen, 1963; Saxén, 1987; Ekblom, 1991; Bard, 1992). The various transformations through "comma-shaped" and "S-shaped" bodies allows the formation of the secretory parts of the nephron. A number of genes are involved in this process, but the extent of their role has not yet been evaluated. Their major use to date has been as markers of the developmental phases of nephrogenesis (reviewed in Bard et al., 1994).

Wnt-4 is first expressed by the mesenchyme as it condenses around the ureteric bud, in the aggregating mesenchyme and later it becomes restricted to the "S-shaped" bodies. The absence of Wnt-4 in the developing mouse leads to failure of pretubular cell aggregations (Stark et al., 1994). **Sonic hedgehog (Shh)** is expressed early throughout the urogenital system (Bitgood and McMahon, 1995) and **Indian hedgehog** is expressed from E14.5 in the kidney (Valentini et al., 1997). In mice with a disrupted Shh gene
there are no kidneys, but this could just be due to the fact that the mid-line patterning has been disrupted and may not reflect a direct role for shh in kidney patterning (Chiang et al., 1996). Several FGFs and their receptors are also found in the developing kidney, but as yet they have no established role either (Orr-Urtreger et al., 1993; Dono and Zeller, 1994).

New nephrons are formed at the periphery or rind throughout organogenesis of the metanephric kidney. This process of nephrogenesis continues until about seven days after birth, with new layers of nephrons being gradually moved towards the medulla of the kidney (Woolf, 1997).

- **Experimental support for reciprocal interactions**

  Transfilter experiments with isolated metanephric mesenchyme cultured adjacent to various potential inducing tissues, with only a millipore filter separating them, were pioneered by Grobstein (1956) and subsequently continued by Saxén (1978). These experiments showed that most other embryonic epithelia are unable to match the ureteric bud in supplying the necessary signals to trigger tubule formation. However, spinal cord could mimic the inductive signals that were normally derived from the ureteric bud epithelium. These experiments also showed that cell-cell contact might be necessary for transmitting these signals since millipore filters with pores too small to allow cell processes through, severely inhibited the inductive signals. In the absence of inductive signals, not only do metanephric mesenchymal cells fail to differentiate, but they all ultimately die, suggesting that the inducing tissue also supplies a trophic or survival factor (Koseki et al., 1992). Similar “catastrophic” death of all metanephric mesenchymal cells is seen in transgenic knockout mouse embryos null for the WT-1 gene (see earlier; Kreidberg et al., 1993), where the ureteric bud fails to grow out from the nephric duct.
Programmed cell death in metanephric development

Even during normal development in vivo, programmed cell death appears to occur as a natural event in the metanephros (Koseki et al., 1992; Coles et al., 1993), but not to the "catastrophic" extent seen in the developing pronephros and mesonephros, or in the metanephric kidney experimentally deprived of an inductive source. Natural death in the metanephros is "trickle-like", with odd cells dying in scattered locations. Two particular zones of cell death are found; one is in the peripheral mesenchyme where the mesenchymal-epithelial transformations are occurring; dead cells are found adjacent to condensations or in the tails of "S-shaped" bodies. The other zone is found much later, just prior to birth and postnatally, in the medullary region, where the ureteric bud is branching and the nephrons are joining up to become patent (Koseki et al., 1992; Coles et al., 1993). It has been suggested that programmed cell death is an important mechanism for balancing cell types and cell numbers in order to yield the correct total of nephrons (Coles et al., 1993). Just as the programmed cell death seen during development of the nervous system is dependent on limiting quantities of trophic factors, namely members of the Nerve Growth Factor (NGF) family (Barde, 1989), it seems that programmed cell death in the developing kidney is also regulated by survival factors. EGF is the favoured candidate for such a survival factor since EGF and TGF-α can both rescue metanephric cell death in vitro (Koseki et al., 1992; Rogers et al., 1992) and intraperitoneal injection of EGF in new born rat pups reduces the apoptotic index in the metanephric kidney (Coles et al., 1993).

The extent of programmed cell death throughout embryogenesis and particularly in organ systems like the metanephric kidney has only recently come to light because wherever cell death is "trickle-like" very few dead cells are ever seen due to very efficient clearing away of any cell debris. In order to determine whether macrophages are the cells responsible for such rapid and efficient clearance, their distribution was studied at
various stages during kidney development, and then experiments were performed to investigate what happens if their numbers are artificially depleted.

**Limb Development**

**Overview**

The forelimbs and hindlimbs develop as paired buds from the lateral plate mesoderm along the antero-posterior axis of the body beginning at about E9 in the mouse. There is about half a day delay in the development of the hind limb but in other respects the interaction and progression of events in both limbs is the same. The limb is a complex structure which itself develops in three axes (Fig. 1.4); proximo-distal (P-D), shoulder to finger, antero-posterior (A-P), thumb to little finger, and dorso-ventral (D-V), back of hand to palm. The limb originates as a mesenchymal mass covered with epithelium that emerges laterally from the region opposite the cervical somites, in the case of the forelimb, and adjacent to the lumbosacral somites in the case of the hindlimb. The overlying epithelium thickens at the distal tip running along the A-P axis to form the apical ectodermal ridge (AER). As the limb grows outwards, skeletal elements are laid down in a P-D direction. P-D patterning is governed by a complex interaction between the AER and the underlying mesenchyme, the remaining overlying dorsal and ventral epithelium interacts with the mesenchymal core to regulate patterning in the D-V axis, and a group of posteriorly located mesenchymal cells, the polarising region, control polarity in the A-P axis. Subsequently, spinal nerves and a capillary network grow into the limb, specific areas of mesenchyme form prechondrogenic condensations which will form cartilage, and two muscle masses form, one dorsal and the other ventral.
Figure 1.4: Overview of genes involved in limb development

Transverse section through the progress zone
Remodelling of the limb, in particular to give rise to the digits, is a partial consequence of cells dying. The limbs are essentially patterned in a miniature form, so that by just after halfway through gestation, they are tiny models of the adult self, which then undergo extensive growth.

**Limb specification and induction**

The factors responsible for the formation of two pairs of limbs at the appropriate level on the axial skeleton are thought to involve homeobox-containing genes and growth factors. Initially in the chick there are ill-defined boundaries of \textit{Hoxb9}, \textit{Hoxc9} and \textit{Hoxd9} in the prospective wing region, but these soon become restricted such that \textit{Hoxb9} and \textit{Hoxc9} form the posterior boundary of the forelimb and \textit{Hoxd9} is expressed throughout the wing bud (Cohn \textit{et al.}, 1997). The flank region expresses \textit{Hoxb9} and \textit{Hoxc9} but not \textit{Hoxd9} and the leg region expresses \textit{Hoxc9} and \textit{Hoxd9}, but not \textit{Hoxb9}. These three \textit{Hox} genes appear to play a crucial role in the positioning of the limb buds (Cohn \textit{et al.}, 1997).

Other data from the chick embryo demonstrates that beads soaked in FGFs - FGF-1, FGF-2 or FGF-4 - and placed in the flank between stages 13 and 17 result in ectopic limbs being formed (Cohn \textit{et al.}, 1995). The extent of re-programming of \textit{Hox} 9 distribution and the eventual type of limb formed, wing or leg, is dependent on the position of the bead in the flank region (Cohn \textit{et al.}, 1995; Cohn \textit{et al.}, 1997). Whilst all FGFs so far tested can ectopically induce limb outgrowth it seems that the crucial initiating FGF may be FGF-10 which is expressed by the prospective limb mesenchyme at the appropriate time; moreover, ectopic expression of FGF-10 induces the formation of a complete limb via FGF-8 in the AER (Ohuchi \textit{et al.}, 1997).
Limb patterning

- Proximo-distal patterning

The AER is thought to play both a mechanical and biochemical role in limb development. The thickening of the distal tip of the limb maintains the shape of the limb bud, while flattening it in a D-V direction. Signals from the AER are important for maintaining the progress zone (PZ), a region of undifferentiated cells at the distal part of the limb bud (Summerbell et al., 1973). The cells in the PZ appear to “count time” and as they are progressively released from this zone, due to population pressure, they are allocated more and more distal positional values, with the most distal encoding the distal most phalanges. The PZ mesenchyme also signals back to the AER to maintain its existence and activity, in a reciprocal fashion.

The homeobox gene Msx-1 is expressed by the mesenchymal cells at the tip of the developing limb in the progress zone (Hill et al., 1989; Robert et al., 1991). It is thought to play a role in maintaining cells in an undifferentiated and proliferative state (Kostakopoulou et al., 1996). A feedback mechanism exists between the AER and Msx-1 expression, and if the ridge is removed then Msx-1 expression is lost. In the limbless mutant which has severely truncated limbs, the AER does not form and Msx-1 expression fails to be maintained in the limb mesenchyme, but can be restored if a normal AER is grafted onto the limb bud (Coelho and Kosher, 1991; Robert et al., 1991).

It seems very likely that members of the FGF family are responsible for the AER signals since they are expressed in the ridge and able to substitute for the AER in ridge removal experiments (Niswander et al., 1993; Fallon et al., 1994). FGF-8 is expressed throughout the AER (Mahmood et al., 1995) while FGF-4 is expressed later and restricted posteriorly (Niswander and Martin, 1992). FGF-2, however, is expressed in both the ectoderm and the mesoderm (Savage et al., 1993).
Antero-posterior patterning

Thirty years ago "cut and paste" experiments revealed that grafting of posterior limb bud mesenchyme to the anterior side of the limb resulted in outgrowth of a mirror image limb. These experiments demonstrated that A-P polarity in the limb is signalled by a small group of posterior cells that release a morphogen and set up a gradient across the limb. These cells, known as the zone of polarising activity (ZPA), have recently been shown to express the short range diffusible factor \textit{Shh} (Riddle \textit{et al.}, 1993). The importance of \textit{Shh} is illustrated in the loss of function mouse mutant which results in a truncated limb with absence of distal structures (Chiang \textit{et al.}, 1996). This phenotype is thought to result from a disruption in the feedback signalling between FGF-4 in the ridge and shh in the ZPA (Laufer \textit{et al.}, 1994; Niswander \textit{et al.}, 1994).

The zinc finger genes \textit{Gli1-3} are expressed in the limb mesenchyme and are potential mediators of hedgehog signalling (Alexandre \textit{et al.}, 1996). \textit{Gli1} is expressed in the posterior mesenchyme, overlapping \textit{Shh} expression, while \textit{Gli2} and \textit{Gli3} are found throughout the limb mesenchyme except in the \textit{Shh} domain. If \textit{Shh} expression is experimentally manipulated then transcription of the \textit{Gli} genes is also affected, with \textit{Gli1} being upregulated in areas of \textit{Shh} expression and \textit{Gli3} is downregulated (Marigo \textit{et al.}, 1996).

It seems that \textit{Shh} also activates the expression of BMPs in the ZPA and cells adjacent to them (Francis \textit{et al.}, 1994). \textit{Bmp2} and \textit{Bmp7} are expressed in the posterior mesenchyme (Francis \textit{et al.}, 1994; Francis-West \textit{et al.}, 1995). \textit{Bmp4} is expressed throughout the chick wing bud mesenchyme. These signalling molecules appear to regulate expression of the homeobox genes, in particular the \textit{HOXD} cluster (Dollé \textit{et al.}, 1989; Dollé \textit{et al.}, 1991; Izpisúa-Belmonte \textit{et al.}, 1991). The resulting expression pattern of \textit{Hoxd} genes (\textit{Hoxd9} through to \textit{Hoxd13}) in overlapping domains across the distal end of the developing limb bud, in domains corresponding to their 3' to 5' chromosomal location in the cluster, may be the means by which limb mesenchymal cells record and act on their
A-P positional cues designated to them by the morphogen gradient (Izpisúa-Belmonte et al., 1991).

• **Dorso-ventral patterning**

The epithelium overlying the limb bud expresses a number of genes some of which are restricted to either the dorsal or ventral compartment, and may be critical in D-V polarity. The gene **Wnt-7a**, which encodes a short range diffusible factor, is expressed in the dorsal ectoderm of the developing mouse limb and in mice null for this gene, double ventral limbs result suggesting that **Wnt-7a** is crucial for specifying the dorsal state (Parr and McMahon, 1995). **Wnt-7a** appears to induce the dorsal mesenchyme to express the LIM-homeodomain containing gene **Lmx-1** and viral overexpression of **Lmx-1** results in the formation of a double dorsal limb (Riddle et al., 1995; Vogel et al., 1995).

Conversely, the transcription factor **Engrailed-1** (**En-1**), which is expressed by the ventral ectoderm, must play a ventralising role since **En-1** null mice have a dorsalised limb phenotype (Loomis et al., 1996). A new candidate gene with a possible role to play in D-V patterning is **Radical fringe** (**R-fng**) which is expressed in the dorsal limb ectoderm of chick embryos (but curiously not in the mouse) and is repressed by **En-1**. In the chick, it seems that the AER forms precisely at the boundary between cells that express **R-fng** and those that do not (Laufer et al., 1997a; Rodriguez-Esteban et al., 1997).

**Programmed cell death in the limb**

Subsequent to the limb patterning mechanisms described above the limb bud is further remodelled and sculpted by programmed cell death or apoptosis. There are four areas of programmed cell death in the developing limb varying in size according to the species.
concerned (Hinchliffe and Johnson, 1982). These were described before programmed cell death had been fully characterised and were thus confusingly called necrotic zones. The first two regions are found on the proximal edges of the limb bud and are called the anterior and posterior necrotic zones (ANZ and PNZ). These zones are particularly pronounced in the bird wing where there is no digit one and a dramatically reduced digit five. The third defined area of cell death is found between the tibia and fibula (or the radius and ulna in the forelimb). The fourth zone of programmed cell death is that in the interdigital web regions. In the chick and the mouse interdigital cell death occurs throughout each interdigit, while in the duck the interdigital cell death is more restricted to the distal margins leading to webbing (Saunders and Fallon, 1967). There is a precise correlation between the appearance of fragmented internucleosomal DNA and the characteristic morphological signs of apoptosis in the chick leg bud (Garcia-Martinez et al., 1993).

Studies in the chick suggest that catastrophic interdigital apoptosis is triggered by signals from the overlying limb ectoderm since removal of small patches of this ectoderm, prior to the onset of death, blocks apoptosis. Cells appear to default to a cartilage phenotype, since cartilage nodules, and even extra digits, are subsequently seen in the normal interdigital space (Hurle and Gañán, 1986; Hurle et al., 1989). Similar experiments were carried out in the duck leg bud and while ectopic cartilages were formed, they were a different shape and fewer extra digits were ultimately produced (Macías et al., 1992). This is consistent with the fact that there is less undifferentiated mesenchyme programmed to die. Prevention of programmed cell death in the interdigital regions by removal of ectoderm has been extrapolated to the PNZ. Local removal of ectoderm results in the failure of cell death in this region (Brewton and MacCabe, 1988).

When the AER is removed from the limb bud prior to the onset of programmed cell death, about stage 30 in the chick, programmed cell death fails to occur in the interdigital areas and ectopic cartilage is found (Hurle and Gañán, 1986; Hurle and Gañán, 1987).
If the AER is removed after stage 30 then programmed cell death proceeds as normal (Hurle and Ganân, 1986).

BMPs - members of the TGF-β superfamily - are expressed in the interdigital spaces prior to the initiation of and during programmed cell death (BMP-2, BMP-4; Francis et al., 1994: BMP-7; Luo et al., 1995). A number of experiments have investigated the role played by BMPs in this process, particularly in the light of the fact that BMP-4 was shown to trigger cell death in the hindbrain (Graham et al., 1994). Beads soaked in BMP-4 and implanted in the interdigital tissue were found to accelerate cell death and digit separation (Ganân et al., 1996). Additionally, when BMP-2 and BMP-4 proteins were added to mesenchymal cells in vitro apoptosis was induced (Yokouchi et al., 1996). Furthermore, if BMP activity is blocked at the level of the receptor by expressing a dominant negative type I BMP receptor (dnBMPR-1B) in chick then interdigital cell death is dramatically reduced and webbed feet were formed (Zou and Niswander, 1996). It was even suggested that the reason why a duck has webbed feet was because it lacked expression of BMP-2, BMP-4 and BMP-7 (Zou and Niswander, 1996), but this statement has since been retracted (Laufer et al., 1997b).

Experiments with heparin beads soaked in TGF-β1 and TGF-β2 inserted into the interdigital tissue resulted in the inhibition of programmed cell death, with cartilage or extra digits being formed (Ganân et al., 1996). Local administration of FGF-2 and FGF-4 also prevents apoptosis in the interdigital tissue (Macias et al., 1996). In the light of this result and what is known about FGF expression patterns in the limb, it has been suggested that interdigital programmed cell death is initiated when AER function stops (Macias et al., 1996).
How are the dead cells cleared?

Not only do cells die during the footplate sculpting process, but they also have to be cleared away. The efficiency of this clearance is just as important as the death itself. Previous studies on the remodelling limb in avian and mammalian embryo, not only describe the distribution of programmed cell death, but speculate as to how these dying cells are cleared away in embryos. Generally it was felt that neighbouring mesenchymal cells were responsible for engulfing the cellular debris (Saunders and Fallon, 1967; Ballard and Holt, 1968). However, more recent studies have shown large numbers of “professional” phagocytes, macrophages, present in the zones of interdigital cell death from the earliest stages when programmed cell death is observed (Hopkinson-Woolley et al., 1994). These cells are clearly swollen with apoptotic bodies. Later studies in the Hammertoe mutant mouse, where there is less cell death, reveal a tight correlation between the numbers of dead cells and the numbers of macrophages (Zakeri et al., 1994). These data suggests that macrophages are “key players”, perhaps the only players, responsible for clearing programmed cell death in the developing limb.
CHAPTER TWO

General Materials and Methods

Mice

• Husbandry

For these studies an outbred strain of Albino mouse, strain CD1 (Olac), was used. Female adult mice have a four day oestrus cycle. Female mice of weight 25 - 30g, ten weeks of age, were checked for signs of being in oestrus, when the vagina becomes pink and swollen, in the early evening, and then each one was mated with a male of the same strain. The following morning each female mouse was checked for a vaginal plug, which is usually white and hard and very obvious, although occasionally deep white plugs were found. Gestational age was calculated on the assumption that conception had occurred at midnight preceding the morning a vaginal plug was seen.

The time-mated female mouse was killed by cervical dislocation, the abdomen was opened and the bicornate uterus exposed. The uterus was removed by cutting its attachments at the ovaries and the cervix, and placed in a petri dish containing phosphate buffered saline (PBS; Oxoid). The uterus was washed twice in PBS before being transferred to a clean dish so that the embryos could be removed. The uterine wall was cut along its entire length on the anti-placental border with a pair of fine iridectomy scissors. The placental side of the embryos were then carefully teased away with fine
The embryo was gently placed into 5ml of foetal calf serum (FCS; Sigma) and culture saline (see Appendix) in a ratio of 1:2 in a 50ml Falcon tube and cultured according to a protocol modified from Cockcroft (1990). The airspace in the Falcon tube was filled with a gas mixture of 95% oxygen and 5% carbon dioxide (O₂ & CO₂; BOC). For an airtight seal the mouth of the tube was coated with a thin layer of silicone grease (Fisons). Tubes were then placed in a roller incubator (BTC Engineering, Cambridge) maintained at 37°C and rolled at 30 revolutions per minute (rpm). The embryos were re-gassed every three hours.

- **Open uterus surgery**

Embryos of E13.5 are unable to survive in roller culture for more than 12 hours on account of requiring more oxygen than can be provided for the embryo in a tube. For studies of macrophage movements in embryos for time-points between 12 and 24 hours post-tagging, a protocol first described by Muneoka and colleagues (1990), which allows for manipulation of embryos in situ, was followed.

Time-mated female mice were weighed and anaesthetised by an intraperitoneal injection of a mixture of Hypnovel (midazolam 5mg/ml; Janssen-Cilag Ltd), Hypnorm (fentanyl citrate 0.315mg/ml and fluanisone 10mg/ml; Janssen-Cilag Ltd) and Hartmann’s solution (Lactated Ringers; Fresenius Ltd; see Appendix) in the ratio of 1:1:2, 0.1ml per 10g body weight. Additional anaesthetic was given intraperitoneally in 0.05ml doses if required. The abdomen was cleared of hair using a depilatory cream (Immac, Reckitt) and washed with 70% alcohol. The first incision was made using fine scissors in the skin in the midline, from the xiphistemum to the pubic symphsis, being careful not to damage the bladder. The skin was pinned back while the muscular layer was cut with large iridectomy scissors (Fig. 5.1). Both layers were then retracted and the abdominal cavity was filled with warmed (37°C) Hartmann’s solution. The uterus was then cut with fine iridectomy scissors along its anti-placental border and the embryos revealed in
forceps. Embryos were then gently freed of their decidua and delivered from their yolk sac by carefully cutting away the yolk sac where it joins the placenta. The amniotic membrane was the last membrane to protect the embryo and this was also removed. The embryos were still alive at this point being attached to their placenta by umbilical vessels. The embryos were then processed accordingly.

Alternatively some females were anaesthetised on the appropriate day before open uterus surgery was performed and eventually killed by cervical dislocation when the embryos were harvested.

The embryos were staged according to Theiller (1989) and Martin (1990), using the development of the forelimbs and hindlimb buds as indicators.

- **Embryo culture**

This procedure is useful for manipulating embryos between embryonic day 9 (E9) and E12.5 outside of the uterus. E13.5 however is a bit more difficult and embryos only live for a maximum of 12 hours without showing any ill-effects.

E13.5 embryos were gently dissected free from the uterus, and the decidua and underlying Reichert’s membrane were carefully trimmed away. Each embryo was then transferred, using the “wrong” end of a glass pipette, to a fresh petri dish containing explant saline (see Appendix). The embryo was delivered from the yolk sac by cutting with fine iridectomy scissors almost all the way round the yolk sac and letting the embryo “fall” out. The embryo was then delivered from the amniotic membrane by making a small hole in the membrane next to the caudal end of the embryo and passing the membrane forward over its head.
their yolk sacs. The left hindlimb was located and an injection made through the yolk sac into the interdigital space. This meant minimal movement of the embryo and no change in its immediate environment as the hole from the injection was so small.

Occasionally an incision was made in the yolk sac to access the limb; the limb delivered and the injection made before returning the limb and suturing closed the yolk sac with 10/0 monofilament suture (Ethicon). The number and position of the embryos treated was carefully recorded. Finally, the abdominal cavity was rinsed with warm Hartmann’s solution, the muscular wall was closed with 6/0 monofilament suture (Ethicon) and the skin was also sutured. The mouse was given an injection of Temgesic (0.3 mg/ml buprenorphine as hydrochloride; Reckitt & Colman) to aid her recovery and prevent any discomfort. She was then placed on tissues in a clean cage with a small dish of water. The whole cage was placed in a warmed box (plant propagator, Midland Oak UK Ltd) until the mouse had regained consciousness, and then she was placed in a warm recovery room (25°C). The mother was closely monitored throughout the recovery period.

After 12, 18 or 24 hours the mother was killed by cervical dislocation and the embryos recovered by cutting through the sutures to re-open the abdomen. The embryos were dissected away from the uterus and their membranes removed, before being killed by immersion in ice-cold PBS and then fixed in 4% paraformaldehyde and processed as for cultured specimens.

- **Kidney organ culture**

Using a dissecting microscope in a tissue culture flow hood, kidney rudiments, which are cylindrical in shape and approximately 400μm in length, were carefully dissected from embryos at 11.5 days of gestation (Fig. 4.1). In a petri dish containing Leibovitz’s L-15 medium (Gibco) the embryo was first transected with small iridectomy scissors midway between the forelimbs and hindlimbs (incision 1); the second cut, again made
using small iridectomy scissors, was just above the hindlimbs (incision 2); most of the tissue anterior to the position of the kidneys was teased away at this stage; the third cut, to remove the developing somites and neural tube, was performed using a pair of 0.6 hypodermic needles on the ends of 1ml syringes (incision 3). The remaining piece of tissue was then placed on its ventral surface exposing the metanephric kidneys lying in close apposition with the mesonephroi (Fig. 4.1 D). At this stage the ureteric bud has branched from the Wolffian duct and just entered the condensed metanephric mesenchyme. It is essential to have both components for the culturing procedure. The metanephric kidney rudiment was gently teased away from the surrounding tissue using watchmakers forceps and a fine bore needle, and placed on a 0.8cm pre-dampened millicell tissue insert (Millipore) in a four well culture dish (Gibco). The organ culture was allowed to flatten down for between 30 minutes to one hour, before 200μl of culture medium was added to the well.

The culture medium used was Dulbecco's Modified Eagle Medium/Nutrient Mix F12 (1:1) with L-Glutamine, 15mM HEPES (DMEM/F12; Gibco; see Appendix) containing 10% FCS and 1% penicillin and streptomycin (Sigma). The organ culture absorbs its nutrients from the medium through the filter. The culture dish was put into an incubator at 37°C with 5% CO₂ and the medium was changed daily. The organ cultures were viewed every day using a Leica Diaplan microscope, and each one was captured digitally via a video camera and the Adobe photoshop programme on a Power Macintosh Computer.

The method was based on the work of Grobstein (1955; 1956) and Adrian Woolf, Institute of Child Health, London.
Resin histology and electron microscopy

Embryos for resin histology, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were rinsed in PBS, fixed in ice-cold half-strength Karnovsky fixative (Karnovsky, 1965; see Appendix) overnight at 4°C and rinsed twice in 0.1M cacodylate buffer for 20 minutes. During this rinsing phase the relevant parts of the embryo were dissected free from the whole embryo. The specimens were then post-fixed in 1% osmium tetroxide (Sigma) in 0.1M cacodylate buffer for between 45 and 75 minutes, depending on the size of the tissue, before being dehydrated through a graded ethanol series, 10 minutes in each.

- Resin histology

Specimens for resin histology were transferred from AnalaR ethanol (BDH) to propylene oxide (BDH) and washed for a further 30 minutes, the solution being changed every 10 minutes. The specimens were then left in a 50:50 mixture of propylene oxide and Araldite resin (see Appendix) for 45 minutes before being transferred to neat, fresh Araldite resin and left for 12 hours on a rotator. The Araldite resin was changed, the specimens rotated for a further five hours before being embedded in fresh Araldite resin in a plastic mould and cured at 60°C for 24 hours. These blocks were cut to the appropriate size and mounted on an Araldite chuck with superglue. Sections of 1-5μm were cut, mounted on poly-L-lysine (1:100; Sigma) coated slides and stained with Toluidine Blue.

- Transmission electron microscopy

A number of these specimens were also examined by TEM. In these cases, ultrathin resin sections were cut using a diamond knife and floated on water prior to being collected on copper mesh grids. Each section was first stained with uranyl acetate for 10
minutes, rinsed thoroughly and allowed to dry before being stained with lead citrate, again for 10 minutes. The sections were rinsed and left to dry prior to viewing using a Jeol 1010 Transmission Electron Microscope.

• **Scanning electron microscopy**

Specimens for SEM were transferred from AnalaR ethanol into AnalaR acetone (BDH) and dried using a Polaron Critical Point Drier, which uses CO₂ as its medium. They were mounted on 10mm cylindrical stubs with electrodag - silver in methyl-isobutyl-ketone (Agar Scientific), and the electrodag allowed to set for 24 hours before each specimen was sputter coated with gold using a SC500 Sputter Coater. The specimens were viewed on a Jeol JSM-5410LV Scanning Electron Microscope.

**F4/80 immunohistochemistry**

• **Sections**

In preparation for wax immunohistochemistry, embryos were fixed overnight in ice-cold Bouin's fixative (70% saturated picric acid (aqueous), 25% formalin, 5% glacial acetic acid), and thoroughly rinsed in 70% alcohol before dehydration through graded alcohols, 10 minutes in each. Specimens were transferred to histoclear (National Diagnostics) at room temperature for 20 minutes, histoclear at 57°C for 20 minutes and then to a 50:50 histoclear and Fibrowax (BDH) mix at 57°C for 20 minutes. The specimens were transferred to fresh Fibrowax for 30 minutes, this was changed and left for another 30 minutes before the specimens were embedded in Fibrowax in plastic 7ml square weigh boats. Each specimen was cut out of the wax, a flat topped pyramid was cut, and this block was mounted on a wooden chuck. 8µm sections were cut on a Leica “Supercut”
microtome, these were floated on distilled water and allowed to dry down on poly-L-lysine coated slides at 40°C.

The sections were rehydrated through graded alcohols, rinsed in PBS and soaked in 0.3% hydrogen peroxide (H$_2$O$_2$; BDH) in methanol (BDH) for 40 minutes to block endogenous peroxidase. They were rinsed again in PBS and then laid out in a humidified box - a plastic tray with lid with water soaked tissue in its base and two pipettes fixed to the tray to lie the slides on. A Pap-pen (Agar Scientific) was used to draw around each section to enable minimal volumes of reagents to be used. Rabbit serum (1:100; Vector Laboratories) was applied to the sections for 30 minutes, to block non-specific binding of the antibody. The sections were then incubated with F4/80, a macrophage specific rat anti-mouse monoclonal antibody (Austyn and Gordon, 1981) for 90 minutes. The sections were washed three times in PBS, and bound antibody was detected using biotinylated mouse adsorbed rabbit anti-rat IgG (5µg/ml; Vector Laboratories), for 45 minutes. The sections were again rinsed in PBS three times, and the avidin-biotin-peroxidase complex was applied for 45 minutes (Vectastain ABC Elite kit; Vector Laboratories). To detect the peroxidase activity 0.5mg/ml of diaminobenzidine tetrahydrochloride (DAB; Sigma) in 10mM imidazole (Sigma) at pH 7.4 and an equal volume of 0.02% H$_2$O$_2$ was used. This was rinsed off with tap water, followed by distilled water and counter-stained with Mayer's Haemalum (BDH).

More recently, a DAB kit (Vector Laboratories) was used, according to the manufacturer’s instructions, to detect peroxidase activity. Two drops of buffer stock solution were added to 5ml of distilled water and mixed thoroughly, plus four drops of DAB stock solution and mixed again, followed by two drops of hydrogen peroxide solution and mixing. The sections were incubated with this solution for between three and five minutes, then rinsed and counter-stained as before.
The sections were dehydrated through graded alcohols and mounted in XAM (BDH).

- **Wholemounts**

  Tissue for wholemount immunohistochemistry was fixed in ice-cold 4% paraformaldehyde (BDH) overnight at 4°C. The specimens were rinsed in PBS, permeabilised with 0.3% Triton and blocked with 10% rabbit serum (Sigma) for 30 minutes. They were then incubated with 10μg/ml F4/80 overnight at 4°C on a rocking platform. The specimens were rinsed in PBS, further permeabilised and rinsed again before incubation with a FITC (fluorescein isothiocyanate) - tagged mouse adsorbed rabbit anti-rat IgG (10μg/ml; Vector Laboratories) overnight, at 4°C and on a shaker. The limbs were rinsed in PBS before being mounted in Citifluor (UKC) in a custom-made well, manufactured by cutting a window in two layers of electrical tape on a microscope slide. A coverslip was then gently placed on the specimen to carefully flatten it, and the coverslip was sealed with nail varnish. The limbs were viewed and photographed using a Leica TCS4D Confocal Laser Scanning Microscope (CLSM).

**Cell death markers**

- **The nuclear dye 7-amino actinomycin D (7-AAD)**

  Specimens were fixed in 4% paraformaldehyde at 4°C for one hour, prior to infiltration with cold 5% sucrose (BDH) for two hours, followed by infiltration with 20% sucrose overnight at 4°C. Specimens were infiltrated with a 7.5% gelatin (BDH) : 15% sucrose mix in PBS at 37°C for five hours, before being embedded in a 7ml square weigh boat in fresh gelatin sucrose mix. Gelatin blocks were cut and mounted on cork with OCT compound (Agar Scientific), and frozen in isopentane (BDH) in liquid nitrogen (BOC).
Sections 5-8μm thick were cut on a Leica cryostat and air dried onto poly-L-lysine (1:100; Sigma) coated slides. The gelatin was removed by rinsing the sections in warm PBS and a Pap-pen was used to draw around each section to enable a minimal volume of reagent to be used. The sections were incubated with 5μg/ml 7-amino actinomycin D (7-AAD; Molecular Probes) in PBS for 20-30 minutes at room temperature. The sections were rinsed in PBS and mounted in Citifluor (UKC).

Alternatively, wholemount preparations were fixed in ice-cold 4% paraformaldehyde overnight, rinsed with PBS, prior to incubation with 2.5μg/ml FITC-phalloidin (Sigma) and 10μg/ml 7-AAD in PBS overnight. These specimens were rinsed thoroughly and mounted on welled slides as previously described. The phalloidin labels filamentous actin which gives more of an overview of the tissue.

To visualise both apoptotic cells and macrophages a double immunofluorescent study was performed on both frozen sections and wholemounts. The sections were first incubated with F4/80 using an identical protocol to that described above for wax section except that rehydration was not required and the secondary antibody was a FITC-tagged mouse adsorbed rabbit anti-rat IgG. The wholemounts followed the protocol previously described. Both were then incubated with 7-AAD in PBS for 20-30 minutes at room temperature. The sections were rinsed in PBS and mounted as before.

In all cases staining was revealed and photographed using the appropriate filter blocks on a Leica Diplan Fluorescent Microscope and the Leica TCS4D CLSM.

- Apoptag in situ apoptosis detection kit - fluorescein (Oncor)  S7110-KIT

The Apoptag protocol consists of three stages: the cut ends of the DNA are labelled with digoxigenin-dUTP (deoxyuridine triphosphate), a fluorescein conjugated antibody binds
to the digoxygenin-dUTP tails on the DNA (deoxyribose nucleic acid) fragments and these are viewed under fluorescence.

The tissue was fixed in ice-cold 1% paraformaldehyde for one hour at 4°C and the protocol above for frozen sections was followed. The specific tissues dissected out and used for this part of the study were the kidneys and liver, which were kept as a unit. 8μm frozen sections were cut and air dried onto poly-L-lysine coated slides.

An ice bath was prepared for holding working strength TdT (terminal deoxynucleotidyl transferase; S7110-3) before it was made. Two humidified chambers were made - each consisting of a plastic tray with lid which has water soaked tissue in its base and two pipettes fixed to the tray to lay the slides across. One was pre-warmed to 37°C in the incubator along with the working strength stop/wash buffer. The working strength antidigoxygenin-fluorescein was also prepared. (See Appendix for details of the solutions used).

The sections were washed twice, for five minutes, in PBS at room temperature, post fixed in ethanol:acetic acid (2:1) for five minutes at -20°C, before being rinsed twice more in PBS at room temperature, five minutes each. The excess liquid was gently shaken off and a cotton bud was used to carefully wipe around each section. A Pap-pen was used to draw around each section to enable minimal volumes of reagents to be used. The sections were incubated with 1x Equilibration Buffer (S7110-1) in a humidified chamber for five minutes at room temperature. A plastic coverslip was placed on each slide to ensure that the sections were totally immersed in the liquid, while keeping volumes to a minimum. The coverslips were removed and the excess liquid tapped off as before. The sections were then incubated with working strength TdT enzyme in a humidified chamber for one hour at 37°C, and covered as noted previously. The coverslips were removed and the slides rinsed in pre-warmed working strength stop/wash buffer for 30 minutes at 37°C, agitating the slides by dipping them in and out
of the solution every 10 minutes. The slides were then washed in PBS, three times, three minutes each. The excess liquid was removed and working strength antidigoxigenin-fluorescein was applied to each section, which was then covered and left for 30 minutes in a humidified chamber at room temperature. The sections were then washed three times in PBS, five minutes per wash at room temperature, and mounted under a glass coverslip with Citifluor. The sections were viewed under fluorescence using a Leica Diaplan Microscope.

**Tracking techniques**

DiI, fluorescent dextrans or F4/80 monoclonal antibody was injected into a single interdigit in the left footplate of the embryo. Using an aspirator tube (Sigma) and applying suction the marker was loaded into a fine bore capillary needle (40μl; Sigma) with a tip diameter of approximately 10μm. With a mouth pipette the marker was injected into the appropriate interdigit under a dissecting microscope. The DiI solution was a 1:9 dilution of 0.5% DiI (Molecular Probes) in AnalalR ethanol (BDH) and 0.3M filter-sterilised sucrose. The fluorescent dextrans (4.4 or 19.6 kiloDaltons, kDa; Sigma) were used at a concentration of 25mg/ml and F4/80 monoclonal antibody was used at 100μg/ml. After culturing (see earlier) for the appropriate time those embryos injected with DiI and fluorescent dextrans were rinsed three times in PBS, mounted and viewed on the CLSM immediately, while those injected with the F4/80 had first to be exposed to a FITC-tagged mouse adsorbed rabbit anti-rat IgG overnight, at 4°C, rinsed in PBS, in order to reveal the location of the primary antibody.
Manipulations of macrophage numbers

• Toxic liposomes

These were made by Graciana Diez-Roux (Skirball Institute, New York) according to established procedures developed by Van Rooijen (1989). For full details see Appendix. The liposome-dependent drug dichloromethylene bis-phosphonate (clodronate) from Boehringer Mannheim GMBH was used.

10µl of either PBS liposomes or toxic liposomes was dripped directly on to the organ culture at twenty four hours. This was washed off with PBS the following day and the cultures were left to develop for a further 36 hours. They were then fixed and processed for resin histology and wholemount macrophage and cell death staining.

• The transgenic mouse PU.1

PU.1 is a member of the ets family of transcription factors and is expressed exclusively by haematopoietic cells. By targeting this gene mice devoid of macrophages are generated. Targeted disruption of the PU.1 gene results in multiple haematopoietic abnormalities (McKercher et al., 1996).

The PU.1 null mouse was generated by standard homologous recombination techniques. The neomycin gene was inserted at the BssH2 site in the exon coding for the DNA binding domain and the thymidine kinase gene was inserted at a ClaI-SalI site, outside of the DNA binding domain. This vector was electroporated into embryonic stem (ES) cells and colonies were selected using a double selection strategy - resistance to G418 and gancyclovir. Positive clones were characterised by polymerase chain reaction (PCR) and three were isolated and injected into C53Bl/6b blastocysts for transfer to the uterus of a
pseudopregnant mother. Resulting chimeric offspring were mated to non-transgenic mice to test for germline transmission of the ES cell allele. The offspring were tested using PCR and only one of the three clones or founders resulted in germline transmission of the mutated allele. Heterozygote mice were interbred to yield homozygotes for the mutated allele. These were produced in the expected Mendelian ratio. The PU.1 null mice are outwardly almost identical to their heterozygous and wild-type littermates, but they die within 48 hours of birth of septicemia (McKercher et al., 1996).

Embryos were genotyped in the host laboratory (Maki/McKercher, The Burnham Institute, La Jolla, California) by PCR using tail DNA as a template.
CHAPTER THREE

The Role of Macrophages in the Clearance of Programmed Cell Death in the Developing Kidney

Introduction

This study investigates the clearance of developmental cell death in the mouse mesonephric and metanephric kidneys between embryonic days 11.5 (E11.5) and E16.5. In particular it addresses whether specialist macrophages or non-specialist neighbouring mesenchymal cells are responsible for phagocytosis and removal of dying cells. To provide an overview picture of these two organs, during the stages when programmed cell death is occurring, scanning electron microscopy (SEM) was used. Numbers and distribution of dying cells were revealed with resin histology and transmission electron microscopy (TEM) has allowed a more precise identification of their location. Using the macrophage specific monoclonal antibody, F4/80 (from Siamon Gordon, Pathology, Oxford), the distribution of macrophages has been identified, and to directly demonstrate that these cells were indeed engulfing dead cells during kidney development sections were double stained with F4/80 and apoptotic markers.

The mammalian kidney develops in three phases (pronephros, mesonephros and metanephros) in a recognised temporal and spatial sequence. Each of these paired organs derives in part from the two rods of intermediate or nephrogenic mesoderm which lie
between the somitic and lateral plate mesoderm of the neural-plate stage embryo. The nephric or Wolffian duct plays a major role during each of these phases, inducing tubules to form in the adjacent mesoderm, serving as the drainage channel for the pronephros and the mesonephros, and then giving rise to the ureteric bud of the metanephric kidney. The two early kidney phases differentiate from the cranial and thoracic nephrogenic mesenchyme but develop to differing extents. The pronephros starts to form basic epithelial structures at E8 but these only survive for a couple of days. The mesonephros progresses further - it develops from E10 and forms simple epithelial tubules in the rostral portion and convoluted tubules with glomeruli more caudally (Lechner and Dressler, 1997). Both of these transient structures eventually undergo a wave of "catastrophic" programmed cell death similar to that found in the interdigit regions of developing limb (Saxén, 1987; Ekblom, 1991).

The third nephric system, which will become the definitive or metanephric kidney, is formed by reciprocal inductive interactions between the ureteric bud and the most caudal nephrogenic mesenchyme which condenses to form the metanephric mesenchyme or blastema. The cells of the metanephric mesenchyme induce the ureteric bud to sprout from the nephric duct and to grow and branch many times to give rise to an elaborate renal collecting system (Jokelainen, 1963; Saxén, 1987; Ekblom, 1991; Bard, 1992). As the ureteric bud branches, the tip of each sprout goes on to induce a small number of neighbouring metanephric mesenchymal cells to proliferate rapidly and then aggregate before undergoing an elaborate mesenchymal-epithelial transformation. This mesenchymally-derived epithelium undergoes invagination and elongation through the characteristic "comma-shaped" and "S-shaped" body phases of tubulogenesis which will eventually give rise to the secretory parts of the young nephron (Saxén, 1987). Throughout metanephric development new nephrons are formed at the periphery of the growing kidney and more mature nephrons are displaced towards the core or medulla of the kidney.
The different parts of the nephron can now be identified by molecular markers. The adhesion molecule E-cadherin is expressed by epithelial cells adjacent to the ureteric bud which will form the distal tubule and the collecting duct, while the transcription factor WT-1 is expressed by those cells which will become the glomerular epithelium. The cells in between these two areas which will form the proximal tubule express K-cadherin (Lechner and Dressler, 1997). The transcription factor BF-2 is expressed by mesenchymal cells which do not undergo this mesenchymal-epithelial transition (Hatini et al., 1996); the fate of these cells is to become either cells of the interstitial mesenchyme or to die.

Significant numbers of cells in close proximity to the forming nephrons undergo programmed cell death or apoptosis (Koseki et al., 1992; Coles et al., 1993). It seems that the metanephric kidney, like the nervous system, undergoes a protracted period of "trickle-like" cell death, which lasts beyond birth, during which time up to 50% of mesenchymal cells in the nephrogenic zone will die. Coles and colleagues (1993) showed in the rat embryo (E19) that between 1 and 3% of all cells were undergoing apoptosis. When extrapolated from what is know about clearance rates in other developing tissues (Perry et al., 1983), which are likely to be in the range of 1-2 hours, it was suggested that as many as one cell from every two that are born may die in this region of the kidney (Coles et al., 1993). As in the developing nervous system, the reason why this extensive cell death went undetected for so long is that as each cell dies it is rapidly phagocytosed and cleared away by other cells.

How, as various organ systems develop, are such large numbers of dying cells so efficiently cleared away? It has previously been reported that neighbouring embryonic mesenchymal cells phagocytose the dead cells (Glücksmann, 1951; Raff, 1992; Coles et al., 1993; Garcia-Martinez et al., 1993), but it has been recently shown that where the cell death is "catastrophic", as in the case of interdigital programmed cell death in the mouse footplate, specialist haematopoietically-derived macrophages are rapidly recruited.
to the site of cell death and seem to be largely responsible for phagocytosis and clearance of dying cells (Hopkinson-Woolley et al., 1994).

The Hopkinson-Woolley (1994) study has been extended to the developing kidney, where cell death occurs in a "trickle-like" fashion. The extent and location of programmed cell death in both the mesonephros and early stages of metanephric kidney development, from E11.5 until E16.5, was examined to establish whether invading specialist macrophages, rather than neighbouring parenchymal cells as suggested by Coles and colleagues (1993), might be responsible for clearing away this death in the way that has been illustrated for dying interdigital cells in the developing limb (Hopkinson-Woolley et al., 1994).
Materials and Methods

An outbred strain of Albino mouse, strain CD1 (Olac) was used for these studies. The gestational age was deduced assuming that conception had occurred at midnight prior to the morning a vaginal plug was found. The stages of the embryos were confirmed using Theiller (1989) and Martin (1990); the shape of the forelimb and hindlimb buds were useful developmental indicators for the stages of interest. Mother mice were sacrificed by cervical dislocation and embryos of stages E11.5 to E16.5 were retrieved from the uterus. These embryos, or just the posterior half of embryos (from the level of the liver) of E14.5 and older, were processed for light and electron microscopy to allow analysis of programmed cell death and macrophage distribution within the developing kidney.

Histology and electron microscopy

Embryos for resin histology, TEM and SEM were dissected out of the uterus in phosphate buffered saline (PBS) and killed by cutting their heads off. Those for resin histology and TEM were rinsed in PBS before being fixed in ice-cold half-strength Karnovsky fixative (Karnovsky, 1965) overnight at 4°C. The embryos for SEM were carefully dissected to reveal the kidneys and pinned out in a petri dish containing Sylgard (Dow Corning), before they were fixed as above. The embryos were then thoroughly rinsed twice in 0.1M cacodylate buffer for 20 minutes, and embryos for resin histology and TEM were dissected to leave the kidneys with a minimal amount of surrounding tissue. The SEM specimens were unpinned and all the prepared tissue was post-fixed in 1% osmium tetroxide (Sigma) in 0.1M cacodylate buffer for an hour at 4°C. The kidney pieces were rinsed in 0.1M cacodylate buffer before being dehydrated through a graded ethanol series, 10 minutes in each.
• **Resin histology**

The specimens for resin histology were washed four times in propylene oxide (BDH), infiltrated with a 50:50 mixture of propylene oxide and Araldite resin (see Appendix) for 45 minutes, infiltrated with neat, fresh Araldite resin and left for 12 hours on a rotator. The Araldite resin was changed, the specimens rotated for a further five hours before being embedded in fresh Araldite resin in a plastic mould and cured at 60°C for 24 hours. These blocks were cut to the appropriate size and mounted on an Araldite chuck with superglue, which was allowed to dry for five hours before sections of 1-5 μm were cut. These were floated on distilled water on poly-L-lysine (1:100; Sigma) coated slides and allowed to dry on a hot plate. The sections were stained with Toluidine Blue and mounted under a glass coverslip with XAM (BDH).

• **Transmission electron microscopy**

Some of these specimens were also examined by TEM. The block was trimmed down to an absolute minimum, and, using a diamond knife ultra-thin resin sections were cut, floated on water and mounted on copper mesh grids. These were first stained with uranyl acetate for 10 minutes, rinsed with distilled water and allowed to dry, before they were counter-stained with lead citrate for a further 10 minutes and then rinsed again. Viewing was by means of a Jeol 1010 Transmission Electron Microscope.

• **Scanning electron microscopy**

Specimens for SEM were transferred from AnalaR ethanol (BDH) to AnalaR acetone (BDH) and dried using a Polaron Critical Point Drier, which uses CO₂ as its medium. They were mounted on 10mm cylindrical stubs with electrodag - silver in methyl-isobutyl-ketone (Agar Scientific), and the electrodag allowed to set for 24 hours before the stub was placed in a SC500 Sputter Coater in order to sputter coat the specimen with gold. The specimens were viewed on a Jeol JSM-5410LV Scanning Electron Microscope.
Embryos for immunohistochemistry were dissected out of the uterus in PBS and cut across the trunk, just below the fore-limbs, before being fixed overnight in ice-cold Bouin’s fixative (70% saturated picric acid (aqueous); 25% formalin; 5% glacial acetic acid). They were rinsed thoroughly in 70% alcohol prior to further dissection to expose the kidneys. The specimens were dehydrated through graded alcohols, 10 minutes in each, transferred to histoclear (National Diagnostics) at room temperature for 20 minutes, histoclear at 57°C for 20 minutes and then to a 50:50 histoclear and Fibrowax (BDH) mix at 57°C for 30 minutes. The specimens were then transferred to fresh Fibrowax for 30 minutes, this was changed and left for another 30 minutes before embedding the specimens in fresh Fibrowax in small weighing boats. Blocks containing the specimens were cut from the wax, mounted on a wooden chuck, and 8μm sections were cut on a Leica “Supercut” microtome. These sections were floated on distilled water on poly-L-lysine coated slides and allowed to dry in an oven at 40°C.

The sections were rehydrated through histoclear and graded alcohols, rinsed in PBS and soaked in 0.3% hydrogen peroxide (H₂O₂; BDH) in Analar methanol (BDH) for 40 minutes to block endogenous peroxidase. They were rinsed twice in PBS before rabbit serum (1:100; Vector Laboratories) was applied to the sections for 30 minutes, to block non-specific binding of the antibody. The sections were then incubated with 8.5μg/ml F4/80, a macrophage specific rat anti-mouse monoclonal antibody (Austyn and Gordon, 1981; Morris et al., 1991) for 90 minutes at room temperature. After three washes in PBS, the bound antibody was detected using biotinylated mouse adsorbed rabbit anti-rat IgG (5μg/ml; Vector Laboratories) for 45 minutes, rinsed three times in PBS and further incubated with the avidin-biotin-peroxidase complex (Vectastain ABC Elite kit, Vector Laboratories) for 45 minutes. This was washed off in PBS and peroxidase activity was detected using 0.5mg/ml of diaminobenzidine tetrahydrochloride (DAB; Sigma) in 10mM imidazole (Sigma) at pH 7.4 and an equal volume of 0.02% H₂O₂. The sections were
rinsed with tap water, followed by distilled water and counter-stained with Mayer's Haemalum (BDH) for five minutes. The sections were dehydrated through graded alcohols and mounted in XAM (BDH).

Double labelling of sections for apoptosis and macrophages

Frozen sections were used for a double immunofluorescent study to visualise both apoptotic cells and macrophages in the same specimens. Embryos were delivered from the uterus and dissected as above, prior to being fixed in 4% paraformaldehyde (BDH) at 4°C for one hour, rinsed three times in PBS, incubated in 5% sucrose in PBS for two hours, and then in 20% sucrose in PBS overnight. The specimens were infiltrated with a gelatin:sucrose (7.5%;15%) mix for four hours at 37°C before being embedded in the fresh gelatin sucrose mixture in small weigh boats. Gelatin blocks were cut and mounted on a piece of cork with OCT compound (Agar Scientific), prior to being frozen in isopentane (BDH) in liquid nitrogen. 10μm thick sections were cut on a Leica cryostat and air dried onto poly-L-lysine coated slides.

These sections were first incubated with F4/80 using an identical protocol to that described above for wax sections except that no rehydration was necessary, the gelatin was removed with warm PBS and the secondary antibody was a FITC-tagged mouse adsorbed rabbit anti-rat IgG (10μg/ml; Vector Laboratories). After the unbound secondary antibody had been washed off in PBS the sections were further incubated with 5μg/ml 7-amino actinomycin D (7-AAD; Molecular Probes) in PBS for 20-30 minutes at room temperature. The sections were rinsed in PBS and mounted in Citifluor (UKC). Double-staining was revealed under fluorescence, using the appropriate filter blocks on a Leica Diaplan Microscope, and specimens were subsequently viewed and photographed using a Leica TCS4D Confocal Laser Scanning Microscope (CLSM).
Apoptag labelling of dead cells

Apoptag in situ apoptosis detection kit - fluorescein (Oncor) was used to label the cut ends of DNA which are numerous in cells undergoing apoptosis. The Apoptag protocol consists of three stages: the cut ends of the DNA are labelled with digoxigenin-dUTP (deoxyuridine triphosphate), a fluorescein conjugated antibody binds to the digoxigenin-dUTP tails on the DNA fragments and these are viewed under fluorescence.

The tissue was fixed in ice-cold 1% paraformaldehyde for one hour at 4°C and the protocol above for preparing frozen sections was followed. The specific tissues dissected out and used for this part of the study were the kidneys and liver, which were kept as a unit. 8μm frozen sections were cut and air dried onto poly-L-lysine coated slides.

An ice bath was prepared for holding working strength TdT (terminal deoxynucleotidyl transferase) before it was prepared. Two humidified chambers were made - each consisting of a plastic tray with lid which has water soaked tissue in its base and two pipettes fixed to the tray to lay the slides across. One was pre-warmed to 37°C in the incubator along with the working strength stop/wash buffer. The working strength anti-digoxigenin-fluorescein was also prepared. (See Appendix for details of the solutions used).

The sections were washed twice, for five minutes, in PBS at room temperature, post fixed in ethanol:acetic acid (2:1) for five minutes at -20°C, before being rinsed twice more in PBS at room temperature, five minutes each. The excess liquid was gently shaken off and a cotton bud was used to carefully wipe around each section. They were incubated with 1x Equilibration Buffer (S7110-1) in a humidified chamber for five minutes at room temperature. A plastic coverslip was placed on each slide to ensure that the sections were totally emmersed in the liquid, while keeping volumes to a minimum.
The coverslips were removed and the excess liquid tapped off as before. The sections were then incubated with working strength TdT enzyme in a humidified chamber for one hour at 37°C, and covered as noted previously. The coverslips were removed and the slides rinsed in pre-warmed working strength stop/wash buffer for 30 minutes at 37°C, agitating the slides by dipping them in and out of the solution every 10 minutes. The slides were then washed in PBS, three times, three minutes each. The excess liquid was removed and working strength anti-digoxigenin-fluorescein was applied to each section, which was then covered and left for 30 minutes in a humidified chamber at room temperature. The sections were then washed three times in PBS, five minutes per wash at room temperature, and mounted under a glass coverslip with Citifluor. The sections were viewed under fluorescence using a Leica Diaplan Microscope.

**Wholemount preparations**

Small numbers of metanephric kidneys between E12.5 and E14.5 were also viewed as wholemount preparations under the CLSM. The kidneys were dissected free of the embryo, fixed in ice-cold 4% paraformaldehyde, before rinsing in PBS, and incubation overnight with 2.5µg/ml FITC-phalloidin (Sigma) and 10µg/ml 7-AAD in PBS. Specimens were rinsed again in PBS before mounting beneath a coverslip in Citifluor. Optical sections through such specimens allowed good observation of apoptosis in developing nephrons. Images were photographed directly from the monitor of the CLSM.
Results

The monoclonal antibody F4/80, which specifically recognises murine macrophages, was used to show that whenever and wherever there was cell death in the developing mesonephric or metanephric kidney there were also haematopoietically-derived specialist macrophages seen. Moreover, in the mesonephros and from E14.5 in the metanephric kidney, there were large numbers of macrophages clearly swollen with phagocytosed apoptotic bodies. Double-labelling experiments, using the nuclear dye 7-AAD, to reveal condensed apoptotic nuclei, and F4/80, to reveal macrophage plasma membranes, show definitively that the majority of dying cells in the developing kidney were engulfed by macrophages.

Location and shape of the mesonephros and metanephros

The mesonephros first begins to differentiate in a rostro-caudal fashion in the mouse at about E10 and soon after this, regression begins in the same direction (Saxén, 1987; personal observations). This was illustrated by SEM pictures at E12.5 which show that the mesonephros is a fairly extensive structure extending from the lower thoracic levels down to the lumbar region (Fig. 3.1 A - C). A SEM of the metanephric kidney at E14.5 shows the “relative” movement of the metanephros from a lumbo-sacral position to a more rostral position, in the thoraco-lumbar region (Fig. 3.1 D). The increase in the overall size of the organ can also be observed.
Programmed cell death spreads as a wave throughout the regressing mesonephros

Sagittal sections of resin histology through the mesonephros reveal aggregations of apoptotic cells in and adjacent to the mesonephric tubules (Fig. 3.2 A & B), in a similar pattern to the large collections of dying cells seen in the interdigital web mesenchyme of the developing mouse footplate (Ballard and Holt, 1968; Hopkinson-Woolley et al., 1994). TEM studies reveal that almost all apoptotic bodies are encapsulated within a phagocytic cell (Fig. 3.2 C). Generally each phagocytic cell appears to contain at least three or four apoptotic nuclei in the plane of the section viewed.

In the metanephric kidney cell death was localised to the mesenchyme and mesenchymally-derived tissues

Resin sections cut through the developing metanephric kidney from E12.5 through to E16.5 revealed the distribution of apoptotic cells. At E12.5, just after the ureteric bud had entered the condensed metanephric mesenchyme, small numbers of apoptotic cells were scattered within the metanephric blastema, but none were observed in the highly proliferating ureteric bud epithelium (Fig. 3.3 A & B).

At E13.5 and E14.5 the kidney undergoes significant morphogenesis with branching of the ureteric bud and induction of mesenchyme to form aggregations at the tips of the ureteric bud's branches (Fig. 3.3 C). At these stages occasional apoptotic cells were seen in the surrounding mesenchyme, as found in earlier stages, but now dead cells were also seen in the mesenchymally-derived epithelial structures, most frequently in the tails of "comma-shaped" presumptive nephrons (Fig. 3.3 D). This was most clear in optical sections through wholemount kidneys where intact developing nephrons were observed (Fig. 3.4 A).
By E16.5, cell death was largely restricted to the outer area or nephrogenic zone of the kidney where the new nephrons develop. The apoptotic cells were seen in and around the developing nephric tubules (Fig. 3.5 A & B). There were no dead cells found within the branching ureteric bud epithelium throughout this period. TEM studies of the developing metanephric kidney show that all but a small number of apoptotic cells appear to have been engulfed by phagocytic cells (Fig. 3.5 C & D). However, in contrast with the mesonephros or developing limb, each phagocyte generally only contains one or two apoptotic bodies in the plane of section.

Confirmation of cell death by labelling the cut ends of the DNA

As clarification of the presence of dead cells in the developing metanephros, frozen sections were stained using the Apoptag kit (Oncor) which labels the cut ends of the DNA. These fragments of multiples of 200 base pairs are numerous in dead and dying cells, and are responsible for the characteristic laddering effect that is seen when DNA preparations are run on standard agarose gels. At E14.5 bright staining apoptotic nuclei were observed using this detection kit in the tails of “comma-shaped” and “S-shaped” bodies (Fig. 3.4 B).

In both the developing mesonephros and metanephros, wherever there was programmed cell death there were also macrophages

The monoclonal antibody F4/80, which recognizes an epitope on the plasma membrane of murine monocytes and macrophages, was used to immunohistochemically reveal macrophages in wax sections of the developing mesonephric and metanephric kidney at stages between E11.5 and E16.5. This F4/80 staining demonstrated that the macrophage
distribution correlates very closely with that of the apoptotic cells as described above (Fig. 3.6 & 3.7).

In the mesonephros there appeared to be a recruitment of macrophages to death zones just as had been previously observed in the limb. The number of macrophages within the kidney was significantly higher than in adjacent regions of loose connective-tissue at these developmental stages. Macrophages were found located adjacent to mesonephric tubules where programmed cell death was occurring as the organ regressed. Many of the macrophages seen in the regressing mesonephros were swollen having recently engulfed cell debris (Fig. 3.6 A & B).

At E12.5, in the early metanephric kidney, there were only a few macrophages found in the metanephric mesenchyme where resin histology had revealed there to be only occasional cell death. No macrophages were found in or immediately adjacent to the ureteric bud epithelium where there was no cell death. Most of the macrophages revealed by F4/80 staining at this stage have a very similar morphology to neighbouring parenchymal cells and are only distinguishable because of the antibody (Fig. 3.6 C & D). At E12.5 swollen macrophages containing many phagocytosed cells were very rarely seen.

By E14.5 macrophages were present in higher numbers both in the kidney and the surrounding loose connective tissue (Fig. 3.7 A). Many macrophages were in close apposition to the developing pre-nephric epithelial aggregates, usually in the tail region of “comma-shaped” bodies where resin histology had previously showed dying cells to be localised.
By E14.5 swollen macrophages containing apoptotic bodies were more commonly found in the metanephric kidney

When the earliest mesenchymal condensations have just started to differentiate into nephrons with immature glomeruli, macrophages were found in and around these epithelial structures (Fig. 3.7 B & C). E14.5 was also the first stage when significant numbers of F4/80 positive cells in the metanephros were noticeably swollen with phagocytosed dead cells (Fig. 3.7 C). At these stages of kidney development, unlike the earlier mesonephric stages, the number of macrophages was not obviously greater within kidney tissues than in adjacent loose connective tissue.

*Double labelling experiments show definitively that macrophages engulf apoptotic cells in the metanephric kidney*

Whilst independent apoptosis and macrophage studies allow correlation of the distributions of these two cell populations, direct evidence that macrophages engulf dying cells requires double labelling experiments. Frozen sections were cut of a number of E14.5 metanephric kidneys and double-stained with 7-AAD to reveal apoptotic bodies and F4/80 for detection of macrophages. These studies reveal brightly stained apoptotic bodies almost always enveloped in an F4/80 positive plasma membrane, suggesting that the majority, if not all, of cell death at these stages is cleared away by haematopoietically-derived macrophages (Fig. 3.7 D). Occasionally single apoptotic nuclei were seen in the interstitial mesenchyme that had not yet been engulfed and similarly there were a few macrophages without obvious apoptotic bodies within them.
Figure 3.1: Scanning electron micrographs of the mesonephros and metanephros in the developing mouse embryo.

A  A low magnification of the E12.5 embryo dissected from the ventral surface to give an overview of the regressing mesonephros (boxed), and its spatial relationship to the forelimb and hindlimb.

B  High magnification of the boxed area in A illustrating the mesonephros (M) adjacent to the indifferent gonad (g) sitting lateral to the metanephros (K).

C  High magnification of the E13.5 embryo dissected from the dorsal surface showing the metanephric kidney (K) sitting medial to the mesonephros (M).

D  A low magnification of the E14.5 embryo dissected anteriorly to illustrate the metanephric kidney (K) with the adrenal glands (a) superiorly and the testis (t) inferiorto laterally.

Scale bars: A = 500µm, B & C = 200µm, D = 1mm.
Figure 3.1

dorsal view
Figure 3. 2: Resin histology and transmission electron microscopy of the mesonephros at E11.5.

A A low magnification longitudinal section (LS) of a resin section through the E11.5 mesonephros. The arrowheads indicate regressing mesonephric tubules.

B High magnification detail of tubules in A illustrating a cluster of apoptotic bodies probably enveloped within a macrophage (arrow) adjacent to a tubule.

C A transmission electron micrograph illustrating a “typical” macrophage within the mesonephros; characteristically containing numerous darkly staining apoptotic bodies.

Scale bars: A = 100μm, B = 15μm, C = 1μm.
Figure 3.2
Figure 3. Resin histology of the developing metanephros.

A  A low magnification view of the early metanephric kidney at E12.5, just after the ureteric bud has first branched within the metanephric mesenchyme.

B  Detail from A revealing small numbers of clustered dying cells (arrows) in the metanephric mesenchyme.

C  A low magnification view of the E14.5 metanephric kidney showing early developing nephrons at its periphery.

D  A high magnification view of the nephrogenic region in the E14.5 kidney. Occasional condensed apoptotic nuclei (arrow) can be seen in the tail regions of “comma-shaped” bodies.

Scale bars: A & C = 100µm, B = 20µm, D = 25µm.
Figure 3.3

A. E12.5

B. B

C. E14.5

D. D
Figure 3. 4: Nuclear staining of apoptotic cells in the tails of developing nephrons.

A Optical section through a wholemount E13.5 metanephric kidney preparation, stained with the nuclear dye 7-AAD (red) to reveal condensed apoptotic nuclei (arrows) in the tail of an “S-shaped” body. The green counter-stain is filamentous actin labelled with FITC-phalloidin.

B A frozen section through the E14.5 kidney stained with the Apoptag kit to reveal an apoptotic cell (bright white) in the tail of a “comma-shaped” body, by labelling the numerous cut ends of DNA formed as a result of apoptosis.

Scale bars: A = 10µm, B = 5µm.
Figure 3.4
Figure 3. 5: Resin histology and transmission electron microscopy of the metanephric kidney.

A A low magnification view of the E16.5 kidney with a well developed peripheral nephrogenic zone.

B A high magnification view of the nephrogenic zone at E16.5 showing individual apoptotic nuclei (small arrows) and a phagocytic cell laden with a number of engulfed cells (large arrow).

C A transmission electron micrograph of a typical macrophage in the less dense mesenchyme adjacent to a developing tubule in the E15.5 metanephric kidney (arrows delineate the margins of the macrophage).

D Another macrophage containing a number of apoptotic bodies, this time closely apposed to surrounding mesenchymal cells (arrows delineate the margins of the macrophage).

Scale bars: A = 200μm, B = 25μm, C = 4μm, D = 2μm.
Figure 3.5
Figure 3. 6: F4/80 immunohistochemistry in the mesonephros and early metanephros.

A A low magnification LS view of the E11.5 mouse embryo showing numerous F4/80-stained (brown) macrophages in the mesonephric region; arrowheads indicate regressing mesonephric tubules.

B Detail from A to reveal macrophages adjacent to mesonephric tubules (arrows) and in the surrounding mesenchyme.

C A low magnification view of the developing metanephric kidney at E12.5 after the ureteric bud has branched just once within the condensed metanephric mesenchyme. Numerous mitotic figures are seen in the epithelium (arrows), but only small numbers of the F4/80-stained macrophages are apparent, and all of these are in the mesenchyme.

D Higher magnification of C shows macrophages (arrow) in the condensed metanephric mesenchyme.

Scale bars: A & C = 50μm, B = 20μm, D = 10μm.
Figure 3.6
Figure 3. 7: F4/80 immunohistochemistry and 7-AAD labelling of macrophages and dead cells in the metanephros.

A A low magnification view of the E14.5 metanephric kidney showing many macrophages throughout the developing kidney tissue.

B A high magnification view of the nephrogenic region showing F4/80 positive macrophages in the mesenchyme and adjacent to developing tubules. The arrow indicates a macrophage in association with a developing glomerulus (g).

C A high magnification view of the nephrogenic zone of the E15.5 kidney showing numerous macrophages (arrows) surrounding developing nephrons and glomeruli (g). Some of the macrophages are swollen after engulfment of apoptotic cells; others appear elongated as though moving into or out of the nephrogenic zone.

D Double-stained section of the nephrogenic region of the E14.5 kidney labelled with the nuclear dye 7-AAD (red) and F4/80 (green) to visualise macrophages. The arrows indicate the two apoptotic bodies within the plasma membrane of an F4/80-positive macrophage.

Scale bars: A = 100μm, B = 50μm, C = 20μm, D = 10μm.
Discussion

At all of the stages studied, wherever there was programmed cell death in the developing kidney there were macrophages also present. This was found to be the case in both the mesonephros and the metanephros (at least from E14.5), and the macrophages that were present were active and capable of phagocytosing cellular debris.

Two groups have recently investigated the presence of apoptosis in the developing metanephric kidney and both show evidence suggesting that the kidney must now be included on a growing list of developing organs where significant programmed cell death occurs and may be playing a key role in tissue patterning and differentiation (Koseki et al., 1992; Coles et al., 1993). Both groups also show that this death may be due to limiting quantities of a trophic or survival factor. A good candidate for this survival factor is epidermal growth factor (EGF), since a proportion of the cells destined to die can be rescued by exogenous EGF (Koseki et al., 1992; Coles et al., 1993). The precise function of such extensive developmental cell death during normal kidney development is not yet clear but it has been suggested that it may assist correct wiring of the various epithelial and mesenchymal cell types during nephrogenesis (Coles et al., 1993). Whatever the function of cell death in the developing kidney, it is impressive that such large amounts of cell debris can be cleared away so efficiently.

In the embryo where there is "catastrophic" cell death, for example as found in the developing limb, it was previously believed that the cells responsible for engulfing the dead cells were simply non-specialists, derived from neighbouring fibroblastic cells (Ballard and Holt, 1968; Garcia-Martinez et al., 1993). However, more recent studies using the F4/80 antibody have shown that the majority of the phagocytes in the developing mouse footplate are specialist macrophages, and that they are recruited at, or shortly after, the initiation of cell death in the interdigital mesenchyme (Hopkinson-
Woolley et al., 1994). This situation was confirmed in the mesonephros where there is also “catastrophic” cell death.

It is possible that the scenario is different in organ systems where the cell death is not “catastrophic”, but is "trickle-like" as in the metanephric kidney. Here it can be argued that where cell death is spread out over a long period, then less efficient, non-specialist phagocytes are capable of clearance. However, it appears that even when the cell death is "trickle-like", as in the metanephros, specialist macrophages are the major “players” in the clearance of apoptosis. The presence of swollen macrophages and the double-staining 7-AAD/F4/80 data showed definitively that the macrophages found here had been actively phagocytosing dying cells. Since the odd apoptotic cell was found free in the tissue, outside of F4/80 positive cells, patrolling macrophages cannot be said to be the only cells responsible for clearing away all programmed cell death in the developing kidney.

In other systems non-specialist cells are capable of phagocytosis. For example, it is well established that endothelial cells lining embryonic blood vessels are phagocytic (Latker et al., 1986), and tissue culture studies show that other non-professional phagocytes including fibroblasts, epithelial cells and tumour cells are all capable of phagocytosing apoptotic cells (Wyllie et al., 1980; Duvall and Wyllie, 1986; Hall et al., 1990;). Moreover, in the adult kidney it has been shown that glomerular mesangial cells, which are not from the monocyte lineage will engulf spent neutrophils at sites of inflammation (Savill et al., 1992).

What might be the signals that lead to macrophage recognition of dying cells in the embryonic kidney? Whilst it is possible that macrophages could be attracted to regions of programmed cell death by medium-range chemotactic signals, it is also likely that recruitment and recognition are driven by short-range or cell-to-cell contact-mediated interactions dependent on macrophages constantly patrolling the tissue. There are
various receptors on the macrophage surface, for example the scavenger receptor membrane glycoprotein (Hughes et al., 1995), which might allow it to recognize the "edible" status of apoptotic cells.

It appears that phosphatidylserine (PS), a key phospholipid of the plasma membrane, that normally faces internally in a cell, becomes exposed as soon as the cell death programme is initiated and is detected before the characteristic morphological signs of cell death are seen (van den Eijnde et al., 1997a). Annexin V, a Calcium ion dependent phosphatidylserine binding protein, has been developed as a method of detecting these apoptotic cells early both in vitro and in vivo (van den Eijnde et al., 1997b). Perhaps macrophages are able to identify the presence of PS on the outside of a cell and this explains why macrophages have been found to contain cells which still appear morphologically healthy (Savill et al., 1989).

In the nematode worm Caenorhabditis elegans, whilst there is no evidence for specialist macrophage-like cells, a series of genes have been identified which are necessary for successful phagocytosis and clearance of apoptotic cells by neighbours (Ellis et al., 1991). Vertebrate homologues of these genes will probably encode at least some of the proteins used by specialist macrophages to recognize, bind to and engulf dying cells in the developing kidney and elsewhere.

The data from the mouse is not the first study to suggest that macrophages play several important phagocytic roles during embryogenesis. It appears that the majority of developmental cell death in Drosophila is cleared away by specialist phagocytes called hemocytes, leaving only a small minority of cells to be engulfed by non-specialist epidermal cells (Tepass et al., 1994). Of direct relevance to this study, Rotello and colleagues (1994), using new antibodies raised against chick phagocytic cells show apoptotic cells in the avian mesonephros being engulfed by what are almost certainly macrophages, and an earlier in vitro study by De Felici and colleagues (1986) showed
that macrophages are indeed present in the murine urogenital ridge from early stages. At later stages of development it has been shown that there is recruitment of F4/80 monocytes into the retina (Hume et al., 1983) and brain (Perry et al., 1985) in response to programmed cell death of neuronal cells.

Recent studies by Lang and colleagues (1993; 1994) in the developing eye, suggest that macrophages are not only responsible for clearing away cell death, but in certain tissues they actually trigger cell death. Lang and Bishop (1993) expressed diphtheria toxin under a promoter specific to a small population of macrophages including the hyalocytes of the developing eye. In resulting transgenic mice they found that hyalocytes were killed and that two collections of capillary blood vessels which are normally transient in the embryonic eye failed to undergo programmed cell death suggesting that hyalocytes were necessary to elicit their cell death. Analogous macrophage depletion experiments in the kidney, either genetic or chemical killing of macrophages, are needed to fully dissect any other roles besides simple clearance that macrophages might be playing during the remodelling phases of kidney development (see Chapter Four).
CHAPTER FOUR

In Vitro Examination of the Metanephric Kidney

Introduction

For many years the metanephric kidney has been cultured and manipulated in vitro in order to dissect out the mechanisms of kidney differentiation and morphogenesis (Saxén, 1987). In this chapter an in vitro kidney culture model is used to extend the observations made in the previous chapter, and to test the function of macrophages during kidney development. Morphogenesis of kidneys cultured in this way is described, illustrating the extensive ureteric bud branching and the formation of tubules during the culture period. Resin histology reveals that mesenchymal-epithelial transitions occur, and wholemount F4/80 staining reveals the presence of macrophages in these cultures, just as in vivo. This system provides an excellent model to manipulate the number of macrophages during the developmental process, and to see whether, by depleting them, the normal pattern of branching and tubule formation is affected.

Kidney organ cultures are an accepted in vitro model for studying kidney development, and were first used as a technique in this regard by Grobstein in the 1950s (Grobstein, 1956). This procedure was later somewhat modified by Saxén and colleagues during the 1960s and 1970s (Saxén et al., 1968; Saxén and Lehtonen, 1978; reviewed in Saxén, 1987). The kidney rudiment, which consists of the ureteric bud and surrounding mesenchyme, is dissected out soon after these two tissues have met in vivo, and is able
to adjust to its new environment. The kidney rudiment develops, presumably in response to the same reciprocal interactions which occur, between these two tissues *in vivo*.

Similar organ culture techniques were utilised by Koseki and colleagues (1992) when they showed that programmed cell death was a naturally occurring phenomenon in the developing metanephric kidney. Even when the metanephric mesenchyme was induced by the ureteric bud in this assay, several cells surrounding each newly formed epithelial nephron exhibited classic signs of apoptosis; by exogenously adding Epidermal Growth Factor (EGF) they were able to rescue some of this cell death (Koseki *et al.*, 1992).

The previous chapter established that most of the naturally occurring cell death in the developing kidney appears to be cleared by macrophages, but these cells may also have other roles to play during kidney morphogenesis besides simply being phagocytes. To test out this possibility it was necessary to consider several strategies to deplete macrophages in organ culture, one being to kill them with toxic liposomes. Liposomes, which are microspheres of one or more lipid bilayers forming aqueous compartments, were pioneered by Gregoriadis (1971) as a means for specific drug delivery. If liposomes containing radioactive tags were intravenously administered to rats, they were removed from the circulation within minutes, and much of the radioactivity was sequestered in the liver, because the liposomes were engulfed by Kupffer cells, the resident macrophages of the liver (Gregoriadis and Ryman, 1972).

Van Rooijen and colleagues (1984, 1985, 1990) showed in both rats and mice, that liposomes were engulfed by macrophages after intravenous injection, and if the liposomes were loaded with a toxin, for example dichloromethylene diphosphonate, then engulfing macrophages were killed, with very little related non-specific cell death. The liposomes are broken down by lysosomal complexes in the macrophages causing release of the toxin within the confines of the engulfing cell. Van Rooijen (1989) found that
large multilamellar liposomes were more efficient at killing macrophages since they were able to "entrapping" the drug within separate concentric aqueous compartments. Most recently the liposome-mediated macrophage elimination technique has been used to test the role of macrophages in the developing rat eye (Diez-Roux and Lang, 1997), where previous genetic studies have suggested macrophages may be involved in cell killing as well as clearance of cell death (Lang and Bishop, 1993). This study successfully depleted the eye macrophages using toxic liposomes and, as in the genetic study, the pupillary membrane appeared not to die. This was shown to be a macrophage specific effect because if macrophage numbers were replenished three days after the toxic liposome killing, then the pupillary membrane began to regress (Diez-Roux and Lang, 1997).
Materials and Methods

An outbred strain of Albino mouse, strain CD1 (Olac) was used. Gestational age was calculated on the assumption that conception had occurred at midnight preceding the morning a vaginal plug was seen. Time-mated females, E11.5, were killed by cervical dislocation, the uterus was removed, and the embryos were carefully dissected out in phosphate buffered saline (PBS; Oxoid) and staged according to Theiller (1989) and Martin (1990), using the shape of the forelimb and hindlimb buds as developmental indicators.

Organ Cultures

Under a dissecting microscope in a tissue culture flow hood, kidney rudiments, which are cylindrical in shape and of approximately 400μm in length, were carefully dissected from embryos at 11.5 days of gestation (Fig. 4.1). In a petri dish containing Leibovitz’s L-15 medium (Gibco) the embryo was first transected with small iridectomy scissors midway between the forelimbs and hindlimbs (incision 1); the second cut, again made using small iridectomy scissors, was just above the hindlimbs (incision 2); most of the tissue anterior to the position of the kidneys was teased away at this stage; the third cut, to remove the developing somites and neural tube, was performed using a pair of 0.6 hypodermic needles on the ends of 1ml syringes (incision 3). The remaining piece of tissue was then placed on its ventral surface exposing the metanephric kidneys lying in close apposition with the mesonephroi (Fig. 4.1 D). At this stage the ureteric bud has branched from the Wolffian duct and just entered the condensed metanephric mesenchyme. It is essential to have both components for the culturing procedure. The metanephric kidney rudiment was gently teased away from the surrounding tissue using
watchmakers forceps and a fine bore needle, and placed on a 0.8cm pre-dampened millicell tissue insert (Millipore) in a four-well culture dish (Gibco). The organ culture was allowed to flatten down for between 30 minutes to one hour, before 200μl of culture medium was added to the well.

The culture medium used was Dulbecco’s Modified Eagle Medium/Nutrient Mix F12 (1:1) with L-Glutamine, 15mM HEPES (DMEM/F12; Gibco; see Appendix) containing 10% foetal calf serum (FCS; Sigma) and 1% penicillin and streptomycin (Sigma). The organ culture absorbs its nutrients from the medium through the filter. The culture dish was put into an incubator at 37°C with 5% CO₂ and the medium was changed daily. The organ cultures were viewed every day using a Leica Diaplan microscope, and each one was captured digitally via a video camera and the Adobe photoshop programme on a Power Macintosh Computer.

The method was based on the work of Grobstein (1955; 1956) and Adrian Woolf, Institute of Child Health, London.
Figure 4.1: Diagram of how E11.5 kidney rudiments were dissected out of the embryo in preparation for organ culture.

This diagram illustrates the position of the kidney rudiment, which consists of the metanephric blastema and the sprouting ureteric bud, relative to the hindlimb bud at E11.5 (A). The kidney rudiment at this stage is the same colour as the rest of the embryo, so it is important to establish some definitive landmarks. As long as the two hindlimbs are attached, the kidneys will be preserved. The liver and gut structures are teased away between B and C, before the somites are removed. The ureteric buds sprout from the mesonephroi, which are large structures at this stage, and these can be used to help locate the position of the kidneys more specifically (D). Once the rudiments have been dissected free from the rest of the tissue, each one can then be transferred to the tissue culture insert with a pipette tip (E).

This diagram is not drawn to scale.
Figure 4.1

A

incision 1

forelimb

hindlimb

metanephric blastema

ureteric bud

B

incision 2

incision 3

D

dorsal view

E

kidney rudiment
Resin Histology

The kidney rudiments were harvested at daily intervals. The culture medium was removed and 0.5ml of ice-cold half strength Karnovsky fixative (Karnovsky, 1965) was dripped on to each culture and left at 4°C overnight. The organ cultures were rinsed in 0.1M cacodylate buffer, and during this time they were removed from their filters and placed in soda vials. The cultures were then post-fixed in 1% osmium tetroxide (Sigma) in 0.1M cacodylate buffer for 45 minutes at 4°C, and further rinsed in 0.1M cacodylate buffer. They were dehydrated through graded alcohols, 10 minutes in each, and rinsed up to four times in AnalaR Ethanol before being rinsed in propylene oxide (BDH) and washed four times. The cultures were then put in a 50:50 mixture of propylene oxide and Araldite resin (see Appendix) for 45 minutes. They were then transferred to neat, fresh Araldite resin and the soda vials containing the cultures were slowly rotated overnight at room temperature. The Araldite resin was changed the following day and returned to the rotator for five hours. The cultures were finally orientated in small rubber moulds, embedded in fresh Araldite resin and baked in the oven at 65°C.

The blocks were cut, mounted on resin stubs with superglue and left to set for 24 hours, before the block was carefully trimmed using a one-sided razor blade. It was important to keep the amount of resin to be cut with the glass knife to a minimum, to ensure that only the sharpest part of the cutting edge was used. Sections of 2 - 5μm were cut and these were water dried onto poly-L-lysine (1:100; Sigma) coated slides. These sections were then stained with Toluidine Blue, rinsed with distilled water and allowed to dry before mounting in XAM (BDH) under a coverslip and viewed using the Leica Diaplan. The images were photographed on tungsten slide film (64T; Kodak).
F4/80 immunohistochemistry

The organ cultures were fixed in their four-well culture dish in 4% paraformaldehyde overnight at 4°C. They were rinsed several times in PBS before being incubated with 20μl of 8.5μg/ml F4/80 per well, for three hours at room temperature on a shaking platform. The cultures were then rinsed in PBS and incubated with FITC-tagged mouse adsorbed rabbit anti-rat IgG (10μg/ml; Vector Laboratories) for two hours. The organ cultures were rinsed in PBS before being mounted in Citifluor (UKC) in a custom-made well, manufactured by cutting a window in two layers of electrical tape on a microscope slide. A coverslip was then gently placed on the top of the organ culture to carefully flatten it. The coverslip was sealed with nail varnish. The organ cultures were viewed and photographed using a Leica TCS4D Confocal Laser Scanning Microscope (CLSM).

F4/80 monoclonal antibody, at a concentration of 50μg/ml, was also added directly to the organ cultures to ensure that macrophages were present in the core of the culture throughout the experiment. The cultures were subsequently fixed as above, rinsed and then exposed only to the secondary antibody (10μg/ml; FITC-tagged mouse adsorbed rabbit anti-rat IgG) for two hours before being mounted, viewed and photographed as previously described.

Double labelling of organ cultures for apoptosis and macrophages

To visualise both apoptotic cells and macrophages in the same culture a double immunofluorescent study was required. Specimens were fixed in 4% paraformaldehyde at 4°C overnight and incubated with F4/80 and then with secondary antibody as described above. The cultures were then incubated with 5μg/ml 7-AAD (Molecular Probes) in PBS for 30 minutes at room temperature. The cultures were rinsed several
times in PBS, and mounted as before, prior to viewing using the FITC and TRITC channels of the CLSM.

**Liposomes**

These were made by Graciana Diez-Roux (Skirball Institute, New York) according to established procedures developed by Van Rooijen (1989b). For full details see Appendix. The liposome-dependent drug dichloromethylene bis-phosphonate (clodronate) from Boehringer Mannheim GMBH was used.

10µl of either PBS-liposomes or toxic-liposomes was dripped directly on to the organ culture at 24 hours. This was washed off with PBS the following day and the cultures were left to develop for a further 36 hours. They were then fixed and processed as above.
Results

Normal development of E11 kidney organ cultures

Light microscopy of kidney organ cultures explanted at E11.5 and photographed at daily intervals, revealed the developmental branching of the ureteric bud within the metanephric mesenchyme, and the formation of condensations at its branching tips (Fig. 4.2). Growth of the organ culture increased each day, in a manner fully reproducible across all specimens making it possible to accurately measure the effects of addition of any reagent to the culture medium (Fig. 4.3 A). The number of tubules also increased at a consistent rate, though at the later stages it became more difficult to distinguish individual condensations (Fig. 4.3 B).

Resin histology showed clearly the gradual branching of the ureteric bud in these kidney rudiments, which does not appear to be affected by the change in environment. The development of in vivo and in vitro kidneys was comparable throughout the period of the culture. After 48 hours in culture, tubules could be clearly seen, and the histology was comparable to that of kidneys from E13.5 mouse embryos. The mesenchymal-epithelial nephric conversion still took place, with tubules appearing just as those in vivo. After 72 to 96 hours in culture it became difficult to distinguish the different parts of the nephron, and the overall shape of the culture was much more rounded than that of its in vivo counterpart (Fig. 4.4 A).

Again just as in vivo, it was possible to observe the odd dead cell in the metanephric mesenchyme after 24 hours of culture. Apoptotic nuclei become more numerous as development proceeds, and by 96 hours numerous apoptotic bodies were found within macrophages in close apposition with the mesenchymal condensations (Fig. 4.4 B & C).
Macrophages labelled with the F4/80 antibody were clearly visible in kidney organ cultures, whether primary antibody was applied during the culture period or after fixation. There was a large increase in the numbers of macrophages observed in individual organ cultures during the culture period. When the kidney rudiment was dissected out of the embryo at E11, approximately 25 macrophages were counted per rudiment (Fig. 4.5 A), but by 96 hours of culture this had increased to between 100 and 150 (Fig. 4.5 B). Clearly these additional macrophages have not derived from a systemic source as is possible in vivo. Either they have derived from proliferation of the initially observed cells or else non-staining macrophage precursors have differentiated into F4/80 expressing cells during the period of culture. Double staining with F4/80 and 7-AAD revealed numerous macrophages containing several apoptotic bodies (Fig. 4.5 C).

Effect of liposome treatment

In the small number of experiments performed it was only possible to analyse at a gross level the morphological effects of this macrophage loss. Wholemount light microscopy revealed that the cultures treated with PBS-liposomes looked just like control cultures, while those treated with toxic-liposomes clearly suffered some effect which became more apparent as the time in culture progressed (Fig. 4.6 Part 1 & 2).

The overall growth of the kidney explants was not affected in cultures treated with PBS-liposomes, but it was significantly reduced in those treated with toxic-liposomes (Fig. 4.7 A). The number of tubules that developed was also dramatically reduced in the toxic-liposome treated cultures, while in the PBS-liposome cultures there was no significant difference (Fig. 4.7 B).
The extent of growth and tubule development of the kidney rudiments exposed to toxic-liposomes appears to slow down and even stop. The presence of the toxic-liposomes and the subsequent death of the macrophages interferes with the normal pattern of development in this system. Resin histology revealed normal tubule structure in PBS-liposome treated cultures, with macrophages seen in the mesenchyme and adjacent to developing tubules (Fig 4.8 A & C). The small number of tubules observed in toxic-liposome treated cultures were less differentiated, with none going beyond the “comma-shaped” body stage. Dead cells were located in the surrounding mesenchyme, but they were free in the tissue and not encased within phagocytic cells as in the control organ cultures (Fig. 4.8 B & D).

To confirm that macrophages had in fact been removed from the cultures treated with toxic-liposomes and not from those exposed to PBS-liposomes, a sample of cultures were exposed to the F4/80 antibody. The F4/80 immunohistochemistry revealed that there were still macrophages present in the control and PBS-liposome treated cultures at 96 hours, but no staining was apparent in the liposome-treated organ cultures (Fig. 4.9). The result of adding toxic liposomes was to remove macrophages from the organ culture, because the macrophages engulf and digest the liposomes which causes the drug inside to be released which then kills the macrophages.
Figure 4.2: Light microscopy of the E11.5 kidney rudiment as it develops in culture over a period of five days.

An overview of the development of the kidney rudiment as photographed at 24 hour intervals from the onset of culture at E11.5. The overall size of the culture gradually increases, as does the number of tubules that can be seen.

Scale bar = 200μm
Figure 4.2

E11.5, 0 hours  24 hours
A       B
48 hours  72 hours
C       D
96 hours
E
Figure 4. 3: Graphs of normal kidney rudiment growth and tubule development over the culture period.

A the area of the growing kidney rudiment over the five days in culture.

B the number of tubules that develop in the organ culture during the five day culture period.
Figure 4.3 A

Figure 4.3 B
Figure 4. Resin histology of an E11.5 kidney rudiment after 96 hours in culture.

A A low magnification view of a section through the kidney rudiment after 96 hours in culture showing normal epithelial tubule development.

B A high magnification view of the periphery of a kidney organ culture (similar to that shown in A), showing clustered apoptotic bodies (arrows) in association with condensing mesenchyme.

C High magnification view of the central region of a 96 hour kidney organ culture, again showing clustered apoptotic cells presumably contained within a macrophage (arrow), adjacent to a developing nephron.

Scale bars: A = 200μm; B & C = 50μm
Figure 4.5: Wholemount F4/80 immunohistochemistry of an E11.5 kidney rudiment at the start and at the end of the culture period.

A  A wholemount E11.5 kidney rudiment fixed and stained with F4/80, immediately upon removal from the embryo. Already there are macrophages (green) found in the rudiment at this early stage of development.

B  An optical section through the kidney rudiment after 96 hours of culture, there are numerous macrophages (green) throughout the tissue.

C  96 hours kidney rudiment similar to that shown in B, but double stained with F4/80 (green) and 7-AAD (red) revealing that the macrophages within the organ culture do indeed contain condensed apoptotic nuclei.

Scale bars: A & C = 100µm; B = 150µm
Figure 4.5

A. E11.5, 0 hours

B. 96 hours

C. 96 hours
Figure 4. 6 (Part 1 and 2): Light microscopy of the kidney rudiments treated with liposomes at 24 hours.

Development of kidney organ cultures explanted at E11.5, and photographed at 24 hour intervals. Cultures were incubated in the presence of toxic liposomes (to kill macrophages), PBS-liposomes as controls or with no liposomes. Even by 48 hours (Part 1) the toxic-liposome treated cultures were beginning to show signs of less tubulogenesis, and development was severely awry by 72 and 96 hours (Part 2).

Scale bar = 200μm
Figure 4.6 (Part 1)

Control   PBS-Liposomes   Toxic-Liposomes

0 hours

24 hours

48 hours
Figure 4.6 (Part 2)

Control  PBS-Liposomes  Toxic-Liposomes

72 hours

96 hours
Figure 4. 7 A: Graph of kidney rudiment growth of organ cultures treated with liposomes at 24 hours.

Daily areas occupied by the liposome-treated organ cultures over the five days of culture, plotted to reveal differences in growth for the three treatment regimes. The toxic-liposome treated cultures appear to stop growing, by comparison to the two control culture categories.
Figure 4. 7 B: Graph of tubules that formed in organ cultures treated with liposomes at 24 hours.

Tubule numbers in the liposome-treated organ cultures over five days of culture, plotted to show differences in branching morphogenesis between the three regimes. The toxic-liposome treated cultures show only a slight increase in tubule number over the period of culture.
Figure 4. 8: Resin histology of organ cultures treated with PBS-liposomes or toxic-liposomes.

A  A low magnification view of an organ culture that has been treated with PBS-liposomes (control), at the end of the culture period (96 hours), to illustrate normal formation of epithelial tubular structures.

B  A low magnification view of an organ culture that has been treated with toxic-liposomes, at the end of the culture period, showing abnormal and reduced numbers of tubules by comparison to control (see A).

C  A high magnification view of a PBS-liposome treated organ culture after 96 hours, showing several apoptotic bodies contained within a macrophage (arrow) in the mesenchyme.

D  A high magnification view from B revealing several apoptotic cells apparently not contained within a phagocyte, in the mesenchyme between aggregations.

Scale bars: A & B = 200µm; C & D = 50µm
Figure 4.8

PBS-Liposomes  Toxic-Liposomes

A  B

C  D
Figure 4. 9: F4/80 immunohistochemistry of liposome treated organ cultures at 96 hours.

A A control organ culture after 96 hours showing the presence of macrophages (F4/80 positive cells - green) throughout the entire rudiment.

B A control PBS-liposome treated organ culture, showing macrophages numbers similar to control untreated cultures (see A).

C An equivalent toxic-liposome treated organ culture, revealing no F4/80 positive cells, thus confirming that macrophages are successfully depleted by this treatment.

Scale bars = 100μm
Figure 4.9

Control

A

PBS-Liposomes

B

Toxic-Liposomes

C
Discussion

One of the benefits of studying kidney development is that this organ can be cultured *in vitro* and many aspects of normal kidney morphogenesis go ahead just as in organs left *in vivo*. Once *in vitro*, these developmental processes are amenable to various surgical and chemical manipulations, which allows an examination of the mechanisms of kidney development. It is possible to test the role played by macrophages in kidney development using this system by simply depleting the macrophages with toxic liposomes, which specifically poisons them.

The answer was “clean” - if the macrophages are killed, then kidney growth and patterning, specifically tubular morphogenesis, is severely disrupted. This suggests that macrophages may not simply be employed to clear up dying cells in the developing kidney, but may also have more proactive roles, perhaps supplying growth factor cues that guide tubule branching. But in an experiment of this type there are clearly other possible explanations for the result which might not be directly linked to the role played by macrophages. It is not possible to rule out the fact that dying macrophages may be toxic to the surrounding tissues or that these large cell corpses could be mechanically disruptive as metanephric mesenchymal cells are condensing or epithelial cells are attempting to branch.

However, there are predecents for macrophages doing more than just clearing up cell debris. A study by Diez-Roux and Lang (1997) used toxic liposomes to see whether rat eye development was affected by the depletion of macrophages. They found that endothelial cells and functional capillaries persisted in areas where programmed cell death would usually occur in the normal pattern of development. They concluded that this provided direct evidence that macrophages play a role in inducing apoptosis as well as being active scavengers. This complemented previous work by Lang and Bishop
(1993), which showed that if a subset of macrophages, found specifically in the eye, were genetically depleted by running a diphtheria toxin under their promoter then the same phenotype was seen.

To confirm that these macrophage killing experiments gave specific results about the roles played by these cells during kidney development, alternative methods of depletion need to be examined. Another method of depleting macrophages in this system would be to use an antibody/complement mediated killing strategy (Stern and Canning, 1990). This method requires an interaction between the primary antibody and the appropriate complement which results in the labelled cells being killed. Certainly, a possible primary antibody/epitope interaction that might be suitable for such a strategy would utilise the F4/80 antibody, since I have shown that it binds to live macrophages. It would be interesting to see whether the same affect was achieved when using the antibody/complement technique. Alternatively, a genetic model could be used to address this question. A mouse is available where the macrophages have been eliminated by disruption of the gene for the PU.1 transcription factor, which is expressed exclusively by cells of the haematopoietic lineage (McKercher et al, 1996), and my work on apoptosis clearance in the footplate of these mice is described in Chapter Six.
CHAPTER FIVE

Investigation of Macrophages in Normal Limb Development

Introduction

This chapter extends the observations reported by Hopkinson-Woolley and colleagues (1994) which suggested a key role for macrophages in the clearance of the programmed cell death that occurs during the sculpting of the developing footplate. Simple histology and scanning electron microscopy (SEM) data reveal what the limbs look like, and illustrate the shape changes that they undergo during the remodelling phase. Wholemount preparations of limb buds at various stages of development, stained with the F4/80 antibody, reveal the numbers and distribution of macrophages involved in the clearance process, whilst transmission electron microscopy (TEM) studies capture the process of clearance in situ. Finally, several strategies for tracking the macrophages in vivo are reported, keeping the embryo alive either in roller culture or by open uterus surgery.

As established in the Introductory Chapter, there is a fairly clear understanding of how the developing vertebrate limb is patterned in each of its three axes. In essence, patterning in the proximo-distal axis is dependent on signals, probably fibroblast growth factors (FGFs), secreted by the apical ectodermal ridge (AER) into the underlying mesenchyme of the progress zone (Cohn et al., 1995); polarity along the antero-posterior
axis is regulated by a morphogen gradient, probably Sonic hedgehog (Shh) and subsequently bone morphogenetic proteins (BMPs), released by the posteriorly located zone of polarising activity (ZPA) cells (Shh, Riddle et al., 1993; BMPs, Francis et al., 1994); and finally the dorso-ventral axis is polarised by short range signals, including Wnt-7a, emitted by the dorsal limb bud ectoderm (Parr and McMahon, 1995).

The signals that specify the basic pattern in each of the limb's three axes are clearly not the whole story. Significant remodelling and growth is super-imposed upon this crude outline to give the eventual shape of the new limb. A major mechanism for remodelling and fine-tuning the limb shape is sculpting with localised programmed cell death. The simplest and most dramatic remodelling by cell death is that which occurs in the footplate to give separate digits (Garcia-Martinez et al., 1993; Zakeri et al., 1994). The synchronous death of hundreds of cells in each interdigit is "catastrophic" and is easily visualised in histological sections as large accumulations of dense and darkly staining apoptotic bodies. This death is classified as apoptosis, since DNA from footplate tissue at the time of interdigital remodelling presents the classic laddering effect of multiples of 200 base pairs (Garcia-Martinez et al., 1993).

A series of experiments by Hurle and colleagues, involving the removal of the dorsal ectoderm overlying the interdigit mesenchyme or the adjacent AER epithelium, just prior to the onset of cell death, resulted in mesenchymal cells not dying, but instead, by default, adopting a chondrogenic cell fate. These experiments suggest that the death "trigger" somehow involves a signal from the overlying ectoderm (Hurle and Ganán, 1986; Hurle et al., 1989). When these experiments were performed on duck embryos rather than chicks, substantially less cartilage was induced, as might be expected, given that fewer cells normally die in the duck foot than in the chick (Macias et al., 1992).

The above experiments point to a relationship between interdigital programmed cell death and signals released by the epithelium. A candidate family of growth factors implicated
as footplate death signals are the FGFs (Macias et al., 1996). FGF-2 is expressed throughout the limb mesenchyme (Savage et al., 1993), while FGF-4 is expressed only by posterior AER (Niswander and Martin, 1992). However, since FGF signals are not restricted to the interdigital regions and receptors for the FGFs are also expressed ubiquitously throughout the limb mesenchyme (Orr-Urtreger et al., 1991), it seems unlikely that this family of FGFs can be the whole story.

Other molecules that may activate the death programme in signalling interdigital cells, are members of the Transforming Growth Factor-beta (TGF-β) superfamily, in particular BMPs 2, 4 and 7 which are all expressed in the interdigital tissue at or around the time of programmed cell death (Francis et al., 1994; Francis-West et al., 1995). Evidence that these signals may interact to initiate death comes from bead grafting experiments: if beads soaked in either TGF-βs (1 or 2) or FGFs (2 or 4) are inserted into chick interdigital zones then BMP-4 expression is downregulated and programmed cell death is inhibited, leading to ectopic cartilage and, in extreme cases, extra digit formation (Ganân et al., 1996). Conversely, grafting of beads soaked in BMP-4 results in an increase in programmed cell death and more extreme digit separation (Ganân et al., 1996).

Subsequently, several studies investigating the molecular nature of the death cues have shown that blocking mesenchymal responsiveness to BMP signals by transfection with viruses encoding the dominant negative type-1 BMP receptor (dnBMPR-1B) leads to less death and more interdigital webbing in treated chick footplates (Zou and Niswander, 1996).

Previous studies on the remodelling limb in avian and mammalian embryos not only describe the distribution of programmed cell death, but speculate as to how these dying cells might be cleared away in the embryo (Saunders and Fallon, 1967; Ballard and Holt, 1968). In both the chick and the mouse the consensus view is that dying cells are simply engulfed by adjacent mesenchymal neighbours. Saunders (1966) also investigated where the cell debris may eventually be taken, and using basic radioactive tracking experiments
he showed some evidence for the limb phagocytes eventually ending up in the subclavian artery and the aorta.

This chapter illustrates the dramatic change in shape of the limb bud between E13.5 and E14.5, when the footplate is being remodelled, and investigates the cells involved by light and electron microscopy. This data clearly shows the role played by macrophages in the phagocytosis of the cell death. A novel way of labelling macrophages in situ is shown, attempting to track their movements in the embryo after they have cleared the interdigital cell death.
Material and Methods

An outbred strain of Albino mouse, strain CD1 (Olac) was used for these studies. Gestational age was calculated on the assumption that conception had occurred at midnight preceding the morning a vaginal plug was seen. Time-mated females at gestational ages E11.5 - E14.5, were killed by cervical dislocation, the uterus was removed, and the embryos were carefully dissected out and staged according to Theiller (1989) and Martin (1990), using the shape of the forelimb and hindlimb buds as developmental indicators.

Embryos were processed in several ways; for light and electron microscopy, for F4/80 immunohistochemistry and stained with the nuclear marker, 7-amino actinomycin D (7-AAD; Molecular Probes), to allow analysis of macrophage distribution and apoptosis within the developing limb.

In order to track macrophage movements with the tissues, E13.5 embryos were either carefully dissected out of the uterus, injected with various marking reagents and prepared for roller culture, or the mother mouse was anaesthetised and open uterus surgery was performed to gain access to the embryos. In this case footplate injections were made through the yolk sac and the mother mouse sewn up and allowed to recover. The embryos were subsequently harvested at either 12, 18 or 24 hours (see later).

Histology and electron microscopy

Embryos for resin histology, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were first thoroughly rinsed in phosphate buffered saline (PBS; Oxoid) and then fixed in ice-cold half-strength Karnovsky fixative (Karnovsky,
1965) overnight at 4°C. The embryos were rinsed in 0.1M cacodylate buffer, during which time they were either bisected (E11.5 and E12.5) or each hindlimb was dissected away with a small piece of flank from the rest of the body (E13.5 and E14.5). The specimens were post-fixed in 1% osmium tetroxide (Sigma) in 0.1M cacodylate buffer before being dehydrated through a graded ethanol series.

- **Resin histology**

The specimens for resin histology were washed four times in propylene oxide (BDH), infiltrated with a 50:50 mixture of propylene oxide and Araldite resin (see Appendix) for 45 minutes, infiltrated with neat, fresh Araldite mixture and left for 12 hours on a rotator. The Araldite resin was changed, the specimens rotated for a further five hours before being embedded in fresh Araldite resin in a plastic mould and cured at 60°C for 24 hours. These blocks were cut to the appropriate size and mounted on an Araldite chuck with superglue, which was allowed to dry for five hours before sections of 1-5μm were cut. These were floated on distilled water on poly-L-lysine (1:100; Sigma) coated slides and allowed to dry on a hot plate. The sections were stained with Toluidine Blue and mounted under a glass coverslip with XAM (BDH).

- **Transmission Electron Microscopy**

Some of these specimens were also examined by TEM. The block was trimmed down to an absolute minimum, and, using a diamond knife ultrathin resin sections were cut, floated on to water and mounted on copper mesh grids. These were first stained with uranyl acetate for 10 minutes, rinsed with distilled water and allowed to dry, before they were counter-stained with lead citrate for a further 10 minutes and then rinsed again. Viewing was by means of a Jeol 1010 Transmission Electron Microscope.
**Scanning Electron Microscopy**

Specimens for SEM were transferred from AnalaR ethanol (BDH) into AnalaR acetone (BDH) and dried using a Polaron Critical Point Drier, which uses CO₂ as its medium. They were mounted on 10mm cylindrical stubs with electrodag - silver in methyl-isobutyl-ketone (Agar Scientific) and the electrodag allowed to set for 24 hours before the specimen was sputter coated with gold using a SC500 Sputter Coater. The specimens were viewed on a Jeol JSM-5410LV Scanning Electron Microscope.

**F4/80 immunohistochemistry**

**Sections**

In preparation for wax immunohistochemistry, embryos were fixed overnight in ice-cold Bouin's fixative (70% saturated picric acid (aqueous), 25% formalin, 5% glacial acetic acid), and thoroughly rinsed in 70% alcohol before dehydration through graded alcohols, 10 minutes in each. The embryos were bisected (E11.5, E12.5) or the limbs dissected away (E13.5, E14.5) during this dehydration stage. Specimens were transferred to histoclear (National Diagnostics) at room temperature for 20 minutes, histoclear at 57°C for 20 minutes and then to a 50:50 histoclear and Fibrowax (BDH) mix at 57°C for 20 minutes. The specimens were transferred to fresh Fibrowax for 30 minutes, this was changed and left for another 30 minutes before the specimens were embedded in Fibrowax in plastic 7ml square weigh boats. Each specimen was cut out of the wax, a flat topped pyramid was cut, and this block was mounted on a wooden chuck. 8μm sections were cut on a Leica “Supercut” microtome, these were floated on distilled water and allowed to dry down on poly-L-lysine coated slides at 40°C.

The sections were rehydrated, rinsed in PBS and soaked in 0.3% hydrogen peroxide (H₂O₂; BDH) in methanol (BDH) for 40 minutes to block endogenous peroxidase. They
were rinsed again in PBS before rabbit serum (1:100; Vector Laboratories) was applied to the sections for 30 minutes, to block non-specific binding of the antibody. The sections were then incubated with 8.5μg/ml F4/80, a macrophage specific rat anti-mouse monoclonal antibody (Austyn and Gordon, 1981; Morris et al., 1991) for 90 minutes. The sections were washed in PBS and bound antibody was detected using biotinylated mouse adsorbed rabbit anti-rat IgG (5μg/ml; Vector Laboratories) and the avidin-biotin-peroxidase complex (Vectastain ABC Elite kit, Vector Laboratories). To detect the peroxidase activity, a DAB kit (Vector Laboratories) was used, according to the manufacturer’s instructions. Two drops of buffer stock solution were added to 5ml of distilled water and mixed thoroughly, plus four drops of DAB stock solution and mixed again, followed by two drops of hydrogen peroxide solution and mixed. The sections were incubated with this solution for between three and five minutes, then rinsed with tap water, followed by distilled water. The sections were counter-stained with Mayer’s Haemalum (BDH), dehydrated through graded alcohols and mounted in XAM (BDH).

• Wholemounts

Tissue for wholemount immunohistochemistry was fixed in ice-cold 4% paraformaldehyde (BDH) overnight at 4°C. The specimens were rinsed in PBS, permeabilised with 0.3% Triton and blocked with 10% rabbit serum for 30 minutes. They were then incubated with 10μg/ml F4/80 overnight at 4°C on a rocking platform. The specimens were rinsed in PBS, further permeabilised and rinsed again before incubation with a FITC-tagged mouse adsorbed rabbit anti-rat IgG (10μg/ml; Vector Laboratories) overnight, at 4°C and on a shaker. The limbs were rinsed in PBS before being mounted in Citifluor (UKC) in a custom-made well, manufactured by cutting a window in two layers of electrical tape on a microscope slide. A coverslip was then gently placed on the top of the specimen to carefully flatten it, and the coverslip was sealed with nail varnish. The limbs were viewed and photographed using a Leica TCS4D Confocal Laser Scanning Microscope (CLSM).
Double labelling of limbs for apoptosis and macrophages

To visualise both apoptotic cells and macrophages in the same specimens double immunofluorescent studies were performed on wholemounts. Specimens were fixed in 4% paraformaldehyde at 4°C overnight and incubated with F4/80 as described above. Prior to mounting, specimens were further incubated with 10μg/ml 7-AAD (Molecular Probes) in PBS for one hour at room temperature. The specimens were rinsed in PBS and mounted in Citifluor as previously described. Double-staining was revealed using the FITC and TRITC channels of the CLSM.

Labelling interdigital macrophages for subsequent “live tracking”

E13.5 embryos were gently dissected free from the uterus, and the decidua and underlying Reichert’s membrane were carefully trimmed away. Embryos were then transferred using the “wrong” end of a glass pipette to a fresh petri dish containing explant saline (see Appendix). The embryo was delivered from the yolk sac by cutting with iridectomy scissors almost all the way round the yolk sac and letting the embryo “fall” out. The embryo was then delivered from the amniotic membrane by making a small hole in the membrane next to the caudal end of the embryo and passing the membrane forward over its head. The embryo was then laid on its right side and DiI, fluorescent dextrans or F4/80 monoclonal antibody was injected into a single interdigit in the left footplate of the embryo. Using an aspirator tube (Sigma) and applying suction the marker was loaded into a fine bore capillary needle (40μl; Sigma) with a tip diameter of approximately 10μm. With a mouth pipette the marker was injected into the appropriate interdigit under a dissecting microscope. The DiI solution was a 1:9 dilution of 0.5% DiI (Molecular Probes) in AnalaR ethanol (BDH) with 0.3M filter-sterilised sucrose. The fluorescent dextrans (4.4 or 19.6 kDa; Sigma) were used at a concentration
of 25mg/ml and the F4/80 monoclonal antibody was used at 100μg/ml. After culturing for either 3, 6, 12, 18 or 24 hours (see later) the embryos were fixed in ice-cold 4% paraformaldehyde for one hour. Those embryos injected with DiI and fluorescent dextrans were rinsed three times in PBS, mounted and viewed on the CLSM straight away, while those injected with the F4/80 had first to be exposed to a FITC-tagged mouse adsorbed rabbit anti-rat IgG (10μg/ml; Vector Laboratories) overnight, at 4°C, rinsed in PBS, in order to reveal the location of the primary antibody.

*Embryo culture of E13.5*

After the embryo was injected with one of the markers it was gently placed into 5ml of foetal calf serum (FCS; Sigma) and culture saline (see Appendix) in a ratio of 1:2 in a 50ml Falcon tube and cultured according to a protocol modified from Cockcroft (1990). The airspace in the Falcon tube was filled with a gas mixture of 95% O₂ and 5% CO₂ (BOC). For an air-tight seal the mouth of the tube was coated with a thin layer of silicone grease (Fisons). Tubes were then placed in a roller incubator (BTC Engineering, Cambridge) maintained at 37°C and rolled at 30rpm. The embryos were re-gassed every three hours.

*Open uterus surgery*

Embryos of E13.5 are unable to survive in roller culture for more than 12 hours on account of requiring more oxygen than can be provided for the embryo in a tube. For studies of macrophage movements in embryos for time-points between 12 and 24 hours post-tagging, a protocol first described by Muneoka and colleagues (1990) was used, which allows for manipulation of embryos in situ.
Time-mated female mice were weighed and anaesthetised by an intraperitoneal injection of a mixture of Hypnovel (midazolam 5mg/ml; Janssen-Cilag Ltd), Hypnorm (fentanyl citrate 0.315mg/ml and fluanisome 10mg/ml; Janssen-Cilag Ltd) and Hartmann’s solution (Lactated Ringers; Fresenius Ltd; see Appendix) in the ratio of 1:1:2, 0.1ml per 10g body weight. Additional anaesthetic was given intraperitoneally in 0.05ml doses if required. The abdomen was cleared of hair using a depilatory cream (Immac, Reckitt) and washed with 70% alcohol. The first incision was made in the skin in the midline from the xiphisternum to the pubic symphsis, being careful not to damage the bladder. The skin was pinned back while the muscular layer was cut (Fig. 5.1). Both layers were then retracted and the abdominal cavity was filled with warmed (37°C) Hartmann’s solution. The uterus was then cut with fine iridectomy scissors along its anti-placental border and the embryos revealed in their yolk sacs. The left hindlimb was located and an injection made through the yolk sac into the interdigital space. This meant minimal movement of the embryo and no change in its immediate environment as the hole from the injection was so small.

Occasionally an incision was made in the yolk sac to access the limb; the limb was delivered and the injection made before returning the limb and suturing closed the yolk sac with 10/0 monofilament suture (Ethicon). The number and position of the embryos treated was carefully recorded. Finally, the abdominal cavity was rinsed with warm Hartmann’s solution, the muscular wall was closed with 6/0 monofilament suture (Ethicon) and the skin was also sutured. The mouse was given an injection of Temgesic (0.3 mg/ml buprenorphine as hydrochloride; Reckitt & Colman) to aid her recovery and prevent any discomfort. She was then placed on tissues in a clean cage with a small dish of water. The whole cage was placed in a warmed box (plant propagator, Midland Oak UK Ltd) until the mouse had regained consciousness, and then she was placed in a warm recovery room (25°C). The mother was closely monitored throughout the recovery period.
After 12, 18 or 24 hours the mother was killed by cervical dislocation and the embryos recovered by cutting through the sutures to re-open the abdomen. The embryos were dissected away from the uterus and removed of their membranes, before being killed by immersion in ice-cold PBS and then fixed in 4% paraformaldehyde and processed as for cultured specimens.
Figure 5. 1: Diagram of the female mouse prepared for open uterus surgery.

The skin and the muscular layers of the abdomen are held back with partly opened staples which are attached to elastic bands (retractors), which are pinned to the operating board. The mouse lies in a gutter between two resin side panels containing Sylgard (shaded area). The uterus is exposed when the abdomen is opened, and at this stage of development it is possible to see the features of the embryos because the uterine wall is translucent.

This diagram is not to scale.
Figure 5.1

- xiphoid process
- skin retracted
- muscle layer
- elastic band with staple
- skin & muscle layer retracted
- embryo in its yolk sac within the uterus
Results

Extensive programmed cell death occurs between E13.5 and E14.5

Scanning Electron Microscopy revealed the morphogenetic shape changes that occurred as the developing footplate was modelled to give separate digits. At E11.5 the limbs were tongue-shaped buds (Fig. 5.2 A), and over the next few days they alter quite significantly. The most dramatic remodelling events occur during the 24 hour period between E13.5 and E14.5, when the footplate is sculpted and the interdigital tissue is removed (Fig. 5.2 B & C).

In resin sections of the E13.5 footplate numerous apoptotic cells were seen within the interdigital areas, most clustered in aggregates as though contained within individual engulfing cells (Fig. 5.3 A). F4/80 staining revealed the presence of macrophages correlating precisely with these zones of interdigital cell death (Fig. 5.3 B). At the TEM level macrophages containing four or more apoptotic cells (within the plane of the section) were routinely found in the interdigits, the largest ones being closest to the regressing epithelium (Fig. 5.3 C).

Macrophages enter the limb at E11.5

Macrophages circulate freely in the blood stream from E10.5 (Morris et al., 1991). Wholemount preparations incubated with the macrophage specific antibody F4/80, showed macrophages entering the limb as early as E11.5, when it is still a bud (Fig. 5.4 A). When the developing limb elongates and flattens at E12.5, and webbing of the
footplate becomes apparent at E13.5, collections of F4/80 positive macrophages were seen first at the anterior and posterior margins of the limb, and then in the interdigital regions (Fig. 5.4 B).

Double-labelling of E13.5 limbs for macrophages and apoptotic cells

Wholemount E13.5 limbs stained with F4/80 and viewed on the CLSM revealed between one and two hundred macrophages within each interdigital space (Fig 5.4 C). They were most densely packed at the distal margin of the interdigit, and the majority of the macrophages showed swollen plasma membranes. To confirm that the macrophages had in fact engulfed dead cells, some specimens were double-labelled with both F4/80 and the nuclear dye, 7-AAD, to reveal condensed apoptotic nuclei. Confocal microscopy of such preparations did indeed reveal numerous F4/80 positive cells containing their own light staining nucleus plus several brighter apoptotic bodies (Fig. 5.4 D). However, there were also some apoptotic cells not within an F4/80 positive membrane (Fig. 5.4 D).

Macrophages can be tracked in vivo

Several strategies were utilised for tagging macrophages in the E13.5 interdigit in order to track their movements after they had engulfed apoptotic debris. Firstly, the lipid soluble dye, DiI, was tried. This is standardly used for tracing cell movements, for example in the developing chick face and limb (Vargesson et al., 1997; McGonnell et al., 1998). However, it turned out not to be useful, largely because it was not cell-type specific; it labelled up all the cells at the injection site, and obscured the movements of any one cell type (Fig. 5.5 A).
Since it is believed that only macrophages within the limb mesenchyme are phagocytic, tagging them by injection of fluorescent dextrans was tried, hoping that they, and not any other cells, would ingest them. This strategy was also unsuccessful. Preliminary experiments showed a bright aggregate of fluorescent dextran at the injection site and it was not possible to determine whether the dextrans had been engulfed or whether any movement of phagocytic cells had occurred (Fig. 5.5 B).

However, the final and most ambitious strategy, to label living macrophages in vivo with the monoclonal antibody F4/80, proved to be fruitful. The effectiveness of macrophage tagging by injecting a single interdigit with F4/80 was assessed by fixation of sample specimens within one or two minutes of the injection. These specimens were then incubated with secondary antibody to reveal labelled cells (Fig. 5.6 A & B). Consistent bright membrane labelling of subpopulations of cells (presumably macrophages) were seen, and these were confined to the interdigital space that had been injected. None of the injections showed marked cells outside of the interdigit loaded. In injected embryos subsequently cultured for six hours and incubated with secondary antibody to reveal macrophage movement, a small number of labelled cells were seen at the site of the injection, but the majority appeared to have moved to the presumptive ankle of the footplate, seemingly moving proximally out of the footplate (Fig. 5.6 C). After nine hours the macrophages were seen moving up into the core of the limb and by 12 hours they were found halfway between the tip and base of the limb (Fig. 5.6 D).

Since E13.5 embryos can only be kept alive in roller culture for 12 hours, these studies were repeated on a small number of embryos operated on while still associated with the maternal circulation. These embryos were maintained for 12, 18 or 24 hours. The 12 hour in utero specimens mirrored very closely what had been observed in the 12 hour roller culture experiments. At the later time-points possible by this technique, labelled cells were seen moving right to the base of the limb and beyond. By 18 hours the tagged
cells were found at the base of the limb (Fig. 5.6 E), and by 24 hours some cells were observed in the flank of the embryo (Fig. 5.6 F).
Figure 5.2: Scanning electron micrographs of the sculpting events that occur during footplate remodelling.

A  Low magnification view of an E11.5 embryo illustrating the tongue-shaped buds of the forelimb (F) and hindlimb (H).

B  In the E13.5 hindlimb footplate the digits are forming with clear webs between them.

C  At E14.5 the digits are beginning to separate from one another and the amount of interdigital tissue is obviously reducing.

Scale bars = 500μm.
Figure 5. 3: Light and electron microscopy of interdigital tissue in the E13.5 mouse footplate.

A  Resin histology through an interdigit of an E13.5 hindlimb, showing clusters of phagocytosed apoptotic nuclei, presumably in macrophages.

B  F4/80 immunohistochemistry from the interdigit of an E13.5 hindlimb illustrating the presence of macrophages, in the same vicinity as the dead cells shown in A.

C  A transmission electron micrograph of a “typical” macrophage found in E13.5 interdigital tissue. This macrophage has a healthy nucleus and intact plasma membrane, and contains four large apoptotic bodies (in this plane of section).

Scale bars: A & B = 25µm, C = 2µm.
Figure 5. 4: Wholemount F4/80 immunohistochemistry of developing mouse limbs at E11.5 and E13.5.

A  Already at E11.5 macrophages can be seen “patrolling” the undifferentiated limb mesenchyme.

B  By E13.5 the number of macrophages has increased dramatically, the majority of which are restricted to the interdigital regions.

C  High magnification detail from B of a single interdigit; the image has been reconstructed by superimposing a number of optical sections that were taken from one interdigital space.

D  A double stained preparation, with F4/80 (green) and the nuclear dye, 7-AAD (red), to demonstrate that macrophages in this region do contain brightly staining apoptotic bodies.

Scale bars: A & B = 250μm, C & D = 50μm.
Figure 5.4
Figure 5.5: Attempts to Dil and dextran label macrophages in the interdigital region of the E13.5 footplate.

A A light microscope view of an E13.5 footplate just after injection with the lipophilic dye, Dil.

B A confocal image of an E13.5 footplate just after injection with fluorescent dextrans.

Scale bars = 250μm.
Figure 5.5
Figure 5.6: Wholemount immunohistochemistry to reveal F4/80 labelled cells in hindlimbs just after interdigit injection and up to 24 hours.

A Wholemount preparation of hindlimb fixed within minutes of the injection of F4/80 into the second interdigit. Subsequent fluorescent immunohistochemistry reveals the labelled macrophages (bright green) in this interdigit.

B High magnification detail of A, to show that F4/80 labelling is of discrete cells and is strictly localised to the interdigit injected.

The inset shows the relative positions of each image in this plate and corresponds to the apparent movement of macrophages proximally with time.

C After the embryo has been cultured for six hours a small number of macrophages remain at the injection site, but the majority appear to have moved into the presumptive ankle region.

D A further six hours later, after 12 hours in culture, macrophages have moved into the core of the limb.

E After 18 hours in culture, the labelled macrophages have moved proximally as far as the base of the limb.

F By 24 hours some of these macrophages have left the limb and are found in the flank region of the embryo.

Scale bars: A & C = 200\,\mu m, B, D & E = 100\,\mu m, F = 50\,\mu m.
Discussion

This chapter shows clear evidence for macrophage presence in the interdigit of the remodelling mouse footplate. The macrophage distribution correlates tightly with the cell death occurring in the footplate, and there is direct evidence that macrophages are engulfing some, if not all, of the dead cells. By tagging macrophages in the remodelling interdigit and following their subsequent movements, the rapid exit of these cells from the death zone is demonstrated, showing them to travel as far back as the base of the limb and into the flank. These observations raise a number of questions:

1. How do macrophages locate and recognise the dead cells?
2. What is the eventual destination of macrophages after they leave the death zone?
3. Are macrophages the only cell type capable of engulfment in the footplate?

Since macrophages were seen within the limb bud from the earliest stages examined, and certainly before the stages of interdigital regression, it is clear that any signals attracting macrophages to the death zones need only be very short range. As described earlier various growth factors, including BMPs are expressed in the interdigit region, and these could well serve as short range chemoattractants for macrophages, although there is, as yet, no study which reports which of the growth factor receptors are expressed by embryonic macrophages. An alternative mechanism whereby macrophages find their apoptotic targets might be by constant patrolling. In this case recognition cues might be membrane bound, for example the vitronectin receptor that triggers macrophage engulfment of senescent neutrophils in the human adult kidney (Savill et al., 1990).

Having recognised several apoptotic cells and engulfed them, the interdigit macrophages could presumably stay in situ and digest this cell debris, but because the interdigit is soon to regress, it is almost essential that the macrophage has some motile capacity. Large numbers of in vitro studies have shown that macrophages are a highly motile cell type.
(Auger and Ross, 1992; Downey, 1994; Lauffenburger, 1996), and indeed in recent years the signalling pathways that regulate this motility have been dissected (Allen et al., 1997).

However, in vivo, there are almost no studies of macrophage movement. These data strongly suggests that the interdigit macrophages are very motile, so much so, that by 24 hours some have left the limb altogether. In an early grafting experiment Saunders (1966) showed that macrophages labelled with tritiated thymidine incorporated into the grafted tip of the chick limb bud could occasionally be found in the circulation of the host embryo, but neither his study or the present one ascertain where these macrophages are finally destined.

With regard to whether macrophages are the only cell type involved in, or capable of, phagocytosis in the interdigit there are various mutant mice which show a reasonably tight correlation between the extent of cell death and numbers of macrophages present in the interdigit. One example is the Hammertoe mutant, where there is a decrease in the amount of cell death and a reduction in the number of macrophages (Zakeri et al., 1994). Very few apoptotic cells appear not to be contained within a F4/80 positive macrophage. However, it could be that macrophages are such efficient phagocytes that any phagocytic capacity of neighbouring mesenchymal cells is masked. The best test of whether neighbouring mesenchymal fibroblasts do indeed possess engulfing capacity would be to deprive the embryo of the macrophage lineage, and this has been done in the PU.1 null mouse. Studies of footplate development in this mouse are reported in Chapter Six.
CHAPTER SIX

Limbs in a Macrophage-less Mouse Embryo

Introduction

Previous chapters have investigated the apparent function and movements of macrophages in wild-type embryos as they remodel their limbs and kidneys. One good way to address the function of macrophages in the embryo is to examine embryos genetically deficient in this cell type, and the PU.1 knockout mouse offers such a model. This chapter describes a study of footplate development in the PU.1 null embryo.

The transcription factor PU.1 is a member of the ets family of transcription factors (reviewed in Janknecht and Nordheim, 1993; Moreau-Gachelin, 1994). The prototypic ets gene, ets-1, was first identified about 15 years ago as an oncogene carried by the avian erythroblastosis virus E26 (Leprince et al., 1983; Nunn et al., 1983). The ETS domain has a highly conserved region of 85 amino acids which binds DNA in a manner similar to the winged helix-turn-helix family of proteins (Kodandapani et al., 1996) allowing it to act as a transcriptional activator (Klemsz et al., 1990). PU.1 is expressed exclusively by cells of the haematopoietic lineage - it has specifically been shown to be expressed by macrophages, B lymphocytes, mast cells, neutrophils and early erythroblasts, but not by T-cells (Galson et al., 1993; Klemisz et al., 1990).
Two recently reported PU.1 knockout mice give rather conflicting evidence as to which of these cell types are absolutely dependent on PU.1 (Scott et al., 1994; McKercher et al., 1996). The first of these papers describes an embryonic lethal phenotype, with foetuses dying at E16.5 apparently of anaemia (Scott et al., 1994). The only haematopoietic cell types which are reported to be normal in this PU.1 deficient mouse are megakaryocytes and platelets. Erythrocyte precursors develop as normal but they seem unable to mature, and there are no lymphoid or myeloid progenitors. All organs studied apparently develop normally, as do other tissues including bone (Scott et al., 1994).

In the most recent paper, a different region of the gene was disrupted and knockout mice were born in the expected Mendelian ratio. If kept in normal conditions, these mice die within 48 hours of birth of septicemia, although they can be kept alive for about two weeks on a strict antibiotic regime (McKercher et al., 1996). Erythrocytes, megakaryocytes, platelets and mast cells are all apparently normal (McKercher et al., 1996). T-lymphocyte development appears to be delayed by eight to ten days in PU.1 null mice, with mature cells not appearing until three days after birth (McKercher et al., 1996). Neutrophil development is also retarded by ten or more days, so that neutrophil-like cells are not seen until three or four days after birth. Of most relevance to this study is that these mice appear to be almost completely missing cells of the macrophage lineage. The only F4/80 positive cells found were in the liver, and they were abnormally large in size and of uncharacteristic shape. No macrophages were seen in any other tissues (McKercher et al., 1996).

These PU.1 null mice are also missing multinucleated osteoclasts and are thus osteopetrotic (Tondravi et al., 1997). Osteoclasts are of myeloid origin. In the wild-type situation marrow macrophages gradually increase their expression of PU.1 as they adopt the osteoclast phenotype. Osteoclast differentiation, like that of macrophages, is affected at the initial myeloid stage (Tondravi et al., 1997). The osteopetrotic effects of PU.1 null
mice can be rescued by bone marrow transplantation (Tondravi et al., 1997), and indeed transplants of this sort will, if delivered in the first week of life, almost completely rescue the mouse, such that it lives for close to the normal life span of a wild-type mouse (personal communication, Scott McKercher, La Jolla, California).

Figure 6.1 summarises the cells involved which are affected by the lack of PU.1, and suggests positions where PU.1 might act in the haematopoietic lineage decision pathway.

In all systems examined to date, cells dying by apoptosis are rapidly cleared by adjacent phagocytic cells. In *C.elegans* it appears that this clearance task is performed by non-specialist neighbours (Ellis et al., 1991). In *Drosophila* non-specific blood cells, called hemocytes, which have macrophage characteristics, engulf the apoptotic cells throughout organogenesis (Tepass et al., 1994). In vertebrate embryo models, various studies have shown that macrophages perform a major role in the clearance of apoptotic cells found in the nervous system (Cuadros et al., 1992), the interdigital zones of the remodelling footplate (Hopkinson-Woolley et al., 1994) and the kidney (Chapter Three; Camp and Martin, 1996).

The PU.1 embryos offer the ideal opportunity to test the situation when macrophages are not available to perform their clearance functions. A remarkable instance of cell redundancy is found. There are absolutely no macrophage-like cells in the PU.1 knockout footplate at stages when interdigits are regressing, and yet this regression proceeds at precisely the same rate as in wild-types or heterozygotes. Closer examination at the transmission electron microscopy (TEM) level shows embryonic mesenchymal cells fulfilling the role of phagocytes in these zones of cell death.
Figure 6.1: A haematopoietic lineage diagram to indicate where PU.1 may be acting.

This diagram was created from the data presented in the McKercher et al. paper (EMBO J 15, 5647-58; 1996) describing the PU.1 knockout mouse and from Paul Martin’s recent (March 1998) discussions with Scott McKercher and colleagues, The Burnham Institute, La Jolla, California.
Figure 6.1

- **PU.1 +/+**
  - T-lymphocyte
  - Plasma cell
  - Erythrocyte
  - Megakaryocyte
  - Basophil/Mast cell
  - Neutrophil
  - Eosinophil

- **PU.1 -/-**
  - Appear late, no mature cells
  - No mature cells
  - Normal
  - Normal
  - Normal
  - No mature cells
  - Neutrophil-like cells, no mature cells
Materials and Methods

For these studies litters of embryos were obtained from crosses of mice heterozygous for the transcription factor PU.1, which gave a Mendelian ratio of wild-type, heterozygote and homozygote offspring. Gestational age was calculated on the assumption that conception had occurred at midnight preceding the morning a vaginal plug was seen. Confirmatory staging of the litters was according to Theiller (1989) and Martin (1990), using the development of the forelimb and hindlimb buds as indicators, although this was potentially only accurate for wild-type and heterozygote embryos.

The transgenic mouse PU.1

The PU.1 null mouse was generated by standard homologous recombination techniques. The neomycin gene was inserted at the BssH2 site in the exon coding for the DNA binding domain and the thymidine kinase gene was inserted at a Clal-SalI site, outside of the DNA binding domain. This vector was electroporated into embryonic stem (ES) cells and colonies were selected using a double selection strategy - resistance to G418 and gancyclovir. Positive clones were characterised by polymerase chain reaction (PCR) and three were isolated and injected into C53Bl/6b blastocysts for transfer to the uterus of a pseudopregant mother. Resulting chimeric offspring were mated to non-transgenic mice to test for germline transmission of the ES cell allele. The offspring were tested using PCR and only one of the three clones or founders resulted in germline transmission of the mutated allele. Heterozygote mice were interbred to yield homozygtes for the mutated allele. These were produced in the expected Mendelian ratio. The PU.1 null mice are outwardly almost identical to their heterozygous and wild-type littermates, but they die within 48 hours of birth of septicemia (McKercher et al., 1996).
Harvesting of embryos

The embryos were harvested by Paul Martin (UCL) in the host laboratory (Maki/McKercher, Burnham Institute, La Jolla, California). Each time-mated female mouse was killed by cervical dislocation, the abdomen opened and bicornate uterus exposed. The uterus was dissected into a petri dish and rinsed several times in PBS before transfer to a clean dish so that the embryos could be removed. The uterine wall was cut along its entire length on the anti-placental border with a pair of fine iridectomy scissors. The placental side of the embryos were then carefully teased away with fine forceps. Embryos were then freed of their decidua and gently delivered from their yolk sac by carefully cutting away the yolk sac where it joins the placenta. The amniotic membrane was the last membrane to protect the embryo and this was also removed. The embryos were still alive at this point being attached to their placental by umbilical vessels. For each embryo the tail was cut off at its midpoint with small iridectomy scissors and transferred to a numbered eppendorf containing 0.7ml of lysis buffer (see Appendix). The head was then cut away from the body with large iridectomy scissors and put into a corresponding numbered eppendorf containing fixative. The abdomen of the embryo was then opened to allow efficient penetration of the fixative to the internal organs, before it was put into the same eppendorf as the head.

Embryos were genotyped in the host laboratory by PCR using tail DNA as a template.

- details of the litters

Litter 1022: Fixed in ice-cold half-strength Karnovsky fixative (Karnovsky, 1965)

12 embryos at E13.5  
- one wild-type (+/+),
- seven heterozygotes (+/-),
- four nulls (-/-)
Litter 1139: Fixed in ice-cold Bouins fixative (BDH)
9 embryos at E13.5 four wild-type (+/+),
  three heterozygotes (+/-),
  two nulls (-/-)

Litter 1065: Fixed in ice-cold half-strength Karnovsky fixative
3 embryos at E14.5 one wild-type (+/+),
  two heterozygotes (+/-),
  zero nulls (-/-)

Litter 1084: Fixed in ice-cold half-strength Karnovsky fixative
7 embryos at E14.5 three wild-type (+/+),
  two heterozygotes (+/-),
  two nulls (-/-)

The head and body of each embryo was received in fixative.

Histology and electron microscopy

Embryos (from Litters 1022, 1065 and 1084) were prepared for resin histology, TEM and scanning electron microscopy (SEM). The bodies of the embryos were transferred to 0.1M cacodylate buffer. Using large iridectomy scissors each embryo was sagitally cut in half down the midline. The right hindlimb was dissected from the right half of the embryo and processed for resin histology and TEM studies. The left half of the embryo was kept intact, with forelimbs and hindlimbs remaining attached to the flank, and processed for SEM. These tissues were then rinsed twice in 0.1M cacodylate buffer, 20 minutes each, and post-fixed in 1% osmium tetroxide (Sigma) in 0.1M cacodylate buffer
for 75 minutes. The specimens were rinsed twice in 0.1M cacodylate buffer for 10 minutes each, and then from 30% alcohol through to 70% alcohol.

- **Resin histology**

Specimens for resin histology (right limbs) were further dehydrated through a graded ethanol series to AnalarR ethanol (BDH), 10 minutes in each before transfer to propylene oxide (BDH) for four rinses of 10 minutes each. Tissues were then placed in a 50:50 mixture of propylene oxide and Araldite resin (see Appendix) for 45 minutes, before transfer to neat, fresh Araldite resin and rotated overnight. The Araldite resin was changed the following day and the specimens were returned to the rotator for a further five hours before being embedded in Araldite resin and cured at 65°C. Sections of 1-5μm were cut, mounted on poly-L-lysine (1:100; Sigma) coated slides and stained with Toluidine Blue.

- **Transmission electron microscopy**

Footplates were further analysed by TEM. In these cases ultrathin resin sections were cut and mounted on copper grids for staining first with uranyl acetate for 10 minutes and with lead citrate for a further 10 minutes, with distilled water rinses between and after stainings. The sections were examined using a Jeol 1010 Transmission Electron Microscope.

- **Scanning electron microscopy**

Specimens for SEM were dehydrated through graded alcohols, 10 minutes in each, transferred to Analar acetone (BDH), dried using a Polaron Critical Point Drier, which uses CO₂ as its medium. They were mounted on 10mm cylindrical stubs with electrodag, which was left to dry for 24 hours. The specimens were coated with gold.
using a SC500 Sputter Coater and viewed on a Jeol JSM-5410LV Scanning Electron Microscope.

Analysis of interdigit regression

Stage of interdigital regression was semiquantitatively compared in PU.1 knockout embryos with wild-type and heterozygote littermates, by measuring interdigit angles of the first and second interdigit of each hindlimb of the SEM specimens (Fig. 6.2). Lines were drawn between the first and second digit, and the second and third digit. For each interdigit, the two angles formed between the regressing tissue and the line were measured. The average of these two angles was calculated for each interdigital space. The angles for each genotype were summed, and the average angle for each interdigital space was calculated and presented in graphical form (Fig. 6.4).
Figure 6. 2: Diagram of the interdigit angle measurements of E13.5 footplate.

For the first and second interdigits lines were drawn between the most distal tips of the digits. The angles formed at the junction of these lines and the regressing epithelium were measured and the mean of these angles calculated. The data are presented graphically in Figure 6.4.
Figure 6.2

E13.5 hindlimb
Results

Sculpting of the footplate occurs at the normal rate in PU.1 null embryos

The specimens for SEM were examined, and at both E13.5 and E14.5 hindlimb footplates from wild-type, heterozygote and PU.1 null embryos appeared outwardly to have identical shape (Fig. 6.3).

To quantify and confirm this observation, the extent of regression was estimated by measuring the regression angles for two of the interdigit regions at E13.5. The results show that the first interdigital region is shallow with a mean angle of 10° for all genotypes of embryo, and the slightly deeper second interdigit is again not significantly different - with a mean of 25° for wild-types and heterozygotes and 23° for knockouts (Fig. 6.4). This study revealed that limb shape for E13.5 PU.1 embryos was identical to that of their wild-type and heterozygote littermates.

Footplate development

When examined by resin histology at low magnification there appeared to be less cell death in the PU.1 null embryos at both E13.5 and E14.5 (Fig. 6.5 A & B; Fig. 6.6 A & B). However, as they were examined in more detail it became apparent that in fact the levels of death were similar, but that apoptotic cells were simply packaged differently. In the wild-type and heterozygotes at E13.5 there are clusters of dead cells within macrophages, which were themselves in groups. In the null embryos the cell death was more widely scattered, an odd apoptotic cell amongst many mesenchymal fibroblasts (Fig. 6.5 C & D). At E14.5 apoptotic bodies in the wild-type embryos still appeared to
be collected within macrophages, but these are becoming more scattered, while in the null embryos the cells still appear to be clustered at the regressing epithelial margin (Fig. 6.6 C & D).

*Fibroblasts can engulf dead cells in the absence of macrophages*

Immediately apparent from TEM studies of PU.1 null embryos was that even in the absence of macrophages many of the dying cells appeared to be engulfed. The phagocytes must be simple fibroblasts. A typical monocyte-derived macrophage in a wild-type embryo had dense chromatin at the edge of its nucleus and a pale cytoplasm, containing “stringy” ribosomal structures, and with many organelles and inclusions. The phagocytic fibroblasts seen in the PU.1 null embryos were lacking strings of ribosomes and generally had an indented nucleus and the cell as a whole ‘looked “strained” by the inclusions.

In an attempt to quantify how efficient these macrophage-replacements were at recognising and engulfing apoptotic cells, the dead cells were categorised according to their relationship with the phagocyte:

- **A “free”** - no obvious associated phagocyte
- **B “noticed”** - filopodial extensions from phagocyte towards dead cell
- **C “owned”** - filopodia almost entirely wrapping around dead cell
- **D “engulfed”** - fully within the body of phagocyte

In the wild-type and heterozygote embryos there was a zone of mesenchyme extending about five or six cell diameters back from the regressing epithelium, where occasional fibroblasts, rather than macrophages, were seen engulfing dead cells - these apoptotic cells were primarily in the “noticed” or “owned” category (Fig. 6.7 A). The majority of dead cells within the interdigit were “engulfed” by macrophages (Fig. 6.7 C), with only
the occasional dead cell found “free” in the mesenchyme or associated with a competing fibroblast. By contrast, PU.1 null embryos contained numerous fibroblasts “noticing” and “owning” dead cells, both directly beneath the epithelium (Fig. 6.7 B) and deeper within the interdigit (Fig. 6.7 D). Preliminary observations from the knockout embryos suggested that up to two-thirds of fibroblasts in any one interdigit were actively involved in cell death clearance, with more activity being concentrated at the distal margin, and a gradual decrease within the depth of the interdigit.

To quantify how efficiently the macrophage-replacements were able to digest dead cells after engulfment, the dead cells were further classified into different categories according to their state of degradation:

1. plasma membrane intact, condensed chromatin
2. blebs in plasma membrane, dense cytoplasm lacks organisation
3. “holes” in plasma membrane
4. small dense digested packages

Macrophages within wild-type interdigits were predominantly found to contain dead cells of the two extreme categories, those that had just become apoptotic (1) or were almost fully digested (4; Fig. 6.7 C), while the phagocytic fibroblasts of PU.1 interdigits were seen to be “struggling” with dead cells in all four stages of degradation. Interestingly, the fibroblasts just beneath the regressing epithelium in the wild-type and heterozygote embryos also contained dying and dead cells in categories 2 - 4, rather than the two extremes (Fig. 6.7 A); consistent with the fibroblastic phagocytes in the PU.1 null embryos (Fig. 6.7 B & D).
Figure 6. 3: Scanning electron micrographs of E13.5 and E14.5 hindlimbs from wildtype and PU.1 null embryos.

A  E13.5 hindlimb from a wild-type embryo.
B  Hindlimb from a PU.1 null embryo, sibling of embryo shown in A.
A & B are very similar in the extent of interdigital regression.

Hindlimb from a wild-type E14.5 embryo (C) and PU.1 null sibling (D) again showing remarkable similarity in the extent of interdigit remodelling.

Scale bars = 200μm.
Figure 6. 4: Graph of the angles of the first and second interdigits in the hindlimbs of wild-type and heterozygote embryos, contrasted with PU.1 nulls.

PU.1 null footplates (purple) show no significant difference in the angles of interdigit regression from their wild-type (WT) and heterozygote (HET) siblings (considered as one group - blue).
Figure 6.4

The bar chart shows the comparison of angles in degrees between WT & HET, KO, WT & HET, and KO groups across the first and second interdigital spaces.
Figure 6. 5: Resin histology of wild-type and PU.1 null hindlimbs at E13.5.

A A low magnification view of the hindlimb of an E13.5 wild-type (+/+ ) embryo, showing aggregations of darkly stained cells in the interdigital zones.

B Low magnification view of the hindlimb of an E13.5 null (-/-) sibling embryo to that shown in A.

C High magnification detail of an interdigit from A, illustrating that the darkly stained dead cells are clustered and packaged within larger cells, probably macrophages.

D High magnification detail of an PU.1 null interdigit from B showing that the darkly stained apoptotic cells are rather more diffusely distributed with only one, or in some cases two, corpses being clustered together.

Scale bars: A & B = 250µm, C & D =25µm.
Figure 6: Resin histology of wild-type and PU.1 null hindlimbs at E14.5.

A Low magnification view of a wild-type (+/-) E14.5 hindlimb showing that digit formation is underway, with apoptotic cells clustered and located in the regressing interdigital zones.

B Low magnification view of an E14.5 hindlimb from a PU.1 null (-/-) sibling of A. Grossly these two footplates look similar.

C High magnification detail of an interdigit from the wild-type limb shown in A revealing a few dead cells clustered within macrophages (arrows), close to the regressing epithelium.

D High magnification detail of an equivalent PU.1 null interdigit illustrates that a considerable amount of cell death still remaining in the interdigit zone.

Scale bars: A & B = 250μm, C & D =25μm.
Figure 6. 7: Transmission electron microscopy of E13.5 hindlimbs in wild-type and PU.1 null embryos.

A  Even in the E13.5 wild-type (+/+) interdigit region occasional fibroblasts are found with one or two apoptotic bodies within them, for example directly beneath the regressing epithelium.

B  Throughout the E13.5 interdigit of a PU.1 null (-/-) embryo fibroblast-like cells are seen stretching out to engulf dead cells.

C  In the core of an E13.5 wild-type interdigit the majority of macrophages characteristically contain large numbers of apoptotic bodies.

D  Phagocytic fibroblasts from the interdigit of a PU.1 null embryo: to the left a fibroblast that has completely enveloped a dead cell, and to the right two other fibroblasts which have already engulfed small numbers of apoptotic bodies.

Scale bars = 2μm.
Figure 6.7

E13.5

[Images of cellular structures labeled A, B, C, D]
Discussion

While macrophages have been shown to be present wherever the clearance of programmed cell death is occurring in the developing mouse limb (Hopkinson-Woolley et al., 1994; Chapter Five) and kidney (Camp and Martin, 1996; Chapter Three), these previous studies had not clarified whether macrophages were essential for this clearance. The current study on PU.1 knockout embryos suggests that they might not be. Rather, in their absence fibroblast-like mesenchymal cells appear to be capable of engulfing the debris. This occurs to such an extent that in both E13.5 and E14.5 hindlimbs, the sculpting of the footplate progresses normally. This is the first clear example of cell redundancy - that is to say, the takeover of a role normally accomplished by one cell, by another cell - to be revealed by transgenic mouse studies.

It is curious that gross tissue regression in PU.1 knockout embryos appears indistinguishable from wild-type embryos, since semiquantitative measures of efficiency of recognition and engulfment, and also digestion rate, suggest that the phagocytic fibroblasts in PU.1 nulls are less effective than their macrophage counterparts in wild-type embryos. A possible explanation for this discrepancy is simply that more fibroblasts in PU.1 nulls, than macrophages in wild-type embryos, take part in clearance in the interdigit. This data suggests that in wild-type embryos mesenchymal fibroblasts would be capable of engulfing dead cells - in fact evidence is shown that a small number of fibroblasts near the regressing epidermis succeed - but they are generally “beaten to it” by the faster macrophages.

The data also largely rules out any possibility that macrophages are specifically required in order to “trigger” cell death in the interdigital zones, as appears to be the case for some tissues in the developing eye (Lang and Bishop, 1993; Diez-Roux and Lang, 1997). Genetic depletion of eye macrophages or killing with toxic-liposomes results in the
hyaloid vasculature and the pupillary membrane remaining intact for up to two weeks after they should have regressed by apoptotic death; there is some evidence that the same phenotype also occurs in newborn PU.1 nulls, although this is difficult to ascertain since PU.1 mice standardly die at the stage for assessing eye cell death. However, unlike the eye, these data suggests quite strongly that macrophages are not needed to "trigger" cell death in the developing footplate interdigit.

An interesting parallel is found in *Drosophila*, where cell death is usually cleared away by hemocytes, multi-functional blood cells with some macrophage-like characteristics (Abrams et al., 1993). However, various mutants exist where there are no macrophages (*Bicaudal D, twist snail, torso*[^402]) and yet similar amounts of cell death still occur, as in the wild-type, suggesting that the phagocytic cells are largely not responsible for "triggering" the cell death in *Drosophila* (Tepass et al., 1994). In such mutants, and others which for some reason have more programmed cell death (*knirps, stardust*), some of the cellular debris is phagocytosed by adjacent epidermal cells, but a considerable amount remains extracellular (Tepass et al., 1994). Further evidence that macrophage-initiated cell death is not a universal mechanism, or even likely to be common place, comes from *C.elegans*, where there are no macrophage-like cells, and yet 131 cells die in particular places and at specific times in every worm (Ellis et al., 1991b). These cells are rapidly engulfed and digested by their neighbours (Ellis et al., 1991a).

In wild-type mouse embryos there is at least one homologue of a *C.elegans ced* gene involved in the engulfment of dead cells, *ABC1* (which has structural homology with *ced-7*), that appears to be expressed by macrophages within the footplate, and may be required for efficient phagocytosis (Luciani and Chimini, 1996). It would be exciting to discover whether this gene, and other "phagocytic" genes, are upregulated by phagocytic fibroblasts in the PU.1 null mouse in the absence of true macrophages, or whether these genes can only be switched on in true cells of the macrophage lineage. There is still more work to be done with this interesting and exciting mouse.
CHAPTER SEVEN

General Discussion

Summary

This thesis has documented the distribution of haematopoietically-derived macrophages in two organ systems, the limb and kidney, and tested their role in the development of these organs using a variety of depletion strategies. In both the developing footplate, where cell death in the remodelling interdigit is "catastrophic", and in the kidney, where cell death is "trickle-like" and extended over a long period, there was evidence that most of the dead cells were cleared by macrophages. Presumably, the clearance of apoptotic cells from these and other organ systems in higher vertebrates is necessary because, if they were not engulfed and removed from these developing tissues, they would release their contents, which may be toxic or act as spurious signals, hindering normal morphogenetic processes.

In *C.elegans*, mutations in a pair of the *ced* engulfing genes, one from each of the two complementary groups, results in worms that fail to phagocytose any apoptotic debris, and yet their development and longevity appears not to be significantly comprised (Ellis *et al.*, 1991a). It is possible that the extra complexity in animals higher than worms would not allow for failure of clearance of this debris. What becomes apparent from studies of the PU.1 knockout footplate, where there are no macrophages, is that there is significant cell redundancy in the mouse with regard to the apoptosis clearance function.

193
Neighbouring mesenchymal fibroblasts are able to engulf the debris, so much so, that interdigital regression appears to progress at the same pace as in wild-type embryos. When looking back at wild-type footplates, it becomes clear that mesenchymal fibroblasts may also play a minor role in clearance. Certainly, close to the regressing epithelial edge there are cells which under transmission electron microscopy resemble more closely the phagocytes of PU.1 knockout footplates, than they do standard wild-type macrophages.

*Do macrophages have further functions in development?*

These studies have focused on macrophage function in limb and kidney development, but clearly other systems may reveal further information about the roles of these cells in development. In the developing eye, Lang and colleagues have shown that when macrophages are genetically or chemically removed from this system, the pupillary membrane and the hyaloid vasculature of the eye persist (Lang and Bishop, 1993; Diez-Roux and Lang, 1997). These experiments suggest that macrophages are responsible for "triggering" the cell death here, and rescue experiments which re-constitute macrophages in the eye confirm this to be the case because the cell death is now re-activated (Diez-Roux and Lang, 1997). The data presented in this thesis shows that macrophages cannot be solely responsible for initiating programmed cell death in this way in either the limb or the kidney.

In kidney organ culture experiments where macrophages were depleted, cell death still occurred but morphogenesis was impaired, suggesting that macrophages might be important for supplying crucial morphogenetic and patterning signals. However, it would be informative to look at the developmental patterning of kidneys in PU.1 null mice since genetic depletion of macrophages in these mice is considerably "cleaner" than the toxic liposome killing strategy.
There is no need to turn to PU.1 null embryos for evidence that immediate clearance of dying cells by "professional" macrophages is not incompatible with normal development. A significant number of morphogenetic events in the mouse occur before the stage when macrophages first make their appearance in the embryo proper at about E10.5. Even in the blastula stage embryo there is a significant amount of programmed cell death, but at this early stage there are no macrophages to clear this debris away (El-Shershaby and Hinchcliffe, 1974). At later stages, but still in the absence of macrophages, neural tube closure begins and involves a line of cell death along the midline seam, when the two tips of the neural plate meet and fuse. This cell death has been shown to be necessary for neural tube sealing because if it is prevented by addition of peptide caspase inhibitors, closure of neural tube fails (Weil et al., 1997). Whether this early cell death is simply lost from the surface of embryos into the amniotic fluid, engulfed by mesenchymal or epithelial neighbours, or whether it waits to be cleared away by professional phagocytes when they eventually differentiate is not yet known.

What molecular machinery allows a macrophage to recognise and engulf a dead cell, and which parts of this machinery might be upregulated by non-macrophage phagocytes?

Phagocytosis of apoptotic debris requires that the phagocytic cell is able to both recognise and to engulf cellular debris. Both of these cell processes are mediated by activation of specific families of receptors on their cell surface which allow interaction between the phagocyte and the cell to be engulfed. A little is already known about the ligands and receptors that allow recognition and which "trigger" the cytoskeletal reorganisations that lead to engulfment. One marker exposed by dying cells that may be important for macrophage recognition is phosphatidylserine (PS), which becomes redistributed within the plasma membrane bilayer at an early stage in the apoptotic programme. In a healthy
living cell PS is not experimentally detectable, being located on the inner leaflet of the plasma membrane. However, when the cell commits to die, PS becomes re-localised to the outer cell surface. New reagents for detecting this shift of PS as a marker of apoptosis are designed around Annexin V, a Calcium ion dependent PS binding protein (van den Eijnde et al., 1997). Whether these "labels" are more than just useful experimental tools and are actually used by macrophages as a means of recognising dying cells and "triggering" binding and engulfment is not yet clear. Other candidate "death labels" include thrombospondin and sugar residues (Savill et al., 1992; 1993).

CD14 is a plasma-membrane glycoprotein molecule involved very early in the phagocytic process; though as yet it is not clear whether the macrophage recognition of apoptotic cells is via PS or anionic phospholipids or polysaccharide-containing molecules (Devitt et al., 1998). CD14 is thought to be involved in binding the dead cell to the phagocyte and then activating other molecules, specifically downstream tyrosine kinases, so that engulfment can take place (Devitt et al., 1998).

CD36, a transmembrane glycoprotein belonging to the scavenger receptor - class B family. Previous studies characterised CD36 as a receptor for collagen and thrombospondin involved in cell-cell and cell-matrix adhesive interactions (Asch et al., 1987). More recently it has been shown to be involved in the phagocytosis of apoptotic cells by macrophages (Ren et al., 1995) and by cells of the retinal pigment epithelium for clearance of photoreceptor outer segments during the remodelling of the visual system (Ryeom et al., 1996).

Macrophage secreted thrombospondin also binds to the vitronectin receptor α₅β₃ integrin, forming a "bridge" between the macrophage and the dead cell that is to be phagocytosed (Savill et al., 1990). This keeps the apoptotic cell "tethered", while cytoskeletal re-arrangements occur so that the dead cell can be engulfed (Savill, 1998).
Class A macrophage scavenger receptors (SR-As) are trimeric membrane glycoproteins, whose properties include the metabolism of lipoproteins and cell adhesion (Hughes et al., 1995). SR-As have now been shown to play a role in phagocytosis (Platt et al., 1996). In vitro, phagocytosis of apoptotic thymocytes is significantly impaired in thymic macrophages taken from mice with a disrupted SR-A gene by comparison to wild-type macrophages (Platt et al., 1996).

The murine ATP binding cassette (ABC) transporter, ABC1, has recently been shown to be expressed by macrophages that are actively involved in the engulfment and degradation of dead cells (Luciani and Chimini, 1996). Moreover, it appears to be essential for macrophages to phagocytose debris, since when ABC1 is blocked, the ability of macrophages to ingest apoptotic cells is significantly disrupted (Luciani and Chimini, 1996). Interestingly ABC1 has structural homology with ced-7, a gene that has been shown to be important for engulfment in C.elegans, and indeed when C.elegans with a mutated ced-7 gene are examined they show impaired phagocytosis of dead cells (Ellis et al., 1991).

Are these “recognition” receptors restricted to the macrophage lineage, or do other cells about to participate in phagocytosis of apoptotic debris have to upregulate the same genes? A good way of addressing this question would be to look in the footplate of PU.1 null mouse embryos, where pure populations of such cells, uncontaminated by macrophages, exist. It would also be interesting to look earlier in embryogenesis when macrophages have not yet appeared in the embryo. Is cell debris phagocytosed by non-macrophage neighbours and, if so, do these cells upregulate the macrophage recognition and engulfment genes described above?
What more will studies of cell death clearance in worms and flies tell us?

There are no true macrophages in *C. elegans*. Rather, the same 131 of 1091 cells reproducibly die, and are always engulfed by neighbours. At least six genes are involved in the efficient removal of these cell corpses, and these genes can be divided into two groups: one is required for “the recognition of specific cell-surface markers expressed by dead cells” and the other is necessary for “non-specific markers” (Ellis *et al.*, 1991a). As described earlier, the vertebrate homologue of *ced-7, ABC1*, is expressed by macrophages and is required for their efficient phagocytic activity (Luciani and Chimini, 1996). Recently, another one of these genes, *ced-5*, which is important for the migration of distal tip cells of the gonad as well as phagocytosis in the worm, has been cloned and sequenced (Wu and Horvitz, 1998). This gene encodes a protein similar to that of human DOCK180 and *Drosophila melanogaster* protein Myoblast City, and appears to be involved in reorganising the cytoskeletal elements to enable lamellipodia to be “sent out” so that the dead cell is surrounded and phagocytosis is possible (Wu and Horvitz, 1998).

There is some way to go in understanding the genetic basis of engulfment in vertebrates, but identifying the homologues of worm engulfment genes seem likely to be a good first step in determining some of the key “players”.

Unlike the worm, the fruitfly *Drosophila* does have semi-specialist macrophage-like cells called hemocytes, which phagocytose apoptotic cellular debris during development (Tepass *et al.*, 1994). The *croquemort* (catcher of death) receptor, which is a member of the CD36 superfamily, is expressed by hemocytes from the start of their phagocytic activity, and is thought to be involved in the ability of hemocytes to recognise and phagocytose dead cells (Franc *et al.*, 1996). Another gene that has been shown to be expressed and required by hemocytes is *glial cell deficient/glial cell missing* (*glide/gcm*). Expression of *glide/gcm* is thought to be sufficient to induce hemocytes to differentiate, enabling them to become phagocytic, and to engulf cells that are about to die (Bernardoni *et al.*, 1997). Macrophage-Derived Proteoglycan-1 (MDP-1) is another *Drosophila* gene
expressed exclusively by hemocytes late in their differentiation pathway, and its product is secreted when they are actively involved in phagocytosis of apoptotic cells (Hortschi et al., 1998). Perhaps homologues of these Drosophila engulfment genes may prove to be as useful in understanding vertebrate phagocytosis, as the worm ced genes have been.

*Evolution of macrophages - what is their primary role in organisms?*

Ezekowitz and colleagues (1996) propose, like Metchnikoff (1905), that the primary role of macrophages is in tissue remodelling, and they suggest that these cells have secondarily become adapted for a role in the immune response in the adult (Franc et al., 1996). The data presented here suggest that almost the reverse might be true, at least during development. Data from PU.1 embryo footplates demonstrates that macrophages are not absolutely essential for the cell clearance events that help sculpt the limb; in fact it seems likely that even in wild-type embryos some clearance is undertaken by neighbouring mesenchymal fibroblasts. Both cell types are able to phagocytose cellular debris, but macrophages are in some way(s) more efficient, so that in wild-type embryos they end up fulfilling most of the clearance role. This cell redundancy suggests that macrophages are unlikely to have evolved specifically for the cell clearance that is required during remodelling events in the embryo.

However, clues as to the primary role of macrophages may come from an understanding of why PU.1 neonates are not able to survive into adulthood. Untreated, these mice die of septicemia within hours of birth, which implies that their immune function is severely compromised. It is likely that this lack of defence against bacterial infections is primarily due to these animals having no neutrophils. When infections are blocked with daily antibiotic injections the mice still only live for approximately two weeks, eventually
dying of kidney failure that McKercher and Maki (personal communication) believe is due to the blood being so heavily overloaded with cell debris. In adult mammals this clearance of cell debris, for example extruded nuclei from red blood cells and other "spent" cells, is performed mainly by resident "specialist" macrophages in the liver, in conjunction with the spleen. Whilst in the embryo, the analogous task of clearing away apoptotic debris can be accomplished by "non-specialist" fibroblasts, this is clearly no longer the case in the neonate. Perhaps, this is because neonatal fibroblasts are less able to adapt to a phagocytic role than their embryonic counterparts or because the neonatal debris load is far greater than in the embryo. Either way, a specialist phagocytic cell - the macrophage - may have evolved to cope with the clearance of this cell debris load, and secondarily been "hijacked" for clearing dead cells in the embryo.

One of the major roles of macrophages has always been thought to be as part of the inflammatory response during adult tissue repair. In the mid 1970s Leibovich and Ross depleted macrophages in the guinea pig by administering a crude anti-macrophage serum together with steroids, and they found that these animals completely failed in wound healing (Leibovich and Ross, 1975). In parallel experiments in which neutrophils were depleted, wound repair was normal as long as the wounds were kept sterile, suggesting that the main function of neutrophils is to keep infection "at bay" (Simpson and Ross, 1972). Since those experiments were done it has become clear that activated macrophages recruited to any wound site express and secrete growth factors and cytokines (Rappolee, 1988; reviewed in Martin, 1997), and it was believed that macrophages are crucial in orchestrating wound closure by directing various cell behaviour (proliferation, migration, contraction) of keratinocytes, fibroblasts and endothelial cells at the wound site, with these growth factor signals. But perhaps even wound healing is not as dependent on macrophages as dogma would have us believe: wound healing studies have been started in PU.1 null neonates, and surprisingly, pilot studies show that tissue repair appears to proceed fairly normally, even in the absence of macrophages (Paul Martin, personal communication).
Macrophages appear to be the “masters” of many cell functions and perhaps are the “favoured” cell type for many critical tasks in the embryo and the adult, but it is beginning to become apparent that, at least in some instances, they are not the only cells that can perform these functions.
REFERENCES


204


206


APPENDIX

1/2 Strength Karnovsky - Karnovsky (1965)

1g Paraformaldehyde (BDH)
25ml distilled water
mix on a heater stirrer
2 - 5 drops of 1M Sodium Hydroxide (BDH)
Allow to cool
18ml 0.2M Sodium Cacodylate buffer
5ml 25% Gluteraldehyde (Agar Scientific)

Araldite resin

10g Araldite resin (Agar Scientific)
10g Dodecenyl Succinic Anhydride (DDSA; Agar Scientific)
0.8g Dibutyl phthalate (plasticizer; Agar Scientific)
mix on a heater stirrer until clear
0.4ml Benzyldimethylamine (BDMA; polymerising agent; Agar Scientific)
mix for a further two minutes
Apoptag in situ apoptosis detection kit - fluorescein (Oncor: S7110-KIT)

1. Working strength TdT (terminal deoxynucleotidyl transferase):
   - 72µl (two drops) Reaction Buffer S7110-2
   - 32µl (one drop) TdT Enzyme S7110-3
   Vortex, can be kept on ice for up to 6 hours

2. Working strength stop/wash buffer
   - 1ml Stop/Wash Buffer S7110-4
   - 34ml Distilled Water
   Prepare in advance, can be stored at 4°C for up to 12 months

3. Working strength antibody solution
   - 56µl (two drops) Blocking Solution S7110-5
   - 49µl Anit-Digoxygenin Fluorescein S7110-6
   Vortex, can be kept on ice for up to 3 hours
Leibovitz's L-15 Medium with L-Glutamine (Gibco)

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inorganic salts:</strong></td>
<td></td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>185.00</td>
</tr>
<tr>
<td>KCl</td>
<td>400.00</td>
</tr>
<tr>
<td>K$_2$PO$_4$</td>
<td>60.00</td>
</tr>
<tr>
<td>MgCl$_2$·6 H$_2$O</td>
<td>200.00</td>
</tr>
<tr>
<td>MgSO$_4$·7 H$_2$O</td>
<td>200.00</td>
</tr>
<tr>
<td>NaCl</td>
<td>8000.00</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>190.00</td>
</tr>
<tr>
<td><strong>Other components:</strong></td>
<td></td>
</tr>
<tr>
<td>D(+) Galactose</td>
<td>900.00</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>10.00</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>550.00</td>
</tr>
<tr>
<td><strong>Amino acids:</strong></td>
<td></td>
</tr>
<tr>
<td>DL-Alanine</td>
<td>450.00</td>
</tr>
<tr>
<td>L-Arginine (freebase)</td>
<td>500.00</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>250.00</td>
</tr>
<tr>
<td>L-Cystine (freebase)</td>
<td>120.00</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>300.00</td>
</tr>
<tr>
<td>Glycine</td>
<td>200.00</td>
</tr>
<tr>
<td>L-Histidine (freebase)</td>
<td>250.00</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>250.00</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>125.00</td>
</tr>
<tr>
<td>L-Lysine (freebase)</td>
<td>75.00</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>150.00</td>
</tr>
<tr>
<td>DL-Phenylalanine</td>
<td>250.00</td>
</tr>
<tr>
<td>L-Serine</td>
<td>200.00</td>
</tr>
<tr>
<td>DL-Threonine</td>
<td>600.00</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>20.00</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>300.00</td>
</tr>
<tr>
<td>DL-Valine</td>
<td>200.00</td>
</tr>
<tr>
<td><strong>Vitamins:</strong></td>
<td></td>
</tr>
<tr>
<td>D-Ca Pantothenate</td>
<td>1.00</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>1.00</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>1.00</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>2.00</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>1.00</td>
</tr>
<tr>
<td>Pyridoxal HCl</td>
<td>1.00</td>
</tr>
<tr>
<td>Flavin Mononucleotide</td>
<td>0.10</td>
</tr>
<tr>
<td>Thiamine Monophosphate HCl</td>
<td>1.00</td>
</tr>
</tbody>
</table>

### Dulbecco’s Modified Eagle Medium/Nutrient Mix F12 (1:1) with L-Glutamine, 15mM HEPES (Gibco)

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/L</th>
<th>Component</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic salts:</td>
<td></td>
<td>Other components:</td>
<td></td>
</tr>
<tr>
<td>CaCl₂ (anhyd)</td>
<td>116.60</td>
<td>D-Glucose</td>
<td>3151.00</td>
</tr>
<tr>
<td>CuSO₄·5 H₂O</td>
<td>0.0013</td>
<td>HEPES</td>
<td>3075.40</td>
</tr>
<tr>
<td>Fe(NO₃)₃·9 H₂O</td>
<td>0.05</td>
<td>Na Hyoxanthine</td>
<td>2.39</td>
</tr>
<tr>
<td>FeSO₄·7 H₂O</td>
<td>0.417</td>
<td>Linoleic Acid</td>
<td>0.042</td>
</tr>
<tr>
<td>KCl</td>
<td>311.80</td>
<td>DL-68 Thiocetic Acid</td>
<td>0.105</td>
</tr>
<tr>
<td>MgCl₂·6 H₂O</td>
<td>61.00</td>
<td>Phenol Red</td>
<td>8.10</td>
</tr>
<tr>
<td>MgSO₄·7 H₂O</td>
<td>100.00</td>
<td>Sodium Putrescine. 2HCl</td>
<td>0.081</td>
</tr>
<tr>
<td>NaCl</td>
<td>6996.00</td>
<td>Sodium Pyruvate</td>
<td>55.00</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1200.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>62.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄·7 H₂O</td>
<td>134.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄·7 H₂O</td>
<td>0.432</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acids:</td>
<td></td>
<td>Vitamins:</td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>4.450</td>
<td>Biotin</td>
<td>0.0035</td>
</tr>
<tr>
<td>L-Arginine. HCl</td>
<td>147.50</td>
<td>D-Ca Pantothenate</td>
<td>2.24</td>
</tr>
<tr>
<td>L-Asparagine. H₂O</td>
<td>7.50</td>
<td>Choline Chloride</td>
<td>8.98</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>6.65</td>
<td>Folic Acid</td>
<td>2.65</td>
</tr>
<tr>
<td>L-Cystine HCl. H₂O</td>
<td>17.56</td>
<td>i-Inositol</td>
<td>12.60</td>
</tr>
<tr>
<td>L-Cystine.2 HCl</td>
<td>31.29</td>
<td>Nicotinamide</td>
<td>2.02</td>
</tr>
<tr>
<td>L-Glutaminic Acid</td>
<td>7.35</td>
<td>Pyridoxal HCl</td>
<td>2.00</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>365.00</td>
<td>Pyridoxine HCl</td>
<td>0.031</td>
</tr>
<tr>
<td>Glycine</td>
<td>18.75</td>
<td>Riboflavin</td>
<td>0.219</td>
</tr>
<tr>
<td>L-Histidine HCl. H₂O</td>
<td>31.48</td>
<td>Thiamine HCl</td>
<td>2.17</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>54.47</td>
<td>Thymidine</td>
<td>0.365</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>59.05</td>
<td>Vitamin B₁₂</td>
<td>0.68</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>91.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>17.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>35.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>17.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>26.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Threonine</td>
<td>53.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>9.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Tyrosine.2Na. 2H₂O</td>
<td>55.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Valine</td>
<td>52.85</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Preparation of multilamellar liposomes composed of phosphatidylcholine and cholesterol, with the drug dichloromethylene diphosphonate (C12MDP) - Van Rooijen (1989)

1. dissolve 75mg of phosphatidylcholine and 11mg of cholesterol in 20ml methanol/chloroform (1:1) in a 500ml round bottom flask
2. remove organic phase by low vacuum rotary evaporation at 37°C
3. dissolve lipid film in 10ml chloroform
4. remove chloroform by low vacuum rotary evaporation at 37°C
5. disperse the thin film, that forms on the interior of the flask, in 10ml PBS, for empty control liposomes by gentle rotation for 15 minutes at room temperature. If C12MDP encapsulation is required 1.8 - 1.9ml of the drug must be dissolved in the 10ml of PBS used in this step
6. after complete removal of the lipid film from the interior of the flask, maintain the milky white suspension for two hours at room temperature
7. sonicate for three minutes at room temperature in a waterbath sonicator
8. maintain suspension for another two hours at room temperature for liposome swelling
9. centrifuge at 100,000x g for 30 minutes at 16°C
10. resuspend in saline or PBS and wash once or twice. Liposomes are finally resuspended in 4ml PBS

0.2ml or 2ml of this suspension given intravenously to mice or rats, respectively, eliminates spleen or liver macrophages
in order to check the functional characteristics of the liposomes, a sample can be stained with fluorochromes and studied with the fluorescent microscope (Van Rooijen, N. and Van Nieuwmegen, R. (1983), *Methods Enzymol* **93**, 83). After incubation of the multilamellar liposomes in a 1% aqueous solution of the fluorochromes acridine orange or eosin Y for several minutes, and washing to remove the free fluorochrome, a drop of liposome suspension can be observed under the fluorescence microscope; 1,000x magnification.

Liposomes containing entrapped C12MDP can be stored at 4°C under a nitrogen atmosphere for some time, depending on the phospholipids used.

If required, the amount of liposome-entrapped C12MDP can be determined using a method based on competition for calcium binding between C12MDP and murexide (Claassen, E. and Van Rooijen, N. (1986). *J Microencapsulation* **3**, 109).
**Explant Saline**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water - AnalaR</td>
<td>500 ml</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl 3.45 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl 0.15 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO₄·7H₂O 0.05 g</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>MgCl₂·6H₂O 0.025 g</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate</td>
<td>NaH₂PO₄·2H₂O 0.05 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>CaCl₂·2H₂O 0.13 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>C₆H₁₂O₆ 0.75 g</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>NaHCO₃ 0.25 g</td>
</tr>
</tbody>
</table>

The chemicals must be added separately in the order listed, dissolved properly after each addition, and filter-sterilised (Sartolab, Sartorius) before use.

**Culture Saline**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water - AnalaR</td>
<td>500 ml</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl 3.45 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl 0.15 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO₄·7H₂O 0.05 g</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>MgCl₂·6H₂O 0.025 g</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate</td>
<td>NaH₂PO₄·2H₂O 0.05 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>CaCl₂·2H₂O 0.13 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>C₆H₁₂O₆ 0.75 g</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>NaHCO₃ 1.00 g</td>
</tr>
</tbody>
</table>

The chemicals must be added separately in the order listed, dissolved properly after each addition, and filter-sterilised before use.

N.B. To convert 500ml of explant saline to culture saline add 0.75g NaHCO₃
**Hartmann’s Solution - Compound Sodium Lactate (Fresenius Ltd)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium</td>
<td>2.5 mM/L</td>
</tr>
<tr>
<td>Sodium</td>
<td>66 mM/L</td>
</tr>
<tr>
<td>Calcium</td>
<td>1 mM/L</td>
</tr>
<tr>
<td>Chloride</td>
<td>56 mM/L</td>
</tr>
<tr>
<td>Bicarbonate (as lactate)</td>
<td>15 mM/L</td>
</tr>
</tbody>
</table>

**Lysis Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M tris-HCl</td>
<td>50ml 100mM</td>
</tr>
<tr>
<td>20% SDS (Sodium dodecyl sulphate)</td>
<td>5ml 0.2%</td>
</tr>
<tr>
<td>0.5M EDTA (Ethylenediaminetetraacetic acid)</td>
<td>5ml 5mM</td>
</tr>
<tr>
<td>4M NaCl</td>
<td>25ml 200mM</td>
</tr>
<tr>
<td>Water</td>
<td>415ml</td>
</tr>
</tbody>
</table>
Programmed Cell Death and Its Clearance in the Developing Mammalian Kidney

Introduction

In recent years it has become clear that programmed cell death or apoptosis plays a key role during development of many organ systems where it appears to be used by the embryo as a tool to get rid of unwanted cells. In some systems apoptosis allows elaborate tissue sculpting of embryonic organs or structures, as for example in the developing amniote footplate where the digits become separated by death of interdigital mesenchyme cells [1, 2]. This almost synchronous death of many cells in a particular zone is 'catastrophic' and is easily visualised in histological sections of these tissues as large accumulations of dense and darkly staining apoptotic bodies. However, in many organ systems programmed cell death is not 'catastrophic'; rather, it occurs in a 'trickle-like' fashion over a relatively long period of development and it is in these situations where it was largely overlooked and its significance in development ignored. Perhaps the best documented example of this 'trickle-like' cell death is seen in the developing nervous system where very small numbers of apoptotic bodies are seen at any one 'snap-shot' time during the developmental history of the tissue, but if cell counts are made before and after the period of cell death and cell division is also taken into consideration, then it becomes clear that cell death probably deletes up to 50% of all cells born [3, 4]. The reason why all this cell death can so easily go undetected is that as each cell dies it is rapidly phagocytosed and cleared away by neighbouring cells, which we think in most instances are almost certainly haemopoetically derived macrophages [5]. Two recent papers [6, 7] that we will discuss in some detail later have now revealed that the metanephric kidney should also be added to the list of developing organs where significant programmed cell death occurs and may be playing a key role in tissue patterning and differentiation.

Apoptotic cell death differs from necrotic cell death stimulated by noxious or toxic signals in that it does not lead to leakage of the cell contents and thus incitement of an inflammatory response. Rather, during apoptosis cells shrink, condense their chromatin and then cleave their DNA into 180–200 bp internucleosomal lengths before being rapidly phagocytosed by other cells and without releasing any of their cell contents into the extracellular space [8]. From extensive work in the nematode worm, *Caenorhabditis elegans*, and in tissue culture cells we are beginning to learn more about the intracellular signalling pathway that is activated as a cell enters this death programme. In *C. elegans* it has been shown that programmed cell death is regulated by a genetic cascade with the main players being *ced3* and *ced4* which are both required for death, and a third gene, *ced9*, which acts as a checkpoint and can block the death pathway [9]. The vertebrate homologues of *ced3* and *ced9* are now known and encode the ICE protease and *bcl2* families of genes, respectively. The function of these genes is almost certainly conserved across the worm/vertebrate evolutionary gap since it has recently been shown that ICE and ICE-related proteases will trigger cell death in many vertebrate cell types [10], and *bcl2* can rescue cells from many normally death-inducing stimuli [11]. The details of this cell-autonomous intracellular death programme are beyond the scope of the current review but it is exciting to know that whilst developmental biologists map out where cell death is...
occurring in the mammalian embryo, what the extracellular cues might be that determine whether cells live or die, how this cell death shapes and patterns organs like the kidney, and how the dead cells are cleared away, the molecular machinery underlying this cell death is being elucidated in other systems.

**The Mammalian Embryo Has Three Kidney Systems which Develop in a Craniocaudal Sequence**

Before reviewing the recent data suggesting a major role for programmed cell death in kidney development we will briefly describe the key events that occur during development of this complex organ system. The kidney develops in 3 phases (pro-, meso- and metanephros), each of which derives at least partially from paired rods of intermediate or nephrogenic mesoderm which lie between the somitic and lateral plate mesenchyme of the neural-plate stage embryo. The nephric duct is a central player during each of these phases, serving as the drainage channel for pro- and mesonephros and then giving rise to the ureteric bud of the metanephric kidney. The two early kidney phases differentiate from the most cranial nephrogenic mesenchyme but are both transient structures and undergo a wave of 'catastrophic' programmed cell death not dissimilar to that described above for the developing footplate [12; our unpubl. data]. The third nephric system, which will become the definitive or metanephric kidney, is formed by reciprocal inductive interactions between the ureteric bud and the most caudal nephrogenic mesenchyme which condenses to form the metanephric mesenchyme or blastema. The cells of the metanephric mesenchyme induce the ureteric bud to sprout from the Wolffian duct and to grow and branch many times to give rise to an elaborate renal collecting system [12]. The branching signal from metanephric mesenchyme to Wolffian duct may be received and transduced by the tyrosine kinase receptor c-ret which is expressed by the early Wolffian duct epithelium and its ureteric bud derivative [13]. In support of this, it has been shown that transgenic knockout mice null for c-ret often fail to form ureteric buds and when they do form, they branch feebly and severe kidney dysgenesis results [14]. Normally, as the ureteric bud branches, the tip of each sprout goes on to induce a small number of neighbouring metanephric mesenchymal cells to proliferate and then aggregate before undergoing an elaborate mesenchymal-epithelial transformation which will eventually give rise to the secretory parts of the young nephron. This transformation involves evolution through the characteristic comma- and S-shaped body phases of tubulogenesis [12] (fig. 1A–D). At each stage during the aggregation and differentiation of a single nephron, various genes are upregulated and downregulated – most of these up- and down-regulations presumably provide us with, as yet, unsolved clues as to the molecular mechanisms underlying these processes but in the meantime have supplied us with good markers of the developmental phases of nephrogenesis [15]. From the earliest stages of metanephric development until after birth new nephrons are formed at the periphery, or nephrogenic rind, of the growing kidney displacing more mature nephrons towards the core or medulla of the kidney (fig. 1A).

**Uninduced Metanephric Mesenchyme Undergoes Catastrophic Programmed Cell Death**

Much of our early understanding of the inductive signals triggering nephron development in the metanephric mesenchyme derive from transfilter experiments of Grobstein [16] and more recently Saxen and Lehtonen [17] who cultured metanephric mesenchyme, isolated from ureteric bud epithelium, adjacent to various potential inducing tissues with only a millipore filter separating them. These studies showed that most other embryonic epithelia fail to supply the necessary signals to trigger tubule formation, but a number of neural tissues, particularly spinal cord, could mimic the inductive signals that were normally derived from the ureteric bud epithelium. From these experiments it also seemed that close cell contact might be necessary for signalling since millipore filters with pores too small to allow cell processes through, severely inhibited the inductive signals. In the absence of an inducer, be it ureteric bud or spinal cord, then early metanephric mesenchyme cells not only fail to differentiate but they die, suggesting that the inducing tissue is supplying a trophic or survival factor as well as the inductive signal [6]. This death occurs rapidly and fairly synchronously within a few hours of setting up the assay and thus resembles the catastrophic wave of cell death seen in the developing footplates of chick and mouse embryos described above. Moreover, when this uninduced mesenchyme is minced and tested for DNA degradation it reveals the classic apoptotic hallmark of cut, low-molecular-weight DNA [6]. This DNA fragmentation can be blocked either by culturing in the presence of an inducer, in this case spinal cord, or by inclusion in the culture
Fig. 1. Cell death in the developing metanephric kidney. A Resin section through an embryonic day (E) 14.5 mouse kidney showing development of new nephrons at the periphery with more mature nephrons displaced towards the medulla. B High-power detail of the periphery of a kidney at the same developmental age as that shown in (A). Note the presence of two apoptotic cells (arrows) adjacent to a newly formed comma-shaped body. C Detail of E14.5 metanephric kidney showing a clear apoptotic cell (arrow) in the tail of an S-shaped body. D Using the confocal laser scanning microscope to cut thin optical sections through an E14.5 wholemount kidney stained with the nuclear dye 7AAD (Molecular Probes), apoptotic bodies can be visualised as bright fluorescent dots (small arrows). Dividing cells can also be seen in the body of the nephron (big arrow). E Resin histology through the periphery of an E16.5 metanephric kidney. Apoptotic cells can be seen singly, either within or adjacent to tubular structures (small white arrows), or in clusters probably within a phagocytic macrophage (black arrow). Note the difference between apoptotic cells and red blood cells (arrowheads). Scale bars: A = 100 μm; B, C, E = 20 μm; D = 10 μm.
media of a calcium chelator which appears to effectively block the endonuclease of apoptosis, or by inclusion of epidermal growth factor (EGF) or phorbol esters in the culture media [6, and see later].

Equally dramatic cell death is seen in vivo in the metanephric mesenchyme of transgenic knockout mouse embryos null for the WTI gene [18]. In these mice the ureteric bud always fails to grow out towards the metanephric mesenchyme and by embryonic day 11 when the two tissues have normally made contact 10–50% of mesenchyme cells are apoptotic; 12 h later there are no mesenchyme cells left. However, this death is not simply due to the absence of an inductive signal because recombination experiments bringing wild-type spinal cord adjacent to WTI null mesenchyme fail to rescue the dying cells [18].

It seems that WTI, which is normally expressed by nephrogenic mesenchyme from 2 days prior to the onset of metanephric kidney formation, may be required in order to make the metanephric mesenchyme competent to respond to inductive signals. The likelihood that the signals coming from the inductive tissue to the metanephric mesenchyme are multistep, involving both trophic and differentiation triggers, is supported by another transgenic knockout mouse – the wnt4 null mouse [19]. Wnt4 is normally first expressed by aggregating cells in the metanephric mesenchyme and subsequently by their epithelial derivatives. In mice null for this gene, mesenchymal aggregation fails but various of the molecular markers associated with aggregation such as c-myec and Pax2 are expressed as normal and there is no obvious phase of ‘catastrophic’ cell death [19]. These observations suggest that the metanephric mesenchyme cells in these mice have been induced to progress some way beyond the normal phase of cell death but have been blocked prior to their successful differentiation. Culture experiments that block cell death provide further evidence that the inductive signal is not merely a survival signal and that surviving metanephric mesenchymal cells are not autonomously programmed to form tubules; if apoptosis is prevented with exogenous EGF, phorbol esters or with calcium chelators, then the rescued cells do not form tubules [6].

**During Normal Development up to 50% of Some Populations of Metanephric Cells May Die**

The studies described above reveal how in transfilter experiments without a suitable inducer or during aberrant kidney development in vivo, catastrophic levels of apoptosis can occur in the metanephric mesenchyme. It appears that in the absence of an ‘inductive’ signal, the default pathway for a mesenchymal cell is to die, but what about in normal development where the inductive signals do not fail? Two recent reports reveal that programmed cell death is very much a part of normal metanephric kidney development, but as in the developing nervous system it occurs in a ‘trickle-like’ fashion and so had previously been missed. The first of these papers [6] showed small numbers of apoptotic bodies adjacent to induced tubules within metanephric mesenchymal rudiments co-cultured with spinal cord in a transfilter assay. Sections of embryonic rodent kidneys from the early stages of nephron development right through to just prior to birth showed islands of apoptotic cells lying between developing nephrons [6] (fig. 1E). These morphological findings in situ were supported by DNA degradation assays which showed a small fraction of cut, low molecular weight DNA within the metanephric mesenchyme throughout the developmental stages analysed. A second paper [7] confirmed these observations of cell death using the DNA stain propidium iodide and also transmission electron microscopy to reveal apoptotic bodies in sections of developing kidney. This paper made a thorough study of the numbers and location of dying cells and describes two major zones of cell death. One zone is the nephrogenic periphery of the kidney where apoptotic cells can account for up to 3% of total mesenchymal cells in any one tissue section at early developmental phases, dropping to 1% or less by the time of birth. This pattern of cell death numbers tailing off during development was mirrored by a parallel reduction in the numbers of mitotic cells in the same nephrogenic zone, suggesting that cell birth and cell death might be tightly linked to one another during nephron formation (fig. 1D). Generally, apoptotic bodies in the nephrogenic zone were seen either adjacent to mesenchymal aggregations, or in the tails of newly formed S-shaped bodies (fig. 1C, D). Whilst a maximum of 3% dying cells does not immediately sound impressive, careful estimates of clearance rate, largely extrapolated from what is known for other developing tissues and likely to be in the range of 1–2 h, suggested that actually up to 1 cell for every 2 that are born may die in this region of the kidney. The second zone of programmed cell death was seen in the medullary papilla and largely peaked after birth. Some of these later cell deaths were associated with the walls of tubular structures including branches of the ureteric bud, which is seldom, if ever, seen in the earlier nephrogenic phase of cell death.
What Is the Role of Programmed Cell Death in the Developing Kidney and Why Do the Cells Die?

So why do so many metanephric cells die during kidney development? There is clearly strong evidence, as outlined above, that mesenchymal cells normally require some trophic or survival signal from the inductive tissue since, if they are denied an inducer, they all undergo catastrophic cell death. It is likely that even during normal development only a proportion of the mesenchymal cells will lie close enough to a branching ureteric bud tip to receive sufficient trophic signal. Certainly, it is clear in transfilter assays where the surface area of inductive tissue is lying adjacent to the metanephric mesenchyme is severely limited that the majority of surviving cells lie close to the millipore filter and the total number of cells surviving is much less than in situ where the ureteric bud can branch and reach more cells. What might such a trophic factor be? The favourite candidates appear to be one of the EGF family of growth factors, either EGF itself or transforming growth factor-α (TGF-α), since exogenously applied EGF can rescue cells from death both in vitro, in transfilter assays [6], and in vivo, by intraperitoneal injection of newborn rat pups [7]. Antibodies against TGF-α completely block tubulogenesis in organ culture studies [21], but curiously transgenic TGF-α deficient mice and waved-2 mice with a point mutation in the gene encoding the common EGF/TGF-α receptor (which severely disables autophosphorylation and thus signalling of this receptor) seem to suffer little, if any, disruption to normal kidney development [22, 23]. Since phorbol esters seem also able to rescue mesenchyme cells from death and staurosporin, an inhibitor of protein kinase C, prevents the rescue effect of an inducer, it is possible that the metanephric trophic signal could be operating via a protein kinase C as well as, or instead of, a tyrosine kinase receptor pathway [6].

Whatever way it turns out that metanephric apoptosis is regulated, there remains the question of its role in normal kidney development. It has been speculated that death may be a way of matching numbers of nephrogenic mesenchymal cells with partner epithelial cells of the ureteric bud [7], but the most direct way to answer this question will be from analysis of kidneys where the extent of cell death has been tinkered with to make it either less than, or more than that which occurs in normal development. In that regard analysis of early kidney development in transgenic knockout mice null for bcl2, the cell death blocking gene, is instructive. The kidneys in these newborn mice are described as having increased levels of apoptosis and apparent hyperproliferation of immature epithelial structures to give a generally polycystic kidney [24]. In a later paper [25] metanephric kidneys were taken from bcl2 knockout embryos at E12 and cultured for several days. Overall growth of the bcl2 null kidneys was very restricted and histological study revealed significantly increased levels of apoptosis when compared to heterozygote and wild-type kidneys, and a much reduced nephrogenic zone with minimal numbers of new nephrons being formed. Having now established the effects of too much cell death on nephrogenesis an obvious, and very important, next step will be to examine kidney development in a mouse where metanephric cell death has been somehow genetically abolished.

How Are the Dying Cells Cleared Away?

Given that about half of all mesenchymal cells in the developing kidney might normally be dying it is of significant interest to discover how they are all cleared away, particularly since it is the efficiency of this clearance that hid the extent of cell death from observers until recently. In embryonic systems where programmed cell death is 'catastrophic', for example in the developing footplate, it was classically believed that the cells responsible for clearing dead cells were simply non-specialists derived from surviving, neighbouring fibroblastic cells [2, 26]. However, we have recently shown using antibodies specific for cells of the haemopoetic monocyte/macrophage lineage, that the majority, if not all, of the phagocytes in the mouse footplate are specialist macrophages and that they are first recruited at or shortly after the onset of cell death in the mesenchyme of the interdigital space [5].

It is possible that the story is quite different in organ systems where cell death is not 'catastrophic', rather is 'trickle-like' as in the kidney. Perhaps if cell death is spread out over a long period of time then less efficient, non-specialist phagocytes can cope with the job of clearance. Indeed, it has been suggested that the cells responsible for phagocytosing dying metanephric mesenchymal cells are probably surviving neighbouring parenchymal cells [7], because it is very unusual to see a cell with the classic morphological appearance of a swollen macrophage in light-microscopic or transmission electron-microscopic (TEM) studies of the developing kidney. However, using the same monocyte/macrophage specific F4/80 monoclonal antibody [27] previously used for our limb studies, we have recently shown the presence of macrophages within the metanephric mesenchyme from the
first stages when cell death is observed [28]. Generally, these macrophages adopt the appearance of mesenchymal fibroblasts and cannot be morphologically distinguished from them except by immunohistochemistry (fig. 2A), but occasionally we find F4/80 positive cells that are clearly swollen with phagocytosed debris (fig. 2B). In TEM sections, we find these cells in very close apposition to neighbouring mesenchymal cells as they patrol the tissue in search of debris (fig. 2C). It seems likely then that just as in the limb, specialist macrophages are responsible for clearing away apoptotic cells in the developing kidney but, quite unlike the limb, because of the relatively small amount of cell death present at any given time, kidney macrophages are rarely caught in the act of engulfing or digesting their lunch. At a more speculative level there is the possibility that macrophages in the developing kidney might be doing more than merely clearing dying cells away. An exciting recent paper [29] shows that targeted killing of macrophages in the developing murine eye results in various populations of cells that normally would die failing to die and suggests that macrophages may actually be primarily responsible for triggering apoptosis in some locations. Similar macrophage depletion experiments, this time targeting the developing kidney rudiment would allow us to more fully dissect out the various roles that macrophages might be playing during metanephric development.

Acknowledgement

V.C. gratefully acknowledges the funding of an MRC studentship.

Fig. 2. Macrophage engulfment of dead cells in the developing metanephric kidney. A F4/80 immunohistochemistry of sections through E15.5 mouse kidney showing macrophages (dark staining) in close apposition to developing glomeruli and tubular structures. Note these macrophages morphologically resemble neighbouring parenchymal cells. B An adjacent section to that shown in (A) reveals a macrophage caught in the act of phagocytosis (arrow). C A transmission electron micrograph of E14.5 kidney, showing a phagocytic cell, probably a macrophage, containing a number of apoptotic bodies. Arrowheads delineate the margins of this cell. Note the filopodial protrusions characteristic of a motile cell. Scale bars: A, B = 20 μm; C 2 μm.
References

ORIGINAL ARTICLE

Victoria Camp • Paul Martin
The role of macrophages in clearing programmed cell death in the developing kidney

Accepted: 28 February 1996

Abstract The metanephric kidney develops from two tissue sources, the metanephric mesenchymal blastema and the ureteric bud epithelium. Following a complex interplay of inductive signals between these two tissues, small groups of metanephric mesenchymal cells aggregate and epithelialise to form young nephrons. As this is happening, significant numbers of cells in close proximity to the forming nephrons undergo programmed cell death or apoptosis. In this paper we investigate the clearance of developmental cell death in the mouse kidney between embryonic days 11.5 and 16.5; specifically, we address the issue of whether specialist macrophages or non-specialist neighbouring mesenchymal cells are responsible for phagocytosis and removal of dying cells. We show, using a monoclonal antibody F4/80 that specifically recognizes murine macrophages, that whenever and wherever there is cell death in the developing mesonephric or metanephric kidney there are also haemopoietically derived specialist macrophages. Moreover, in the mesonephros and from E14.5 in the metanephric kidney, we see large numbers of macrophages clearly swollen with phagocytosed apoptotic bodies. Double-labelling experiments using the DNA dye 7AAD to reveal condensed apoptotic nuclei and F4/80 to reveal macrophage plasma membranes show definitively that the majority of dying cells in the developing kidney are engulfed by macrophages.

Key words Mouse embryo • Macrophages • Mesonephros • Metanephros • Apoptosis

Introduction

Apoptosis, or programmed cell death, is now believed to be of fundamental importance during development of all multicellular organisms, where it appears to be used by the embryo as a tool to get rid of unwanted cells (Glucksman 1951; Saunders 1966; Wyllie et al. 1980; Hinchliffe 1981; Clarke 1990; Ellis et al. 1991; Raff 1992). In some systems apoptosis allows elaborate tissue sculpting of embryonic organs or structures, as for example in the developing amniote footplate, where the digits become separated by death of interdigital mesenchyme cells (Hinchliffe and Johnson 1980; Garcia-Martinez et al. 1993; Zakeri et al. 1994). This almost synchronous death of many cells in a particular zone is "catastrophic" and is easily visualised in histological sections of these tissues as large accumulations of dense and darkly staining apoptotic bodies. However, in other tissues, such as the developing nervous system, programmed cell death is not "catastrophic"; rather, it occurs in a "trickle-like" fashion over a relatively long period of development (Oppenheim 1991; Raff 1992; Barres et al. 1992) and in these situations it had until recently been largely overlooked and its significance in development ignored. Two recent papers (Koseki et al. 1992; Coles et al. 1993) have shown that the metanephric kidney, like the nervous system, undergoes a protracted period of "trickle-like" cell death that lasts beyond birth, during which time up to 50% of mesenchymal cells in the nephrogenic zone will die. As in the developing nervous system, the reason why this extensive cell death went undetected for so long is that as each cell dies it is rapidly phagocytosed and cleared away by other cells.

Our interest lies in how such large numbers of dying cells are so efficiently cleared away as various organ systems develop. The general dogma has been that neighbouring embryonic mesenchymal cells phagocytose the dead cells (Glucksmann 1951; Raff 1992; Garcia-Martinez et al. 1993; Coles et al. 1993), but we have recently shown that, where the cell death is "catastrophic", as in the case of interdigital programmed cell death in the mouse footplate, specialist haemopoietically derived macrophages are rapidly recruited to the site of cell death and are responsible for phagocytosis and clearance of dying cells (Hopkinson-Woolley et al. 1994). In this paper we extend our study to the developing kidney, where cell death occurs in a "trickle-like" fashion.
Fig. 1A–J Resin histology of developing mouse kidneys from E11.5 to E16.5. A A low magnification longitudinal section (LS) through the regressing E11.5 mesonephros. B High magnification view of a similar section to that in A to show numerous dying cells (arrows) in and directly adjacent to the epithelium of a degenerating mesonephric tubule. C Another higher magnification detail from the E11.5 mesonephros showing a cluster of apoptotic bodies probably enveloped within a macrophage (arrow) adjacent to a tubule. D A low magnification view of the early metanephric kidney at a stage just after the ureteric bud (ub) has first branched within the metanephric mesenchyme (E12.5). E Detail from D revealing small numbers of dying cells (arrows) in the metanephric mesenchyme. F A low magnification view of the E14.5 metanephric kidney showing early developing nephrons. G A high magnification view of the nephrogenic region in an E14.5 kidney. Occasional condensed apoptotic nuclei (arrow) can be seen in the tail regions of comma-shaped bodies. H Another E14.5 detail showing a cluster of dead cells (arrow) in the mesenchyme adjacent to a developing tubule. I A low magnification view of an E16.5 kidney with a well developed peripheral nephrogenic zone. J A high magnification view of the nephrogenic zone at E16.5 showing individual apoptotic nuclei (small arrows) and a phagocytic cell laden with a number of engulfed cells (large arrow). Bars A,D 100 μm; B 50 μm; C 15 μm; E,G,H,J 25 μm; F,J 200 μm
The kidney develops in three phases (pro-, meso- and metanephros), each of which derives at least partially from paired rods of intermediate or nephrogenic mesoderm that lie between the somitic and lateral plate mesoderm of the neural plate stage embryo. The nephric or Wolffian duct is a central player during each of these phases, serving as the drainage channel for pro- and mesonephros and then giving rise to the ureteric bud of the metanephric kidney. The two early kidney phases differentiate from the most cranial nephrogenic mesenchyme but both are transient structures, undergoing a wave of "catastrophic" programmed cell death not dissimilar to that described above for the developing footplate (Saxen 1987; Ekblom 1991).

The third nephric system, which will become the definitive or metanephric kidney, is formed by reciprocal inductive interactions between the ureteric bud and the most caudal nephrogenic mesenchyme, which condenses to form the metanephric mesenchyme or blastema. The cells of the metanephric mesenchyme induce the ureteric bud to sprout from the Wolffian duct and to grow and branch many times to give rise to an elaborate renal collecting system (Jokelainen 1963; Saxen 1987; Ekblom 1991; Bard 1992). As the ureteric bud branches, the tip of each sprout goes on to induce a small number of neighbouring metanephric mesenchymal cells to proliferate and then aggregate before undergoing an elaborate mesenchymal-epithelial transformation that will eventually give rise to the secretory parts of the young nephron. This transformation involves evolution through the characteristic comma- and S-shaped body phases of tubulogenesis (Saxen 1987, and see Fig. 1C–J). From the earliest stages of metanephric development until after birth new nephrons are formed at the periphery, or nephrogenic rind, of the growing kidney, displacing more mature nephrons towards the core or medulla of the kidney. Coles et al. (1993) analysed the extent of cell death in the metanephric kidney of the rat embryo from a stage equivalent to about embryonic day 17 (E17) in the mouse. Their data showed low indices of apoptosis over a relatively long developmental period extending up to and beyond birth. Any one tissue section during this period revealed that between 1 and 3% of all cells were apoptotic and this cell death was largely restricted to the nephrogenic zone. Whilst a maximum of 3% dying cells does not immediately sound impressive, careful estimates of clearance rates, largely extrapolated from what is known for other developing tissues (Perry et al. 1983), and likely to be in the range 1–2 h, suggested that as many as one cell from every two that are born may die in this region of the kidney.

In this paper we have examined the extent and location of programmed cell death in both the mesonephros and early stages of metanephric kidney development from E11.5 until E16.5, and have gone on to investigate whether invading specialist macrophages, rather than neighbouring parenchymal cells as suggested by Coles et al. (1993), might be responsible for clearing away this death in the way that we have previously shown for dying interdigital cells in the developing limb (Hopkinson-Woolley et al. 1994).

Materials and methods

For these studies we used an outbred strain of Albino mouse, strain CD1 (Olac). Gestational age was calculated on the assumption that conception had occurred at midnight preceding the morning a vaginal plug was seen. The embryos were staged according to Thaller (1989) and Martin (1990), using the shape of the fore- and hindlimb buds as developmental indicators. Embryos from E11.5 to E16.5 were processed for light and transmission electron microscopy, and for F4/80 immunohistochemistry in order to allow analysis of apoptosis and macrophage distribution within the developing kidney.

Resin histology and transmission electron microscopy

Embryos for resin histology and transmission electron microscopy (TEM) were fixed in ice-cold half-strength Karnovsky fixative (Karnovsky 1965), rinsed in 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide before being dehydrated through a graded ethanol series. Specimens for resin histology were embedded in Araldite and sections of 1–5 pm cut, mounted on poly-L-lysine coated slides and stained with toluidine blue. Dissection and trimming of specimens for resin histology was performed in buffer prior to post fixation in osmium tetroxide. A number of specimens were also examined by TEM. In those cases ultrathin resin sections were cut, stained with uranyl acetate and lead citrate in the usual way and examined using a Jeol 1010 transmission electron microscope.

F4/80 immunohistochemistry

In preparation for immunohistochemistry, embryos were fixed overnight in ice-cold Bouin's fixative (25% formalin; 70% saturated picric acid; 5% acetic acid), and thoroughly rinsed in 70% alcohol before dehydration through graded alcohols. Specimens were then embedded in Fibrowax (BDH) and 8 pm sections were cut on a Leica microtome and mounted on poly-L-lysine coated slides. The sections were rehydrated, rinsed in PBS and soaked in 0.3% hydrogen peroxide (H2O2) in methanol for 30 min to block endogenous peroxidase. They were rinsed again in PBS before rabbit serum (1:100; Vector Laboratories) was applied to the sections for 30 min, to block non-specific binding of the antibody. The sections were then incubated with 8.5 μg/ml F4/80, a macrophage specific rat anti-mouse monoclonal antibody (Austyn and Gordon 1981; Morris et al. 1991) for 90 min. The sections were washed in PBS and bound antibody was detected using biotinylated mouse adsorbed rabbit anti-rat IgG (5 μg/ml; Vector Laboratories) and the avidin-biotin-peroxidase complex (Vectorstain ABC kit, Vector Laboratories). To detect the peroxidase activity 0.5 mg/ml of diaminobenzidine tetrahydrochloride (DAB; Sigma) in 10 mM imidazole at pH 7.4 and an equal volume of 0.02% H2O2 was used. This was rinsed off with tap water, followed by distilled water and counter-stained with Mayer's haemalum. The sections were dehydrated through graded alcohols and mounted in XAM (BDH). Those sections in which either primary antibody, secondary antibody or ABC reagents were omitted were negative.

Double labelling of sections for apoptosis and macrophages

To visualise both apoptotic cells and macrophages in the same specimens we performed a double immunofluorescent study on frozen sections. Specimens were fixed in 4% paraformaldehyde at 4°C for 1 h, rinsed in PBS, incubated first in 5% sucrose in PBS
and then in 20% sucrose in PBS prior to embedding in a gelatin-sucrose (7.5%:15%) mix. 10 μm thick sections were cut on a Leica cryostat and mounted on poly-L-lysine coated slides. These sections were first incubated with F4/80, using an identical protocol to that described above for wax sections except that the second antibody was a FITC-tagged mouse adsorbed rabbit anti-rat IgG (10 μg/ml; Vector Laboratories). Sections were further incubated with 5 μg/ml 7-aminoactinomycin D (7AAD; Molecular Probes) in PBS for 20-30 min at room temperature. The sections were rinsed in PBS and mounted in Citifluor (UKC). Double-staining was revealed using the appropriate filter blocks on a Leica Diaplan fluorescent microscope and specimens were subsequently viewed and photographed using a Leica TCS4D confocal laser scanning microscope (CLSM). Small numbers of metanephric kidneys between E12.5 and E14.5 were also viewed as wholemount preparations under the CLSM. These specimens were dissected free of the embryo, fixed in 4% paraformaldehyde as above, rinsed in PBS, incubated overnight with 2.5 μg/ml FITC-phalloidin (Sigma) and 100 μg/ml 7AAD in PBS and rinsed again before mounting beneath a coverslip in Citifluor. Optical sections through such specimens allowed good observation of apoptosis in intact developing nephrons. Images were photographed directly from the monitor of the CLSM.

Results

A wave of programmed cell death spreads through the regressing mesonephros

The mesonephros first begins to differentiate at about E10 in the mouse and soon after begins to regress (Saxen 1987; V. Camp and P. Martin, unpublished observations). By E11.5, although regression is well underway, resin histology shows a fairly extensive mesonephros extend-
soon after the ureteric bud had first entered the condensated metanephric mesenchyme, we saw small numbers of apoptotic cells scattered within the mesenchyme (Fig. 1D,E) but none in the highly proliferating ureteric bud epithelium.

At E13.5 and E14.5 the kidney is undergoing significant morphogenesis with branching of the ureteric bud and induction of nephrogenic tubules from condensed metanephric mesenchymal aggregates at the tips of the ureteric bud’s branches. At these stages we see occasional apoptotic cells in the surrounding mesenchyme, as in earlier stages, and now we also see dead cells in the mesenchymally derived epithelial structures, most frequently in the tails of comma-shaped presumptive nephrons (Fig. 1F–H). This is perhaps most clear in optical sections through wholemount kidneys where we have been able to observe intact developing nephrons (Fig. 3H). By E16.5 cell death has become largely restricted to the outer area or nephrogenic zone of the kidney, where apoptotic cells are seen in and around the developing nephric tubules (Fig. 1L,J), as previously reported by Coles et al. (1993). Throughout this period we found no sign of dead cells within the branching ureteric bud epithelium. TEM studies of the developing metanephric kidney show that all but a small number of apoptotic cells have been engulfed by phagocytic cells, but by contrast with the mesonephros or developing limb, each phagocyte generally only contains one or two apoptotic bodies (Fig. 2B,C).

Throughout mesonephric and metanephric development, wherever there is programmed cell death there are also macrophages.

We have used the monoclonal antibody F4/80, which recognizes an epitope on the plasma membrane of murine monocytes and macrophages, to immunohistochemically reveal macrophages in wax sections of the developing mesonephric and metanephric kidney at stages between E11.5 and E16.5. F4/80 staining demonstrates that macrophage distribution correlates very closely with the distribution of apoptotic cells as described above. In the mesonephros we see a pronounced recruitment similar to that found in the limb. The numbers of macrophages are far higher here than in adjacent regions of loose connective-tissue at this developmental stage. The macrophages are found located next to the mesonephric tubules where programmed cell death is occurring as the organ is regressing (Fig. 3A–C). Many of the macrophages we see in the regressing mesonephros are apparently swollen, having recently engulfed cell debris (Fig. 3C).

A day later (E12.5) in the early metanephric kidney, we find relatively few macrophages in the metanephric mesenchyme where our resin histology had revealed there to be only occasional cell death (Fig. 3D,E). No macrophages were found in or immediately adjacent to the ureteric bud epithelium where there was no cell death. Most of the macrophages we observe at this stage have a very similar morphology to neighbouring parenchymal cells and are only distinguishable because of the F4/80 antibody. At E12.5 we rarely find obviously swollen macrophages containing many phagocytosed cells (Fig. 3E).

By E14.5 macrophages are present in higher numbers both in the kidney and the surrounding loose connective tissue. Macrophages are found no longer just in the metanephric mesenchyme but also in close apposition to the developing pre-nephric epithelial aggregates, usually hugging the tail region of comma-shaped bodies where our resin histology had previously showed dying cells to be localized (Fig. 3F–H).

Swollen macrophages containing apoptotic bodies become more frequent in the E14.5 metanephric kidney at E14.5, when the earliest mesenchymal condensations have just started to differentiate into nephrons with immature glomeruli, macrophages were found in and around these epithelial structures (Fig. 3F,G). E14.5 was also the first stage when we found significant numbers of F4/80-positive cells in the metanephros that were noticeably swollen with phagocytosed dead cells (Fig. 3G,I). At these stages of kidney development, unlike the earlier mesonephric stages, numbers of macrophages were not obviously greater within kidney tissues than in adjacent loose connective tissue. However, at all of the stages we looked at, wherever there is cell death in the developing kidney there are macrophages also present, and in both the mesonephros and the metanephros (at least from E14.5) those macrophages that are present are active and capable of phagocytosing cellular debris.

Double labelling experiments show definitively that macrophages engulf apoptotic cells in the metanephric kidney.

Whilst our independent apoptosis and macrophage studies allow us to correlate the distributions of these two cell populations, direct evidence that macrophages engulf dying cells requires double-labelling experiments. We cut frozen sections of a number of E14.5 metanephric kidneys and double-stained them with 7-AAD to reveal apoptotic bodies and F4/80 for detection of macrophages. These studies reveal brightly staining apoptotic bodies almost always enveloped in an F4/80 positive plasma membrane (Fig. 3I), suggesting that the majority, if not all, of cell death at these stages is cleared away by haemopoietically derived macrophages. Occasional single apoptotic nuclei are seen that have not yet been engulfed, as are a number of macrophages without obvious apoptotic bodies within them (data not shown).
is “trickle-like” as in the metanephric kidney; it could be argued that in developmental situations where cell death is spread out over a long period, then less efficient, non-specialist, phagocytes could cope with the job of clearance. However, our studies reported in this paper suggest that whether cell death is “catastrophic”, as in the mesonephros, or “trickle-like”, as in the metanephros, specialist macrophages are the major players in clearance of apoptosis. However, since we do occasionally find apoptotic bodies outside of F4/80-positive cells we cannot definitively say that all programmed cell death in the kidney is cleared away by patrolling macrophages. It is certainly true that non-specialist cells are capable of phagocytosis. For example, it is well established that endothelial cells lining embryonic blood vessels are phagocytic (Latker et al. 1986), and tissue culture studies show that other non-professional phagocytes including fibroblasts, epithelial cells and tumour cells are all capable of phagocytosing apoptotic cells (Wyllie et al. 1980; Duvall and Wyllie 1986; Hall et al. 1990). Moreover, in the adult kidney it has been shown that glomerular mesangial cells, which are not from the monocyte lineage, will engulf spent neutrophils at sites of inflammation (Savill et al. 1992). However, at all stages and all locations where there is cell death in the developing metanephric kidney we find that macrophages are also present. In fact from E14.5 the presence of swollen macrophages and our double-staining 7AAD/F4/80 studies show definitively that these macrophages have been actively phagocytosing dying cells.

What might be the signals that lead to macrophage recognition of dying cells in the embryonic kidney? Whilst it is possible that macrophages could be attracted to regions of programmed cell death by medium-range chemotactic signals it is also likely that recruitment and recognition are driven by short-range or cell-to-cell contact-mediated interactions dependent on macrophages constantly patrolling the tissue. In this regard there are various receptors on the macrophage surface such as the scavenger receptor membrane glycoprotein (Hughes et al. 1995) that might allow it to recognize the “edible” status of apoptotic cells. In the nematode worm Caenorhabditis elegans, whilst there is no evidence for specialist macrophage-like cells, a series of genes have been identified which are necessary for successful phagocytosis and clearance of apoptotic cells by neighbours (Ellis et al. 1991). Presumably vertebrate homologues of these genes will encode at least some of the proteins used by specialist macrophages to recognize, bind to and engulf dying cells in the developing kidney and elsewhere.

Ours is certainly not the first study to suggest that macrophages play several important phagocytic roles during embryogenesis. It appears that most developmental cell death in Drosophila is cleared away by specialist phagocytes called hemocytes, leaving only a small minority of cells to be engulfed by non-specialist epidermal cells (Tepass et al. 1994). Of direct relevance to this study, Rotello et al. (1994), using new antibodies raised against chick phagocytic cells, show apoptotic cells in the avian mesonephros being engulfed by what are al-
most certainly macrophages, and an earlier in vitro study by De Felici et al. (1986) showed that macrophages are indeed present in the murine urogenital ridge from early stages. At later stages of development it has been shown that there is recruitment of F4/80 monocytes into the retina (Hume et al. 1983) and brain (Perry et al. 1985) in response to programmed cell death of neuronal cells. Recent studies by Lang and Bishop (1993) and Lang et al. (1994) suggest that macrophages might even be responsible, not only for clearing away cell death, but in some tissues of the developing eye for actually triggering cell death. Lang and Bishop (1993) expressed diphtheria toxin in a promoter specific to a small population of macrophages including the hyalocytes of the developing eye. In resulting transgenic mice they found that hyalocytes were killed and that two collections of capillary blood vessels that are normally transient in the embryonic eye failed to undergo programmed cell death, suggesting that hyalocytes were necessary to elicit their cell death. Analogous macrophage depletion experiments in the kidney, either genetic or by antibody-targeted or chemical killing of macrophages, are needed to fully dissect any other roles besides simple clearance that macrophages might be playing during the remodelling phases of kidney development.

Acknowledgements We are grateful to the MRC and UCL for funding this study. We also thank Professor Sianmon Gordon, for his gift of F4/80 antibody, Dr. Adrian Woolf for guiding us in the way of kidneys, Mark Turmaine and Suhel Miah for help with TEM, and Professor Sianmon Gordon and Dr. Sue Nodder for their critical comments on the manuscript.

References