Programmed Cell Death in the Developing Kidney and the Ubiquity of the Programme

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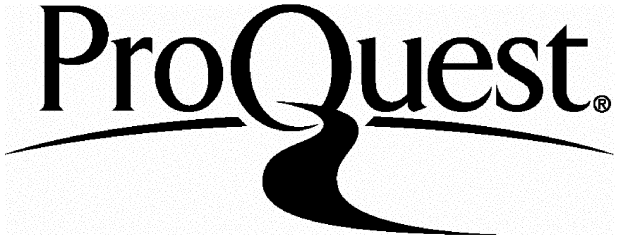

Abstract

It has recently been suggested (Raff 1992) that all cells except blastomeres die by programmed cell death unless signalled to survive. In this thesis I explore implications of this idea by asking whether there are cases of developmental cell death that have been overlooked, and whether cell death is induced in many cell types by blocking protein kinase activity and protein synthesis.

Normal cell death was not considered to be important in mammalian kidney development. I have found, however, that cell death occurs with distinct time courses, in the nephrogenic region and medullary papilla of the developing rat kidney. Up to 3% of cells in these areas are apoptotic, and are cleared within 1-2 hours by phagocytosis. These values are similar to those in vertebrate neural tissues where 50% or more of the cells die during normal development, suggesting that large scale death is a normal feature of kidney development. *In vivo* treatment with epidermal growth factor or insulin-like growth factor inhibits kidney cell death suggesting that this normal cell death may reflect insufficient survival factors.

Raff (1992) suggested that all cells depend on survival factors in order to avoid cell death. Blocking protein kinase activity (and consequently cell signalling) and protein synthesis in a variety of neonatal rat tissue explants and preimplantation blastocysts induces 90% cell death within 18 hours. In contrast, blocking protein kinases and protein synthesis in 2-4 cell stage blastomeres does not induce apoptosis. These findings suggest that most cells, except blastomeres,

constitutively express the protein components of the cell death programme.

I conclude that cell death during vertebrate development is more extensive than was previously thought, that normal cell death may often reflect limiting supplies of survival factors, and that blastomeres differ from even their earliest derivatives in the way cell survival and death are controlled. These findings support the idea that all cells except blastomeres require constant signalling from other cells in order to avoid programmed cell death.

"Oh well, no matter what happens, there's always death."

Napoleon, 1817

"If there wasn't death, I think you couldn't go on."

Stevie Smith, 1969

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Chapter 1

General Introduction

Vertebrate development, from the fertilised egg to the adult animal, has been described mainly in terms of cell proliferation and differentiation; there is, however, increasing evidence that programmed cell death (PCD) has an equally important role in this process. The research community has been slow to consider PCD as a part of the cell biology of multicellular organisms and it is likely that there are still cases of PCD during development that have been overlooked. In this thesis I describe one such case - PCD in the development of the rat kidney. I also ask whether all cells in developing multicellular animals are capable of undergoing PCD.

Apoptosis and cell necrosis

Cells that undergo PCD usually die with morphological features collectively called *apoptosis*. This type of cell death is morphologically distinct from most cell deaths that result from acute injury, a process called *cell necrosis* (Kerr et al., 1972; Wyllie et al., 1980). In necrosis, the cell, its organelles and nucleus usually swell and lyse, spilling cytosolic components into the extracellular space, which elicits an inflammatory response in which the cellular debris is phagocytosed by macrophages. In apoptosis, the cell and its nucleus shrink and often fragment, and the cell or its fragments (called apoptotic bodies) are rapidly phagocytosed by either neighbouring cells or macrophages before there is any leakage of cytosolic contents. Thus in apoptosis unwanted cells are removed discretely and tissue integrity is maintained. While necrosis usually affects a group of cells in the affected region, apoptosis often occurs selectively in cells scattered throughout a tissue.

The term "apoptosis" was introduced to distinguish between accidental cell death that results from injury (cell necrosis) and normal active, or programmed cell death (Kerr et al., 1972). Considerable variations in the morphology of cells undergoing PCD have been reported (Clarke, 1990; Lockshin and Zakeri, 1991). In some cell types, for example, the nuclear DNA is cleaved into oligonucleosomal fragments (Wyllie 1980; Wyllie et al., 1984), while in other cell types it is not (Howell and Martz, 1987; Lockshin and Zakeri, 1991, Martin 1993). These variations are likely to be due to different cell phenotypes and tissue organisations, although they could, in principle, reflect distinct mechanisms of cell death. Therefore, I shall use "normal cell death" (NCD), "apoptosis" and "programmed cell death" (PCD) as general and interchangeable terms for cells undergoing active cell death.

The study of normal cell death: an overview

Compared with the extensive research on the control of cell proliferation, attention has only recently been turned to the control of cell survival and death. For the past 100 years, the potential relevance of cell death to the control of body size has been debated, and researchers in developmental biology, pathology and histology have noticed predictable and discrete cell death occurring throughout vertebrate development. In 1951, Glucksmann catalogued the diverse observations that implicated normal cell death in almost all developing vertebrate tissues; his aim was to "stimulate interest in the study of these degenerations as one of the mechanisms of the integration of cells into tissues and organs". He also emphasised the significance of normal cell death as a controlled phenomenon. In 1966, Saunders raised the important question of

whether these predictable cell deaths were executions or suicide. In 1972, Kerr, Wyllie and Currie defined a group of morphological characteristics that distinguish cells dying by normal cell death from cells dying by necrotic cell death. Thus they provided a framework for the systematic investigation of cell death in the absence of a molecular marker for normal cell death; also, they named the process "apoptosis" and proposed that it is an active form of cell death - a cell suicide.

In the absence of a molecular markers for apoptotic cells the evidence for a suicide programme in vertebrate cells remained circumstantial consisting mainly of the predictable timing, location and common morphology of apoptotic cells, and, in some cases the dependence of normal cell death on *de novo* protein synthesis (Wyllie et al., 1980; Martin et al 1988; Oppenheim et al., 1990; Schwartz et al., 1990). Furthermore, the pattern and extent of developmental cell death varies between closely related species, indicating that the regulation of cell death is open to selection during evolution and therefore has a genetic basis (Snow 1987). In contrast, studies on nematodes clearly defined the genetic basis of programmed cell death in invertebrates. In *Caenorhabditis elegans,* 131 of the 1090 somatic cells in hermaphrodites undergo programmed cell deaths, which are cell autonomous, morphologically similar to apoptosis, and affect the same cells in all individuals (Sulston and Horvitz, 1977; Hedgecock et al., 1983). Mutants defective in these cell deaths have identified 14 genes that are involved in: (1) the specification of cells to the cell death pathway (Trent et al., 1983; Ellis and Horvitz, 1986); (2) the mechanism and regulation of cell death (Ellis and Horvitz, 1986; Hengartner et al., 1992); and (3) the clearance of dead cells by phagocytosis and degradation (Hedgecock et al., 1983; Ellis

et al., 1991). The discovery of mammalian homologues of one of the genes required for normal cell death in worms (Tsujimoto et al., 1985; Yuan et al., 1993) and of a family of mammalian, cell death suppressor genes (Boise et al., 1993; Oltavi et al., 1993) has confirmed that normal cell death in vertebrates depends on an intrinsic suicide programme that has been highly conserved in evolution from worms to humans.

Recently it has been proposed that the programme for cell death is a fundamental feature of the cells of multicellular animals, and that all cells (except blastomeres) will kill themselves unless continuously signalled not to do so (Raff 1992). The implications of this idea are that PCD may occur more extensively and on a larger scale than previously thought and that limiting supplies of survival factors may be a common mechanism for regulating cell populations. The experiments presented in this thesis explore these possibilities.

The significance of programmed cell death

Programmed cell death occurs in many animal tissues at some time in their development (Glucksmann, 1951), where it serves to eliminate unwanted cells (Ellis et al., 1991). This programmed cell death underlies the fusion or separation of tissues such as neural tube closure (Glucksmann 1951) and palatal shelf formation (Hassell and Pratt, 1977; Shah 1979), the sculpting of digits from the limb-bud of amniotes (Saunders and Fallon, 1967), the numerical matching of interacting populations of cells such as the innervation of muscle by motor neurones (Hamburger and Levi-Montalcini, 1949; Oppenheim 1985), the restriction of cell location such as during the migration of primordial germ cells along the gonadal ridge (Godin et al., 1991), the negative selection of self-

reactive lymphocytes (Kappler et al., 1987; Kislielow et al., 1988), the regression of exclusively embryonic or vestigial structures such as the pronephros and metanephros and the Wolffian duct in females (Clarke 1982), and the reabsorption of larval structures during metamorphosis in holometabolous insects and amphibia (Goldsmith 1966; Kerr et al., 1974).

PCD also occurs in adult organisms where it also serves to eliminate unwanted cells. It is important in limiting inflammatory responses (Savill et al., 1993), in cytotoxic T-lymphocyte killing of target cells (Russell et al., 1983; Golstein 1987), in the prevention of oncogenesis, in the disposal of virally infected cells (Bursch et al., 1992), and in the preparation of the uterine epithelium for implantation of the embryo and placenta formation (El-Shershaby and Hinchliffe, 1974; Enders et al., 1981). PCD also underlies the involution of cyclically stimulated endocrine tissues, such as the involution of the endometrium during the menstrual cycle (Claman 1972; Verhage et al., 1984). It may also be important in the homeostasis of organ size in general: for instance, the liver, after experimentally induced hyperplasia, returns to its original size by a large increase in PCD (Bursch et al., 1984; Columbano et al., 1985). PCD in the adult organism has, however, been less intensively studied than in the embryo, and many examples of PCD may have been overlooked.

The diversity of functions to which PCD has been applied during evolution underlies its importance in the biology of multicellular organisms. An inevitable consequence of this, however, is the subversion of PCD in the evolutionary arms race between parasite and host. Just as there are viral genes that hijack the cell cycle to allow successful viral reproduction in host cells, so there are viral genes, such as BHRF1 that

suppress PCD and so prolong the life-span of its host cell (Henderson et al, 1991). Moreover, while some oncogenes promote cell proliferation thus contributing to oncogenesis, others, such as *bcl-2* suppress PCD thus contributing to both oncogenesis (Vaux et al., 1988; Strasser et al., 1991a) and possibly to metastases (Raff 1992).

The cell death programme and its intracellular regulation

Studies on both nematodes and vertebrates are beginning to sketch a cell death pathway within dying cells. Two nematode genes - *ced-3* and *ced-4* - are essential for PCD since loss of function mutations in either, block all PCDs (Ellis and Horvitz 1986); genetic mosaic analysis shows that both act in the dying cell or its mother cell (Yuan and Horvitz, 1990; Yuan and Horvitz, 1992; Yuan et al., 1993). A third gene - *ced-9* - is a suppressor of PCD, since gain-of-function mutations of *ced-9* block all PCDs. Normally, *ced-9* acts to prevent *ced-3* and *ced-4* activity: loss of function *ced-9* mutations are lethal if *ced-3* and *ced-4* are intact (Hengartner et al., 1992). Details of the cell death pathway are being investigated using nematodes in which *ced-9*, *ced-3* and *ced-4* have been inactivated by mutation. *Ced- 3* or *ced-4* is then expressed ectopically under the control of the *mec-2* promoter, which targets expression of these genes specifically to touch cells. In these transgenic worms *ced-3* expression kills the cells, while *ced-4* does not; therefore, *ced-3* apparently acts downstream of *ced-4* in the PCD pathway (Horvitz and Yuan, unpublished results).

Mammalian homologues of both *ced-3* and *ced-9* have been found: *ced-3* is homologous to the human cysteine protease interleukin-1β converting enzyme (ICE) (Yuan et al., 1993; Miura et al., 1993) and *ced-9*

has homology with the protoncogene *bcl-2* (Hengartner and Horvitz, 1994). A mammalian homologue of *ced-4* has not been found yet; the nematode gene, however, encodes a protein with 2 possible calcium-binding domains (Yuan and Horvitz, 1992). The beginnings of a cell death pathway are now emerging: a cysteine protease (*ced-3* / ICE) may either activate other cell death proteins or cleave proteins required for cell viability. In either case, the protease seems to be either directly or indirectly negatively regulated by *Ced-9* / *bcl-2* family members and positively regulated by *Ced-4*. The findings that the human *bcl-2* gene acts in nematode cells as it does in human cells to suppress PCD (Vaux et al., 1992; Hengartner and Horvitz, 1994), and that the nematode gene *ced-3* acts in mammalian cells as it does in nematode cells to induce cell death (Miura et al, 1993), show that the cell death programme has been highly conserved through evolution from nematodes to mammals, and confirms that programmed cell death is a fundamental feature of multicellular animal cells.

The control of programmed cell death by extracellular signals

The regulation of normal cell death by extracellular signals was first demonstrated by Hamburger and Levi-Montalcini in their work on nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1949; Hamburger and Yip, 1984). As further examples of cell death have been discovered some general principles of the extracellular regulation of cell survival and cell death are now emerging. Cells seem to die *in vivo* either due to the lack of survival factors - for example, in development of the vertebrate CNS (Cowan et al., 1984) - or due to the presence of PCD-inducing factors - for example during the reabsorption of the Mullerian duct in

male mammalian embryos (Clarke 1982). In general, cell death seems to be regulated by limiting supplies of survival factors during tissue homeostasis and histiogenesis, when only a proportion of the total cells die; while cell death is induced by "killing" signals either when whole structures are reabsorbed, or when cell deletion must over-ride other circumstances - for example, when cytotoxic-T-lymphocytes (CTL) kill target cells (Russell 1983; Golstein 1987).

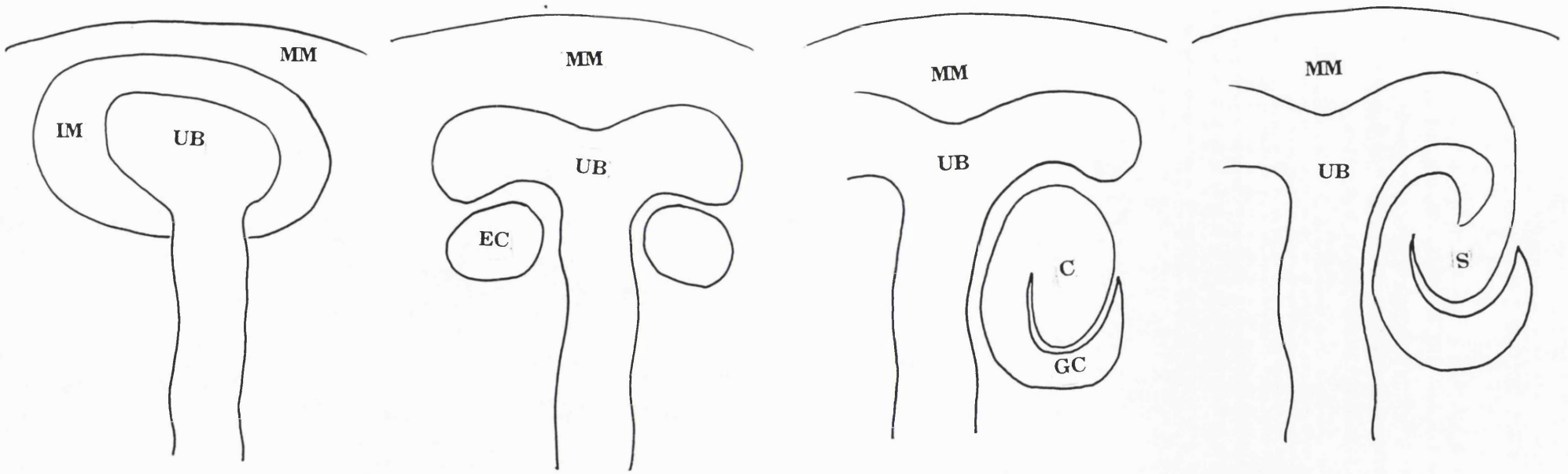
It is likely that the capacity to die by PCD is widespread among animal cells. Virtually any vertebrate cell can be induced to die by CTLs with no requirement for new gene expression in the target cell (Russell 1983; Landon et al., 1990; Ucker 1991). Moreover, specific signalling proteins (survival factors) seem to be required by many cell types in order to avoid PCD *in vitro* and recent studies show that long-term survival of cells may require more than one survival factor, *in vitro* at least (Bottenstein et al., 1980; Arakawa et al., 1990; Barres et al., 1993). It has been suggested that PCD is the default state of all cells (except blastomeres) and therefore that cell survival is a continuously signalled event (Raff 1992). This would allow constant regulation of cell populations by limiting supplies of survival factors and the rapid and discrete disposal of potentially misplaced cells that would fail to get the survival factors that they require to live. Signalling molecules that induce cell survival or cell death in one cell type can, however, have different effects on other cell types. Thyroid hormone induces apoptosis and therefore tail reabsorption during amphibian metamorphosis, while simultaneously stimulating the differentiation of skin, gut and liver cells from larval to adult types (Nishikawa et al., 1989).

An introduction to kidney development

In chapters 2 and 3 I describe studies on PCD in the developing kidney; the following is a brief account of kidney development.

On day 11 of rat development, a caudal outgrowth of the Wolffian duct - the ureteric bud - invades the caudal end of the nephric cord - the metanephric mesenchyme. The metanephric mesenchyme induces the ureteric bud to branch repeatedly; in turn, the ureteric bud induces groups of mesenchymal cells to condense, divide and differentiate into epithelial cells, which then assemble into nephrons. Thus, the nephrons are derived from metanephric mesenchyme which undergoes conversion into epithelial cells, while the collecting ducts are derived from the ureteric bud (Ekblom et al., 1987). The nephrogenic zone is the region of active nephron induction and is found at the outer edge of the developing kidney. During the earliest stages of nephron formation, the tips of the branching ureteric bud induce metanephric mesenchyme cells to undergo a series of clearly identifiable morphological changes (Fig 1.1): they condense to form a renal vesicle which then epithelialises, elongates (to form a comma-shaped body), and hollows to form a folded tube (the s-shaped body). At one end, this tube fuses to the tip of the ureteric bud (which forms a collecting duct), and at the other end it interacts with capillaries to form a glomerulus (Saxèn, 1987; Bard, 1992).

Many growth factors have been implicated in kidney development, including epidermal growth factor (EGF), insulin-like growth factors (IGFs), transforming growth factor β (TGFβ), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and fibroblast growth factors (FGFs) (for a review see Mendley and Toback, 1988). This diversity reflects the many cell types found in the kidney and



The ureteric bud (**UB**) invades the metanephric mesenchyme (**MM**) and induces **MM** at its tip to enter the nephrogenic pathway (**IM**).

The induced mesenchyme aggregates to form early condensates (**EC**) at the tips of the **UB**.

After a period of rapid cell division, the first signs of the glomerular crevice (**GC**) form in the induced mesenchyme furthest from the **UB**. The nascent nephron is now called the comma-shaped body (**C**).

A second slit forms close to the **UB** (the nascent collecting duct). The nascent nephron, now called the S-shaped body (**S**), consists of epithelial cells enclosing a central lumen. It fuses with the UB and differentiates into a mature nephron.

Figure 1.1 Diagram of early nephrogenesis in the rat metanephric kidney

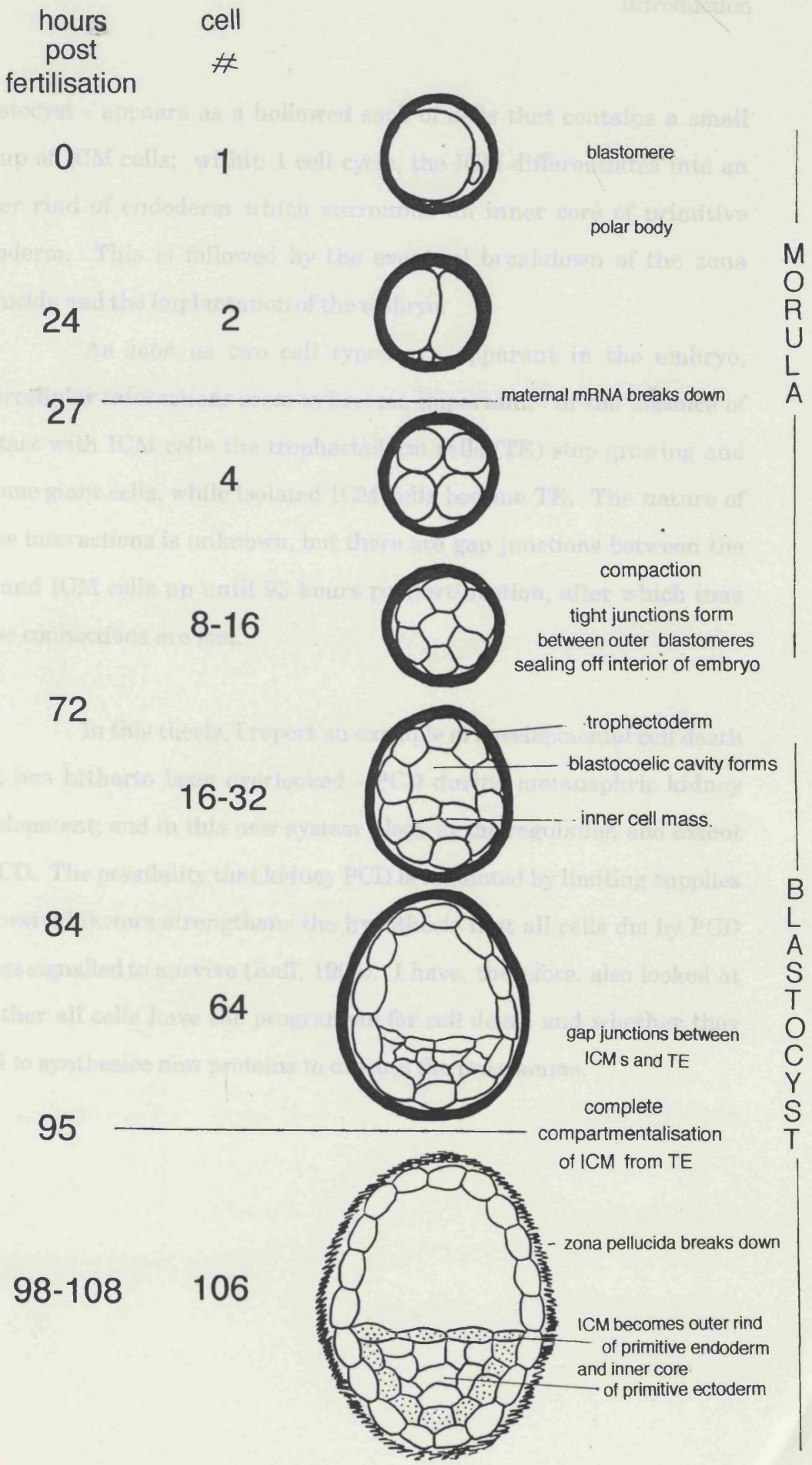
experiments suggest distinct roles for each factor. For example, PDGF and FGF may be important in angiogenesis (Mendley and Toback, 1988); HGF may stimulate branching of the ureteric bud (Montesano et al., 1991) and the mesenchymal to epithelial transition (Tsarfarty et al., 1994); and TGF β may be important in transforming mitogenic stimuli into hypertrophic stimuli (Fine et al., 1985). While EGF and IGFs are traditionally regarded as mitogens for several renal cell types (Fisher et al., 1989; Rogers et al., 1991), in Chapter 3 I suggest a new role for both EGF and IGFs.

An introduction to pre-implantation mammalian development

In Chapter 4 I describe experiments on cell types from the preimplantation mouse embryo; the following is a brief account of early mammalian development.

Soon after fertilisation, the vertebrate egg undergoes repeated cleavage divisions to produce successively smaller cells, called blastomeres, which are contained within the zona pellucida (Fig. 1.2). These blastomeres are equipotent until the early 8-cell stage: isolated 2- and 4-cell stage blastomeres can form a whole mouse, while early 8-cell stage blastomeres can contribute to a wide range of tissues in chimeras (Kelly, 1977). During subsequent development there is a progressive restriction of cell potency. Late 8-cell stage embryos undergo compaction: blastomeres increase intercellular contact and become polarised with the formation of distinct apical and basal surfaces. During the 16-cell stage 2 populations of cells develop: the outer cells form the trophectoderm and the inner cells, with no contact with the zona pellucida, become the inner cell mass. At this stage the embryo - or

Figure 2.1 (overleaf) Diagram showing preimplantation development of the mouse embryo


hours post fertilisation
cell #
0
1
blastomere
polar body
24
2
27
maternal mRNA breaks down
4
8-16
compaction
tight junctions form between outer blastomeres sealing off interior of embryo
72
trophectoderm
blastocoelic cavity forms
16-32
inner cell mass.
84
64
gap junctions between ICM s and TE
95
complete compartmentalisation of ICM from TE
zona pellucida breaks down
98-108
106
ICM becomes outer rind of primitive endoderm and inner core of primitive ectoderm
MORULA
BLASTOCYST

blastocyst - appears as a hollowed sack of cells that contains a small group of ICM cells; within 1 cell cycle, the ICM differentiates into an outer rind of endoderm which surrounds an inner core of primitive ectoderm. This is followed by the eventual breakdown of the zona pellucida and the implantation of the embryo.

As soon as two cell types are apparent in the embryo, intercellular interactions seem to become important: in the absence of contact with ICM cells the trophectoderm cells (TE) stop growing and become giant cells, while isolated ICM cells become TE. The nature of these interactions is unknown, but there are gap junctions between the TE and ICM cells up until 95 hours postfertilization, after which time these connections are lost.

In this thesis, I report an example of developmental cell death that has hitherto been overlooked - PCD during metanephric kidney development; and in this new system I look at the regulation and extent of PCD. The possibility that kidney PCD is regulated by limiting supplies of survival factors strengthens the hypothesis that all cells die by PCD unless signalled to survive (Raff, 1992). I have, therefore, also looked at whether all cells have the programme for cell death and whether they need to synthesise new proteins to execute the programme.

Chapter 2

Programmed Cell Death in the Developing Rat Kidney

Introduction

PCD is only now becoming accepted as part of the repertoire of animal cells along with division and differentiation. Until recently, PCD was thought to occur only in highly specialised circumstances such as in negative selection in the thymus (Kislielow et al., 1988; Kappler et al., 1987) and in metamorphosis (Goldsmith 1966; Kerr et al., 1974). In developmental biology, for example, cell death had only been studied in detail in the developing nervous and immune systems, and in a few cases of morphogenesis in the early embryo; this was in spite of Glucksmann's review (1951) implicating cell death in the development of most vertebrate tissues and organs. Now, however, it is becoming clear that most, and perhaps all, cells can kill themselves by PCD (Raff et al., 1993).

Why has it taken so long for normal cell death to be viewed as a general property of animal cells? Cell death *in vitro* has often been regarded as an artefact of culture conditions. Cell death *in vivo* was also initially viewed as an artefact, in this case, of tissue preparation; more recently, although considered real, it has often been regarded as quantitatively insignificant, since in most cases the dead cells are rare. However, this is because cells that undergo PCD are cleared so rapidly by phagocytosis, that there is no leakage of cytosolic contents and hence no inflammatory response. Therefore, even large scale normal cell death is usually histologically inconspicuous. Only over the last 20 years or so, for example, has it gradually been recognised that many types of vertebrate neurones are overproduced and up to 50% or more of them die during normal development (Barde, 1989; Cowan et al., 1984; Hamburger and Levi-Montalcini, 1949; Oppenheim, 1991; Purves, 1988). And only in

the past two years has it been recognised that 50% of the oligodendrocytes produced in the developing rat optic nerve normally die (Barres et al., 1992). This massive death of newly formed neurones and oligodendrocytes was initially missed because the dead cells constitute less than 1% of the cells in the developing tissue. As the proportion of dead cells in the developing nervous system is not very different from that seen in many other developing vertebrate organs, it is possible that large scale normal cell death occurs in many non-neural organs, even if it has not yet been recognised.

In the present study I have looked for cell death in the normal developing rat kidney, where cell death was not thought to play an important role, despite intense study of kidney development. In this chapter I show that cell death plays an important part in normal kidney development. It occurs in two regions, the nephrogenic zone of the cortex and the medullary papilla, and in each it follows a distinct developmental time course. The dead cells have a typical apoptotic morphology, as shown by electron, fluorescence, and phase contrast microscopy. DNA end-labelling studies *in situ* show that the DNA in the dead cells is nicked. The majority of the dead cells in the cortex of the kidney seem to be either uninduced blastemal cells or stromal cells; a minority are cells of developing nephrons.

Results

Apoptotic cells in the developing kidney

To identify apoptotic cells in the developing kidney, I stained frozen sections of embryonic and postnatal perfusion-fixed rat kidneys with propidium iodide to label nuclei and then examined the sections in a fluorescence microscope. Pyknotic nuclei, which often appeared as a cluster of 2 or more brightly stained fragments, were readily recognised in such sections (Fig. 2.1A, B). Whereas normal nuclei were generally oval in shape and had a mean diameter of 8 ± 0.2 mm (n = 83), pyknotic nuclei, or their fragments, were roughly spherical, had a mean diameter of 3 ± 0.2 mm (n = 27) and were more brightly stained than normal nuclei. With phase contrast optics, pyknotic nuclei and their fragments appeared darker than normal or mitotic parenchymal cell nuclei (Fig. 2.1C). In embryonic non-perfused kidneys it was necessary to differentiate between nucleated red blood cells and pyknotic cells (Coggeshall et al., 1993): the red blood cell nuclei were readily distinguished as they were more spherical, larger, more textured, and less brightly stained than apoptotic nuclei, and they were never fragmented (Fig. 2.1 D). Mitotic figures were also clearly identifiable (not shown).

Apoptosis is characterised by profound ultrastructural changes, including condensation of the chromatin and fragmentation of the cell to form small, membrane-bound apoptotic bodies (Wyllie et al., 1980; Clarke, 1990). To confirm that the cells that die in normal kidney development do so by apoptosis, I examined thin sections from newborn rat kidneys by electron microscopy (EM). All the dead cells seen had the

characteristic features of apoptosis. Of the 22 apoptotic cells examined by EM, 20 had the form of typical apoptotic bodies contained within normal cells (Fig. 2.2A, B), while 2 were in the lumen of developing nephrons (Fig. 2.2C). The cells that contained apoptotic bodies were morphologically indistinguishable from their neighbours. Thus most of the apoptotic cells in the developing kidney fragment into apoptotic bodies, which are very quickly phagocytosed, by neighbouring parenchymal cells rather than by macrophages.

A typical, but not invariable, characteristic of apoptosis is internucleosomal cleavage of nuclear DNA by endonucleases (Wyllie, 1984). To determine whether the pyknotic nuclei seen in the kidney had fragmented DNA, I used the terminal transferase-mediated dUTP-biotin nick end-labelling (TUNEL) method on P0 rat kidney sections (Gavrieli et al., 1992). Biotinylated dUTP was incorporated into the 3' cut ends of genomic DNA; the incorporated nucleoside was visualised with avidin-peroxidase, using DAB as a substrate. All the cells that appeared pyknotic by phase-contrast microscopy had DAB+ nuclei (Fig 2.3), confirming that these cells die by apoptosis.

Timing of cell death in the developing kidney

I found pyknotic nuclei mainly in two regions of the embryonic and postnatal rat kidney: in the nephrogenic zone, which is the region of the developing kidney cortex where new nephrons are produced, and in the developing medullary papilla (Fig. 2.4A). The time course of cell death was different in the two regions. In the nephrogenic zone, cell death was highest (pyknotic index of 2.7%) in embryonic kidneys and decreased thereafter, reaching a low basal level (pyknotic index of 0.15%) at

postnatal day 14 (P14), where it remained for at least 50 days postnatally (Fig. 2.4B, open circles). In the most proximal region of the papilla, cell death peaked at around P6-7 (pyknotic index of 3.2%) and fell to less than 0.1% by P14 (Fig. 2.4C, open circles). In the most distal regions of the papilla, cell death was especially high in the embryo, but I did not quantitate the death in this region.

Identity of the dead cells in the nephrogenic zone

The nephrogenic zone contains epithelial cells of the ureteric bud, epithelial cells derived from the metanephric mesenchyme and uninduced mesenchymal cells. To get an idea of which cell types were dying in the nephrogenic zone, frozen sections of P3 rat kidney were double-labelled with PI and anti-Pax-2 antibodies, which specifically recognise the nuclei of early nephrogenic cells (condensing mesenchymal cells and their early epithelial derivatives) and of ureteric bud cells (Dressler and Douglass, 1992). It is important to use a nuclear and not a cytoplasmic marker to avoid ambiguity, since most dead cells in the kidney are present as phagosomes inside other cells. I found that 70% of the pyknotic nuclei in the nephrogenic zone were Pax-2 $^-$ (n=50; Fig 2.5). This suggests that the majority of dead cells were blastemal or stromal, rather than epithelial.

Antigenic markers may be degraded early in the cell death programme. In the developing optic nerve, for example, where 90% of the dead cells are newly-formed oligodendrocytes, the oligodendrocyte-specific monoclonal antibody RIP labelled only 15% of the dead cells, whereas a different oligodendrocyte-specific monoclonal antibody that recognises galactocerebroside (GC) labelled 90% of the dead cells (Barres et al., 1993). Therefore, I examined propidium-iodide-stained cryosections of

kidneys from E19 rats and recorded the location of over 400 pyknotic nuclei in the nephrogenic zone. Most of the pyknotic nuclei (60%) were found among metanephric mesenchymal cells, many of which were close to, but not in, nephrogenic structures (Fig. 2.6A); they were not seen among the densely packed metanephric mesenchymal cells at the outer edge of the embryonic kidney. The remaining 40% of pyknotic nuclei were mainly found in condensed mesenchymal and tubular epithelial structures, which could be readily identified as developing nephrons; they were either in the walls of the developing nephrons or in their lumen (Fig. 2.6B, see also Fig. 2.2C). It was common to see dead cells among the proximal-most cells of s-shaped bodies (Fig. 2.6C), easily recognisable epithelial structures that will eventually form glomeruli. Pyknotic nuclei were rarely seen in the branches of the ureteric bud in the nephrogenic zone. In the maturing cortex at the inner edge of the nephrogenic zone, the incidence of cell death decreased abruptly.

The papilla of the developing kidney contains epithelial cells of both the ureteric bud and the loops of Henle, as well as interstitial cells. In embryonic animals, when cell death is highest in the most distal region of the papilla, the majority of dead cells were in the walls or lumen of tubular structures, at least some of which seemed to be branches of the ureteric bud (Fig. 2.7A). In postnatal animals, when the majority of cell death in the papilla is in the most proximal region, (where tubule structures were less clearly defined), dead cells were scattered throughout the tissue (Fig. 2.7B).

At all ages examined I found mitotic figures mainly in the nephrogenic zone, where their proportion paralleled that of pyknotic nuclei in this zone (Fig. 2.4B, closed circles). By contrast, mitotic figures were rare in the

papilla at all ages examined (Fig. 2.4C, closed circles). The close relationship between mitosis and apoptosis in the nephrogenic zone (Fig. 2.4B) suggests that many of the cells that die in this region are newly generated (while the cells that die in the papilla apparently are not). To examine further the relationship between cell division and cell death, I injected the thymidine analogue bromodeoxyuridine (Brd-U, 4 intraperitoneal injections given 8 hours apart) into P0 animals. Two hours after the last injection, the rats were perfusion-fixed and frozen sections of their kidneys were prepared and stained with both propidium iodide and monoclonal anti-Brd-U antibody, as described previously (Barres at al., 1992; Gonchoroff et al., 1985). In these experiments about 50% of the live cells and 25% of the pyknotic cells in the nephrogenic zone were Brd-U labelled (data not shown), indicating that at least one quarter of the dead cells had synthesised DNA sometime in the 26 hour period before their death. Unfortunately this experiment could not be extended for a longer period as Brd-U was toxic to kidney cells at later time points (data not shown).

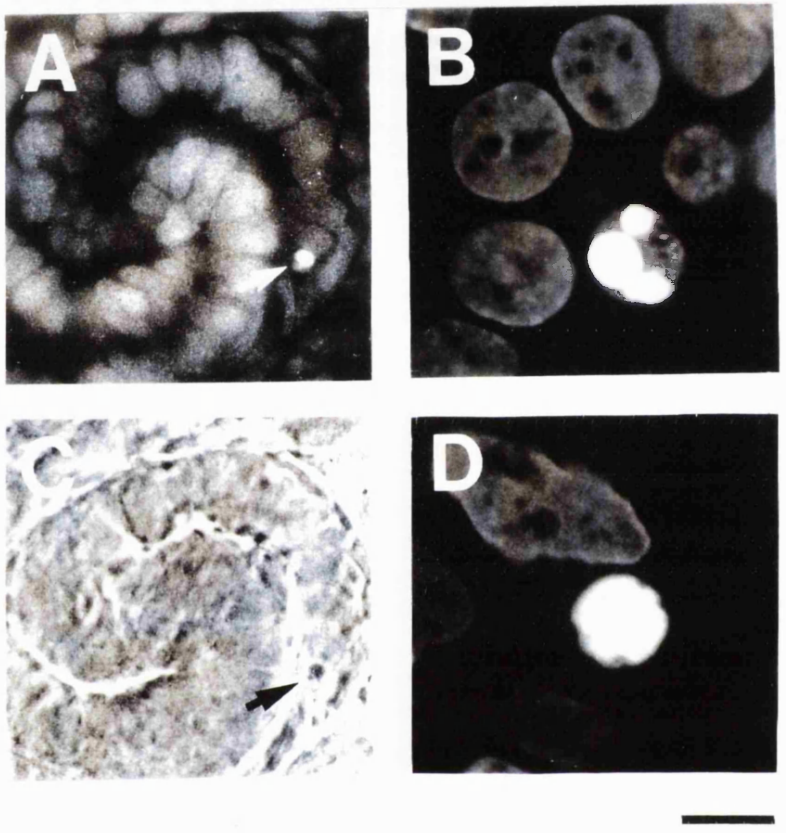


Figure 2.1 Propidium iodide staining of nuclei in developing rat kidney. (A) and (C) Fluorescence and phase contrast micrographs, respectively, of the same field, showing a pyknotic nucleus (arrow) in the nephrogenic zone of a P1 kidney. (B) Confocal fluorescence micrograph of a fragmented pyknotic nucleus in the medullary papilla of an E19 kidney. (D) Confocal fluorescence micrograph of a normal nucleated red blood cell in the papilla of an E19 kidney. Bar = 20 μm in (A) and (C), 6 μm in (B), 4 μm (D).

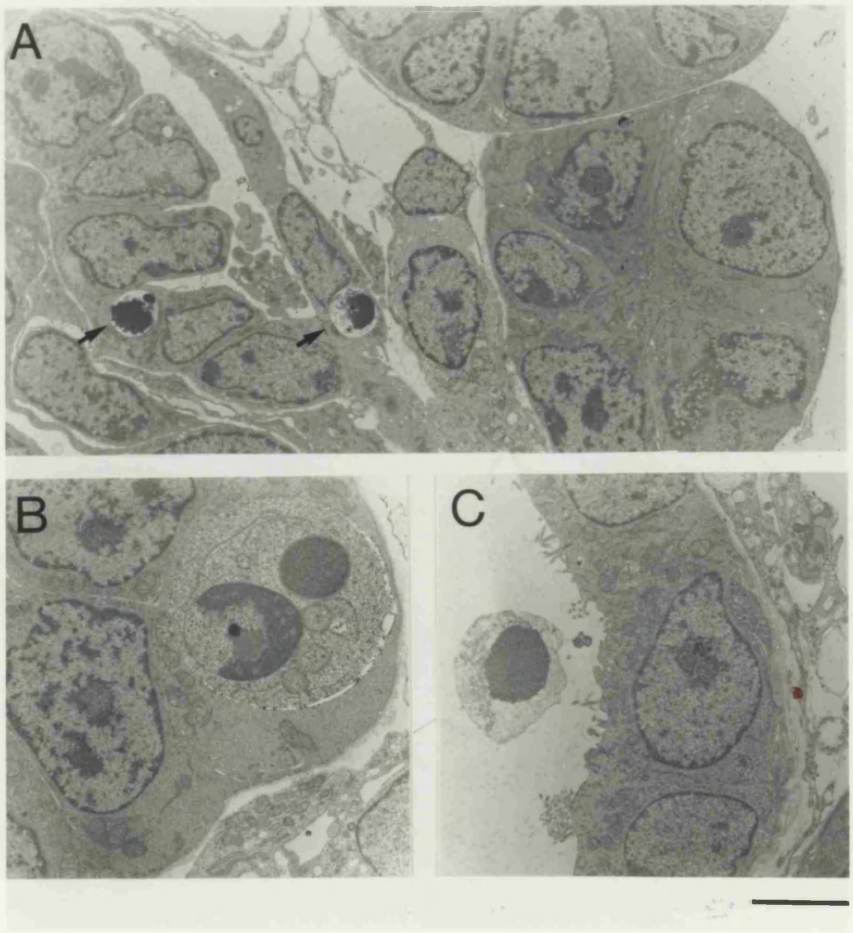


Figure 2.2 Electron micrographs of apoptotic bodies in the nephrogenic zone of a newborn rat kidney. Note that the apoptotic bodies in (A) and (B) are within neighbouring parenchymal cells, while the apoptotic body in (C) is in the lumen of a developing nephron. Bar = 5 μm in (A), 1.5 μm in (B) and (C)

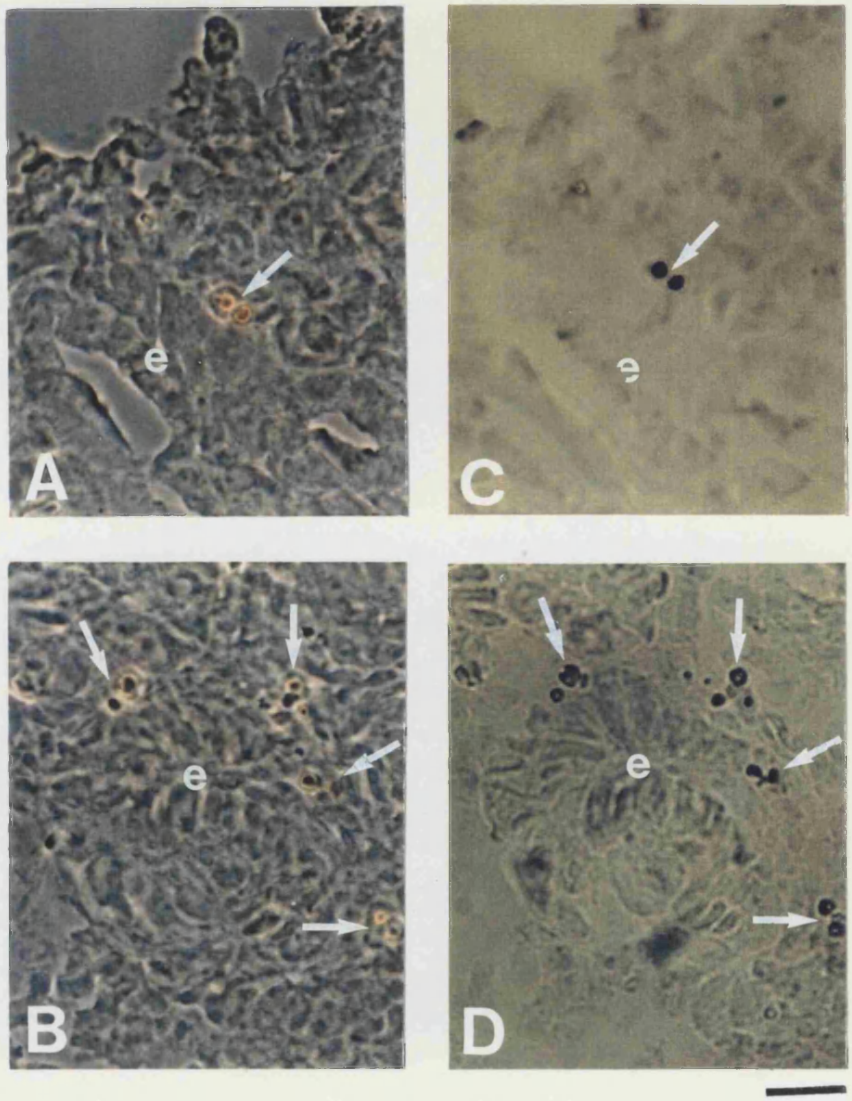


Figure 2.3 End-labelling of nicked DNA *in situ*, in the nephrogenic zone of neonatal rat kidney. Phase contrast (A) and (B) and bright field (C) and (D) micrographs of the nephrogenic zone of P0 rat kidney. Pyknotic nuclei (arrows) contain nicked DNA and so incorporate biotinylated dUTP; they are labelled with avidin-peroxidase and visualised with DAB. The pyknotic nuclei in (A) and (B) are clearly DAB positive (arrows) in (C) and (D) respectively. All of the pyknotic nuclei are close to epithelial structures (marked **e**). Bar= 18μm

Figure 2.4 (overleaf) Pyknotic and mitotic indices in the nephrogenic zone (B) and medullary papilla (C) of the developing rat kidney at different ages. A schematic drawing of a midsaggital section through a developing kidney is shown in (A) to indicate the regions where normal cell death was assessed. In (B) the nuclei in the outer-most 100 μm of cortex were counted in every second field (total of 10-30 fields) of 3 non-serial sections for each pup. In (C) the nuclei in a 300 x 300 μm grid at each side of the proximal papilla (shown in A) were counted in 4 non-serial sections for each pup. The results are presented as mean ± s.e.m. of the results from 4-5 pups at each age.

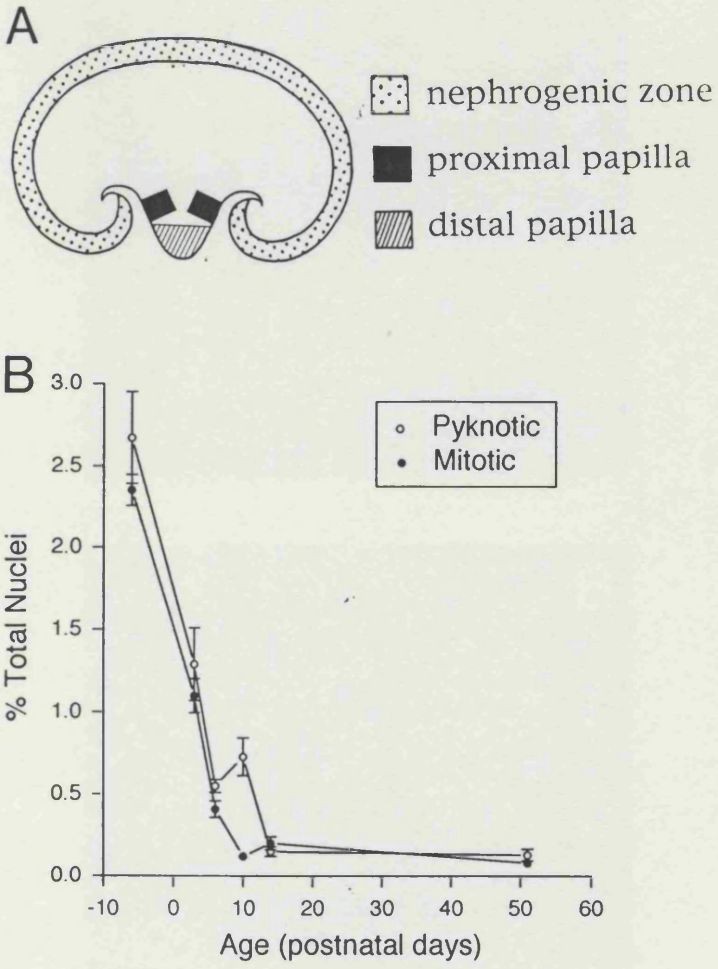
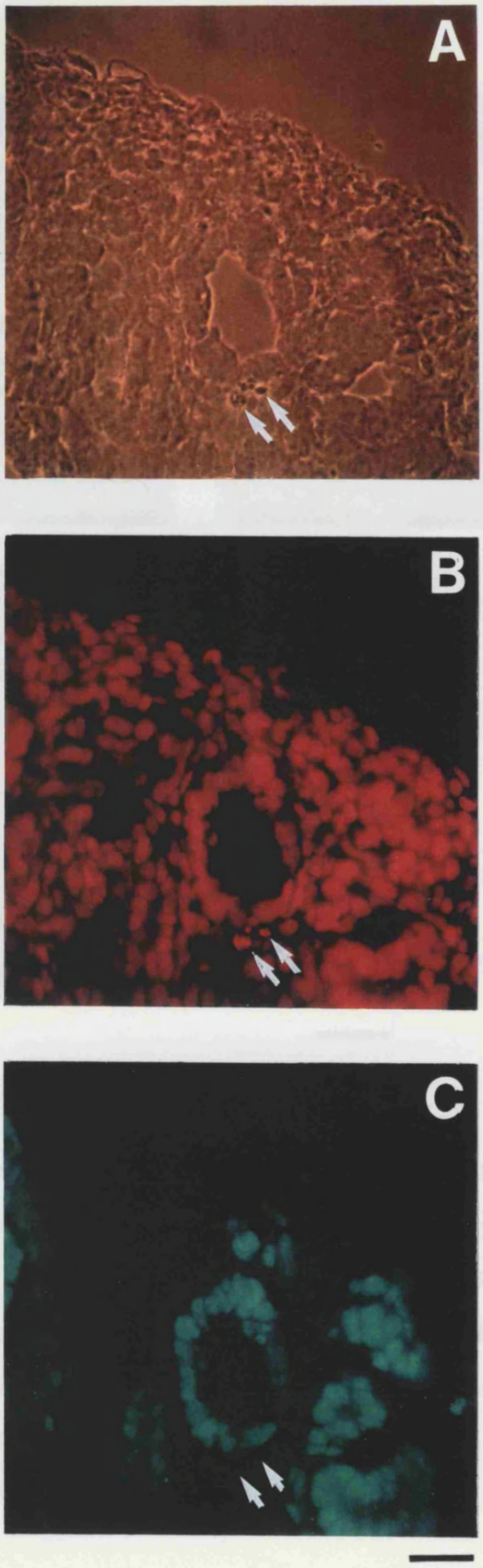

A
nephrogenic zone
proximal papilla
distal papilla
B
Pyknotic
Mitotic
% Total Nuclei
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-10
0
10
20
30
40
50
60
Age (postnatal days)
C
Pyknotic
Mitotic
% Total Nuclei
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-10
0
10
20
30
40
50
60
Age (postnatal days)

Figure 2.5 (overleaf) Pax-2 and propidium-iodide double labelling of the nephrogenic zone of developing rat kidney. Phase contrast (A) and fluorescence micrographs (B) of the same field of propidium-iodide-stained sections of P0 rat kidney showing pyknotic nuclei (arrows) next to an epithelial structure in a P3 rat kidney. (C) fluorescence micrograph of the same field stained with an anti-Pax-2 antibody and visualised with a FITC-conjugated secondary antibody, showing that the pyknotic nuclei in (A) and (B) are not Pax-2 positive (arrows). Bar= 22μm


A
B
C

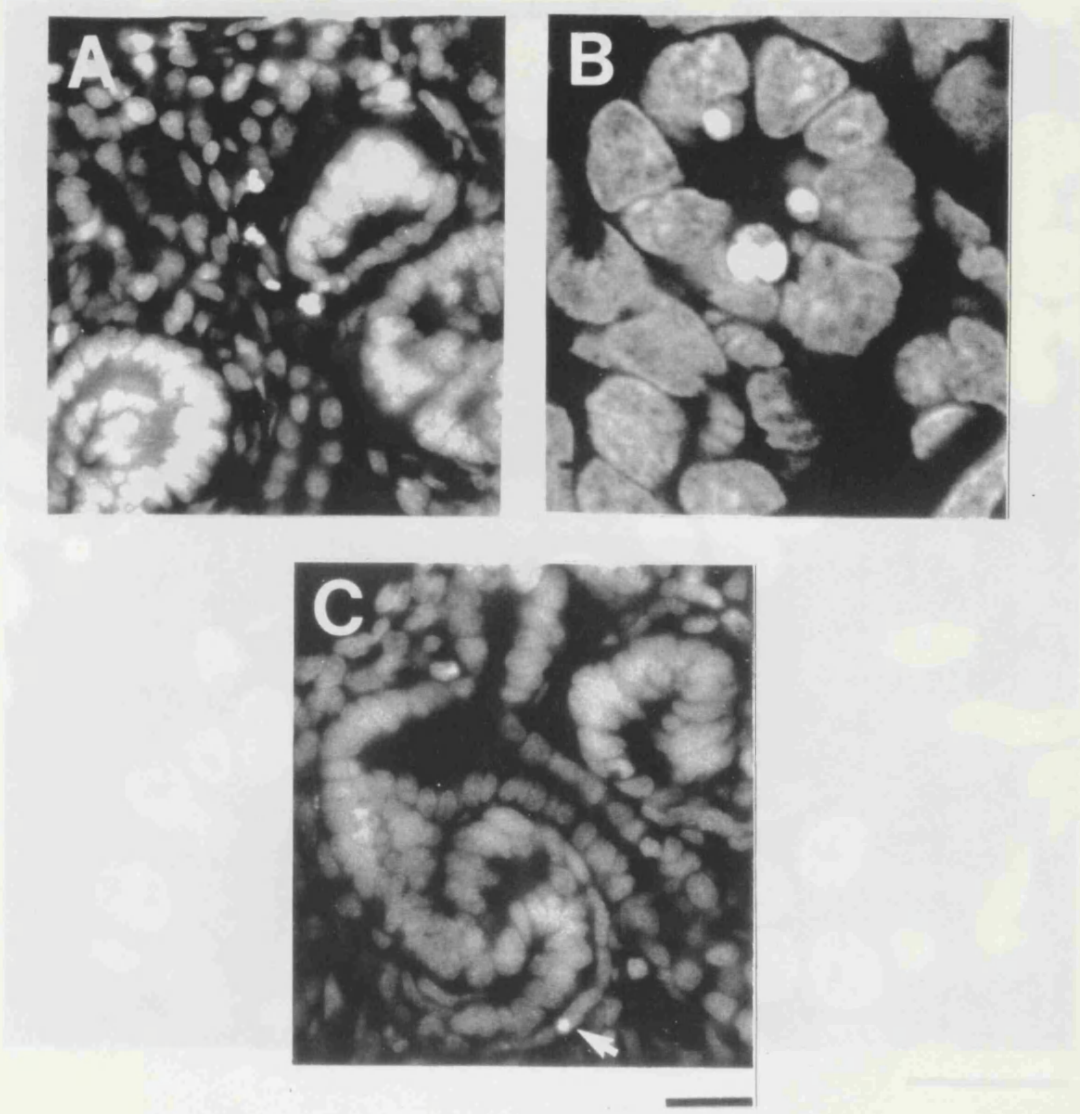


Figure 2.6 Localisation of propidium-iodide-stained pyknotic nuclei in the nephrogenic zone of a P1 kidney. In (A) most of the pyknotic nuclei are close to, but not in, developing nephrons. In (B), which is a confocal micrograph, and (C), the pyknotic nuclei are in the wall or lumen of developing nephrons. Note the pyknotic nucleus (arrow) in the proximal part of an s-shaped body in (C).
Bar = 25 μm in (A) and (C), 7 μm in (B).

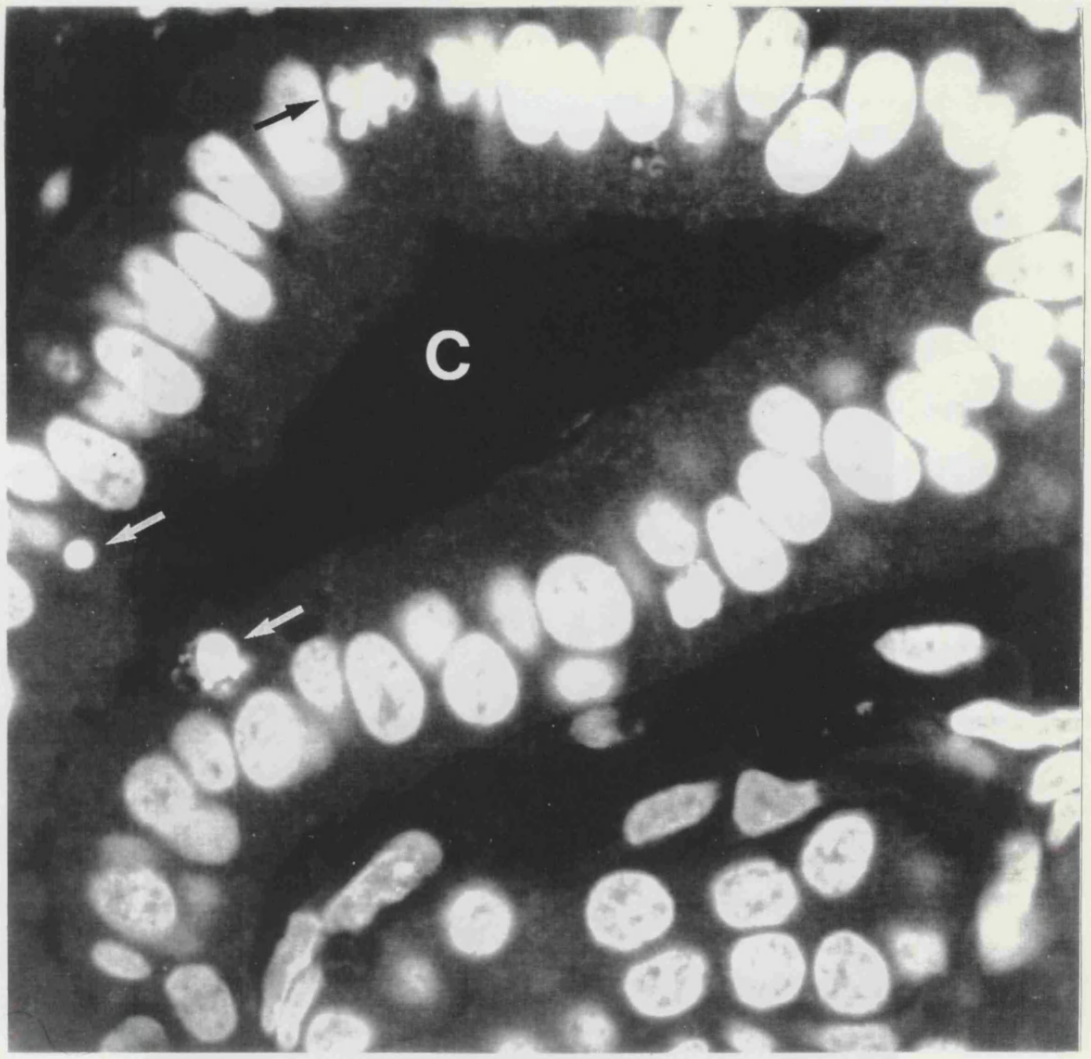


Figure 2.7 (this page and overleaf) Confocal fluorescence micrographs of propidium-iodide-stained pyknotic nuclei (white arrows) in (A) the most distal part of the papilla of an E19 kidney and (B) the proximal part of the papilla in a P7 kidney. In (A) a mitotic nucleus (black arrow) is also seen and the tubular epithelial structure shown is a collecting duct (marked **c**).
Bar =15 μm in (A) and 22 μm in (B).

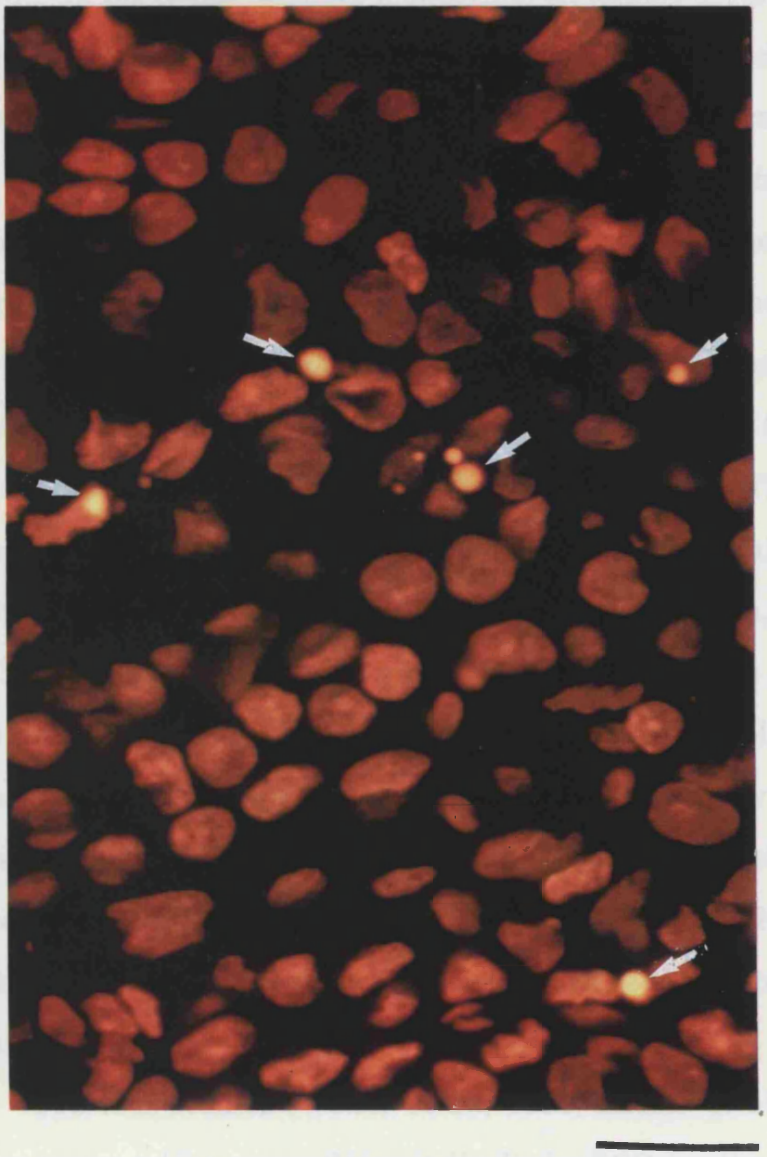


Figure 2.7 (B) Pyknotic nuclei (white arrows) in the proximal part of the medullary papilla of a P7 rat kidney. Bar = 22 μm.

Discussion

Cell death has not been thought to play an important role in kidney development. In his comprehensive monograph on kidney development, for example, Saxèn (1987) does not mention cell death, and Glucksmann (1951) refers only to a study that implies that the first generations of nephrons degenerate during metanephric development (Kampmeier 1926). I find, however, that there is substantial cell death in the normal developing rat kidney, both in the nephrogenic zone and in the medullary papilla. It can be readily detected by electron microscopy in thin plastic sections, or in frozen sections, either by fluorescence microscopy following propidium iodide staining or by phase contrast microscopy. The dead cells show the characteristic features of apoptosis (Wyllie et al., 1980; Clarke, 1990), including nuclear condensation and fragmentation, nicking of the DNA, and cell shrinkage. Furthermore, the dead cells or their fragments are phagocytosed by neighbouring parenchymal cells. Cell death during renal development may have been previously missed because many of the recent significant studies on kidney development (review see Saxèn 1987) have used the transfilter technique (Grobstein 1953 and 1956), in which dead cells could easily have been interpreted as artefactual.

Previous studies (Weller et al., 1991; Koseki et al., 1992) showed that metanephric mesenchyme cells die by PCD *in vitro* and my study shows that a proportion of metanephric mesenchyme cells die by PCD during normal kidney development *in vivo*. Both Pax-2 staining for early induced nephrogenic cells and studies in which the location of dead cells was scored show that the majority of dead cells are either stromal or blastemal metanephric mesenchyme cells and not epithelial cells. I also

find that a considerable proportion (40%) of the dead cells are in nephrogenic structures.

Glucksmann (1951) distinguished three kinds of normal cell death during development: (1) **phylogenetic death**, associated with the loss of vestigial structures or of larval organs during metamorphosis; (2) **morphogenetic death**, associated with the sculpting of specific structures such as digits or the separation or fusion of epithelia; and (3) **histiogenetic death**, associated with cell differentiation. Most of the deaths that I see in the nephrogenic zone in the developing kidney fit best into the category of histiogenetic deaths: they are scattered and seem to occur mainly in metanephric mesenchymal cells at a time when a proportion of these cells is being induced to differentiate into nephrogenic epithelial cells. Furthermore, our findings suggest that many of the cells that die in the nephrogenic zone have recently divided: there is a strong temporal correlation in this region between the pyknotic and mitotic indices (see Fig. 4B), and Brd-U incorporation studies indicate that at least 25% of the dead cells in this region synthesised DNA sometime in the 26 hour period before they died. This observation could not be extended to cells that had divided within the last two days as Brd-U was toxic at longer time points.

What is the function of metanephric mesenchyme cell death in kidney development? Cell death in the developing nervous system is thought to help match both the numbers of neurones to the numbers of target cells they innervate (Cowan et al., 1984) and the number of oligodendrocytes to the number (and length) of axons that require myelination (Barres et al., 1992; Barres and Raff, 1993). In the kidney, cells of two lineages are involved in early nephrogenesis - nephrogenic

mesenchymal cells and epithelial cells of the ureteric bud - and it is possible that cell death helps to match the numbers of cells in these two lineages.

It is less clear how to categorise the cell deaths in the developing nephrons. Developmental cell death contributes to the fusion of epithelia in, for example, palatal shelf formation (Farbman, 1986; Shah 1979) and neural tube closure (Glucksmann 1951); it is also suggested that cell death could contribute to lumen formation (Snow 1987), by hollowing out structures. The dying cells that I observe at the ampullae (the tips of the ureteric bud) may be instrumental in the fusion of two epithelia - the nascent nephrons and the collecting ducts. Although it is not known how the glomerular crevice is formed in the S-shaped body, it is thought that changes in cell adhesion may play a role (Saxèn 1987). My observation that dying cells are commonly found in the proximal tip of the S-shaped body raises the possibility that cell death may participate in glomerular crevice formation. Finally the dying cells in the medullary papilla may be involved in the fusion of the major collecting ducts (Potter, 1972). Thus the epithelial cell deaths seen are probably best considered examples of morphogenetic deaths.

De-regulation of genes involved in cell death may contribute to some diseases. The mammalian gene *bcl-2*, for example, was first described as an oncogene in follicular B-cell lymphomas (Tsujimoto et al., 1985): translocation brings the gene under the control of an Ig enhancer so that it is over-expressed in B-cells, where it suppresses PCD, thereby promoting B-cell survival and tumorigenesis by increasing the probability of further oncogenic mutations (Vaux et al. 1988; Chen-Levy et al., 1989; Strasser et al., 1990). Experimental over-expression of the *bcl-2* gene

product also prevents PCD in many other vertebrate cell types (Vaux et al., 1988; Sentman et al., 1991; Strasser et al., 1991; Garcia et al., 1992). My finding that cell death is a normal feature of kidney development raises the possibility that defects in cell death might play a part in some developmental abnormalities of the kidney. This was first suggested by Kampmeier (1923 and 1926), who proposed that congenital cysts arose from vestigial renal vesicles that did not degenerate during development. The Bcl-2 protein is expressed in the developing kidney where it is preferentially located in the metanephric cap and the developing nephrons (Veis and Korsmeyer, 1993; my unpublished observations). In homozygous *bcl-2* knockout mice, polycystic kidney disease is an invariable feature and may be the cause of death in at least some of the mice (Veis et al., 1993), supporting the idea that de-regulated PCD may be involved in some developmental kidney disorders.

Wilms' tumour is a second kidney disease that may involve abnormal renal PCD. It is a nephroblastoma in which control of nephrogenesis is aberrant (van Heyningen and Hastie, 1992). The Wilms' tumour-suppressor gene *wt-1* is a zinc-finger, DNA-binding protein that acts as a putative repressor of various genes, including the IGF-2 gene (Drummond et al., 1992) and possibly the IGF-1 receptor genes (Werner et al., 1993). *wt-1* is often inactivated in Wilms' tumours (van Heyningen and Hastie, 1992), which may result in increased expression of IGF-2 (Drummond et al., 1992) and the IGF-1 receptor (Werner et al., 1993). My finding that IGF-1 decreases the pyknotic index in the nephrogenic zone of the kidney (see chapter 3) raises the possibility that decreased PCD may contribute to Wilms tumour formation and

progression, just as it does in follicular B-cell lymphoma (Vaux et al., 1988; Strasser et al., 1990).

Methods

Tissue preparation

Sprague-Dawley rats were bred in the UCL animal facility. Postnatal rats were deeply anaesthetised with pentobarbitone and perfused through the heart with phosphate buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4. The kidneys were removed and fixed over night at 4°C and cryoprotected with 30% sucrose in PBS until equilibrated. Embryonic kidneys were dissected from embryos that had been removed from the uterus and put immediately on ice; they were then fixed as above. Whole kidneys were frozen in OCT compound (Miles), and 6.5 μm cryosections were cut on a Bright cryostat. Sections were collected on gelatinised glass microscope slides, air dried, and post-fixed with 70% ethanol at -20°C for 10 minutes.

Propidium iodide labelling

Sections were incubated with 4 μg/ml propidium iodide (Sigma) and 100 μg/ml RNase (Sigma; DNase-free) in PBS for 30 minutes at 37°C (Barres et al., 1992; Rodriguez-Tarduchy et al., 1990). The slides were washed in PBS, mounted in Citifluor (City University, London), examined in a Zeiss Universal fluorescence microscope using a 40x oil immersion phase contrast objective, and photographed with Tri-X film, ASA 400. In some cases sections were examined with a Biorad MRC-600 laser-scanning confocal imaging system in conjunction with a Nikon Optiphot microscope and the images were printed on a Mitsubishi CP100 printer.

Pyknotic nuclei in propidium-iodide-labelled sections were readily recognised by fluorescence microscopy: they were smaller and

more brightly stained than normal nuclei, and they were often fragmented. Clusters of nuclear fragments or apoptotic bodies occurring within one normal nuclear diameter were counted as one pyknotic nucleus (Wyllie, 1975). Pyknotic nuclei (or apoptotic bodies) could also be readily recognised by phase contrast microscopy by their small, phase dark appearance. Propidium-iodide-labelled mitotic figures were also easily distinguished by fluorescence microscopy.

For each age examined, 3 non-serial 6.5 μm midsagittal sections were examined from at least 3 animals. In each section pyknotic nuclei were counted in every second field. In the kidney, cells were counted in the outer-most 100μm of the cortex and in a 300 x 300 μm area on each side of the proximal part of the medullary papilla (see figure 3A). Both pyknotic and mitotic nuclei were counted and the numbers obtained were corrected for split cell counts (Abercrombie, 1946) and expressed as a percent of total nuclei.

Abercrombie correction

Propidium iodide-stained cryosections of P0 rat kidney (6.5 μm) were prepared. To measure the diameters of pyknotic and normal nuclei, I traced nuclear profiles on a digitising tablet (Summa graphics) and used *cigal* software (J. Voyvodic, unpublished) for the morphometry. The average diameter of non-dividing, non-pyknotic nuclei was 8 μm $\pm$ 0.2 (n=83) compared to that of pyknotic nuclei which was 3 μm $\pm$ 0.2 (n= 27) and mitotic nuclei which was 10 μm $\pm$ 3.2 (n=5). To determine the relationship between profile and particle numbers in the kidney and thymus, I obtained correction factors for split cell counts using the equation derived by Abercrombie (1946):

$$P = A \frac{M}{L + M}$$

where:

P = corrected number of nuclei
A = raw count (profile count)
M = section thickness
L = average diameter of the nucleus

Correction Factor	Tissue	
	Kidney	**Thymus**
Normal	0.45	0.46
Pyknotic	0.687	0.59
Mitotic	0.45	---

Immunohistochemistry

Cryosections of paraformaldehyde-fixed P1 and P6 rat kidney were rehydrated with PBS, permeabilised with 70% ethanol at -20°C for 10 minutes and then washed in PBS containing 0.05% Triton-X100. Non-specific antibody binding was blocked by incubation the sections in 50% goat serum and 20 mM L-lysine in PBS for 30 minutes. After washing, the sections were labelled with Rabbit anti-Pax-2 IgG antibody (10 μg/ml; Dressler and Douglas, 1992) in 2% goat serum, followed by flourescien-coupled goat-anti-rabbit antibody (Welcome, diluted 1:100), both for 1 hour at room temperature. The slides were stained with PI, mounted and examined as described above.

Brd-U incorporation

Five new born (P0) rat pups were given 4 intraperitoneal injections of Brd-U (0.1 mg/g body weight; Boehringer Mannheim) at 8 hour intervals.

Two hours after the last injection, the pups were perfused and the kidneys fixed and processed as described above. Air dried sections were rehydrated with PBS and post-fixed with 70% ethanol for 10 minutes at -20°C. The DNA was denatured by incubation in 2 M HCL for 10 minutes and the and was then neutralised by incubation in 0.1 M sodium borate (pH 8.5) for 10 minutes. To block non-specific binding, the sections were incubated in 50% sheep serum, containing 1% bovine serum albumin (Sigma) and 100 mM l-lysine . The sections were then labelled with BU-1, a monoclonal anti-Brd-U antibody (Gonchoroff et al., 1985; Greipp et al., 1985; concentrated supernatant diluted, 1:10), followed by biotinylated sheep anti-mouse IgG (Amersham; diluted 1:50) and then fluorescein-coupled strepavidin (Amersham; diluted 1:100). Finally, the sections were stained with propidium iodide and examined as described above. The pyknotic index in the kidneys was not increased in Brd-U-treated rats, suggesting that the Brd-U treatment was not toxic to kidney cells at the concentrations used.

Electron microscopy

P0 rat pups were deeply anaesthetised with pentobarbitone and perfused with PB, followed by 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (Yun and Kenney, 1976). The tissue was cut into 1 mm slices and fixed for a further 2.5 hours at room temperature and then post-fixed with 1% osmium tetroxide in PB for 1 hour. After dehydrating in acetone and embedding in Epon, thin sections were cut on an LKB Ultratome, counter stained with uranyl acetate and then lead citrate, and examined in a JEOL 100-CX II electron microscope at 80kV.

The TUNEL technique

To determine whether pyknotic cells in the kidney have fragmented DNA, 6.5 μm cryosections of P0 rat kidney were collected on APES treated slides, air-dried and rehydrated with 10 mM Tris.HCL pH8. Proteins were stripped from the sections with proteinase K (20 μg/ml, Sigma) for 15 minutes at room temperature, and endogenous peroxidases were inactivated with 3% hydrogen peroxide. After extensive rinsing in double distilled water, the slides were treated with biotinylated dUTP (Bio-16-dUTP, 3 nM, both Boehringer Mannheim UK.) in the presence of terminal deoxynucleotidyl transferase (TDT, 10 u / l in TDT buffer, both GIBCO BRL) at 37°C for 1 hour to end-label any nicked DNA in the sections. The reaction was terminated by 300 mM NaCl and 30 mM sodium citrate.

The slides were washed with PBS for 5 minutes at room temperature, blocked with 2% BSA for 10 minutes and then washed again. Biotinylated-dUTP endlabelled DNA was labelled with and Avidin DH:biotinylated horseradish peroxidase H complex for 30 minutes at room temperature. Labelled nuclei were then visualised by a 7 minute incubation with the peroxidase substrate diaminobenzidine tetra-hydrochloride (DAB, Amersham UK.) in the presence of 30% peroxide. The slides were then rinsed, lightly counter stained with Toll Blue, mounted in citifluor (City University, London) and examined with phase contrast and bright field microscopy. Pretreatment of sections with DNase I (0.2 mg/ml, 10 minutes at room temperature) or omission of the dUTP from the end-labelling reaction destroyed all DAB reactivity in the sections.

Chapter 3

Factors Affecting Kidney Cell Survival

Introduction

The extensive death of developing vertebrate neurones is thought to result, at least in part, from a competition among neurones for limiting amounts of survival (neurotrophic) factors secreted by the target cells that they innervate. The best documented example of this is nerve-growth-factor-dependent sympathetic and sensory neurones; increasing the availability of either target tissue or nerve growth factor (NGF; Hamburger and Yip 1984) *in vivo* decreases the amount of neuronal cell death; conversely, decreasing the availability of either target tissue (Levi-Montalcini, 1987) or NGF (Levi-Montalcini, 1972; Gorin and Johnson, 1979) *in vivo* increases the amount of neuronal cell death. It is thought that many other cases of neuronal cell death in the developing central nervous system are due to a similar mechanism (Cowan et al., 1984; Purves 1988; Oppenheim 1991); for example, brain-derived neurotrophic factor (BDNF) rescues quail nodose ganglion neurones *in vivo* (Hofer and Barde, 1988). Recently it was found that the death of newly formed oligodendrocytes in the developing optic nerve may also reflect a competition for limiting amounts of survival factors (Barres et al., 1992), suggesting that this cause of cell death is not confined to neurones.

The overproduction of neurones and their subsequent selection due to limiting availability of survival factors is thought to match the sizes of synaptically connected populations of cells and eliminate erroneous neuronal projections during vertebrate neural development (Cowan et al., 1984; Purves 1988; Oppenheim, 1991). Similarly, normal cell death of oligodendrocytes may match the numbers of these cells to the size and length of the axon that they myelinate (Barres et al., 1992). It seems unlikely that such an elegant mechanism

for regulating the co-ordinated development of cell populations is unique to the developing nervous system. Indeed, it has recently been proposed that all cells (other than blastomeres) in higher animals may require survival factors to avoid killing themselves and that many cases of normal cell death may reflect limiting supplies of survival factors (Raff 1992). I have looked at the possible role of survival factors in normal cell death in the developing kidney in order to test this possibility.

Regulation of cell survival has not been considered to be a part of normal kidney development, and therefore most studies have investigated the mitogenic but not survival effects of growth factors on kidney cells. Epidermal growth factor (EGF), for example, is known to be important in metanephric development and has been generally considered to be a co-mitogen (Mendley and Toback, 1988, Fisher et al., 1989). However, EGF maintains the integrity of kidney rudiment explants grown in serum- and inducer-free conditions without stimulating DNA synthesis (Weller et al., 1991). Furthermore, EGF decreases the amount of DNA degradation seen in early kidney rudiments cultured in serum- and inducer-free medium (Koseki et al., 1992). These studies suggest that EGF may act as a survival factor for metanephric mesenchyme *in vitro*.

A second growth factor of interest is insulin-like growth factor 1 (IGF-1), which is the main mediator of the effects of growth hormone in the body (Mathews et al., 1988). While IGFs 1 and 2 are known to be important in the regulation of mammalian growth, it has been assumed that they promote growth by promoting cell proliferation. However, IGF-1 promotes survival and not proliferation in vertebrate CNS neurones (Svrzic and Schubert, 1990), Balb/c 3T3 murine fibroblasts (Tamm and

Kikuchi, 1990), neuroepithelial cells (Drago et al., 1991), oligodendrocytes, and their precursor cells (Barres et al., 1992). Furthermore, IGF-1 promotes survival of Rat-1 fibroblasts, Swiss 3T3 cells and primary vascular smooth muscle cells when these cells are driven through mitosis by overexpression of *c-myc*, a stimulus that would normally induce PCD in these cell types (Evan et al., 1992; E. Harrington and G. Evan, personal communication). Endogenously produced IGF-1 is important for kidney development since anti-IGF-1 neutralising antibodies block the growth of kidney rudiment explants (Rogers et al., 1991), although whether it is primarily a mitogen or a survival factor is unknown.

To determine whether EGF and IGF-1 are survival factors for kidney cells *in vivo* I have tested the affects of EGF and IGF-1 treatment on the pyknotic and mitotic indices in the nephrogenic zone, and looked at which cells are affected by these factors; in addition, I have studied the affects of reducing the levels of IGF-1 *in vivo* with anti-IGF-1 antibodies. I have used EGF treatment to estimate the clearance time of dead cells in the nephrogenic zone and consider the extent of cell death during normal kidney development.

Results

If cell death during kidney development is due to limiting amounts of survival factors, then increasing the amount of available factors should decrease the death. I did these experiments in order to determine whether EGF and IGF-1 act as survival factors *in vivo*, and whether increasing the amount of EGF and IGF-1 available to the developing kidney decreases the amount of cell death.

Effects of increased EGF and IGF-1 on kidney cell death *in vivo*

Cohen (1962) showed that EGF injected intraperitoneally (i.p.) induces premature eye opening in newborn rats. I injected EGF or vehicle alone into P1 rat pups (4 i.p. injections given 3 hours apart). Two hours after the last injection the rats were perfusion-fixed and frozen sections of their kidneys were prepared and stained with propidium iodide. Whereas EGF treatment reduced the pyknotic index in the nephrogenic zone by 75% (Fig 3.1A), it did not significantly affect the mitotic index in this region (Students t-test, $P > 0.05$; Fig 3.1B). Similarly, EGF treatment reduced the pyknotic index in the medullary papilla by 85% (Student's t-test, $P > 0.05$; Fig 3.1C), but it did not significantly affect the mitotic index in this region (Fig 3.1D). I subsequently discovered that a single injection of EGF had the same effect after 3 hours as did the multiple injections described above.

To study the effects of IGF-1 and maximise its biological activity *in vivo* I used human, recombinant truncated IGF-1 (tIGF-1). This form of IGF-1 lacks the amino-terminus tripeptide and therefore binds less well than full length IGF-1 to IGF-binding proteins, which might

be expected to enhance its availability to cells *in vivo*. I gave a single injection of tIGF-1 or vehicle alone to P0 rat pups. Three hours later the rats were perfusion-fixed. Frozen sections of their kidneys (6.5 μm thick) were prepared and stained with propidium-iodide, and the pyknotic and mitotic indices in the nephrogenic zone were determined. While tIGF-1 treatment did not significantly affect the mitotic index in the nephrogenic zone, it reduced the pyknotic index in this region by 63% (Student's t-test, $P>0.02$; Fig 3.2).

The nephrogenic zone contains epithelial cells of the ureteric bud, epithelial cells derived from the metanephric mesenchyme, uninduced mesenchymal cells and mesenchymally derived stromal cells. To get an indication of which of these cell types were responding to increased levels of EGF and IGF-1, I injected P1 rat pups with either IGF-1 or EGF; 3 hours later they were perfusion fixed, and frozen sections of the kidneys were prepared and stained with propidium iodide. EGF reduced the proportion of pyknotic nuclei by 90% in the mesenchymal compartment of the nephrogenic zone, but by only 50% in the epithelial compartment of this region. Therefore the main effect of EGF was on cells found in the mesenchymal compartment of the nephrogenic zone. On the other hand, IGF-1 reduced the proportion of pyknotic nuclei in both the mesenchymal and epithelial compartments equally.

A change in the proportion of pyknotic cells in a tissue at any one time (the pyknotic index) may be due to a change in either the number of cells dying or the rate of clearance of the dead cells. If EGF was preventing cells from dying then the total DNA content of EGF kidneys should be greater than that of controls. Therefore, I delivered extra EGF or vehicle alone to neonatal rat pups for 3 days and then

measured the DNA content of the kidneys; there was no difference in total kidney DNA content between pups treated with EGF or vehicle. After I finished these experiments it was reported that extra EGF *in vivo* inhibits DNA synthesis in neonatal rat kidneys (Gattone et al., 1992); this affect would counteract a cell-saving effect of EGF.

In principle, a second way of possibly distinguishing between an effect on cell clearance and an effect on cell survival is to use the recently developed TUNEL technique (Gavrieli et al., 1992). This method end labels nicked DNA *in situ* and has been reported to be more sensitive in the detection of apoptotic cells than conventional methods for looking at nuclear morphology (Chang et al., 1993), because cells in the earliest stages of apoptosis may have nicked DNA before they appear pyknotic with propidium iodide. If EGF prevents cell death, then EGF treatment should decrease the proportion of nuclei in early pyknosis before it decreases the proportion of nuclei in late pyknosis. I used the TUNEL technique to stain kidney sections from EGF-treated and untreated pups, and compared this to propidium iodide stained sections from the same pups. Contrary to the reports of Chang et al. (1993), I found no enhanced sensitivity for detection of pyknotic nuclei with the TUNEL technique as compared to propidium iodide. This is in agreement with subsequent studies on the optic nerve and cerebellum (B.A. Barres, and B.K. Krueger personal communication).

Effect of decreasing the levels of survival factors *in vivo*

The observation that an increase in either IGF-1 or EGF decreases the pyknotic index in the nephrogenic zone of neonatal rat kidney is consistent with the idea that kidney cells die due to limited supplies of

survival factors. To test whether survival factors normally control cell survival in the developing kidney I looked at the effects of reducing the levels of IGF-1 *in vivo*. I could not easily manipulate signalling through the EGF receptor as it is activated by two ligands: EGF and TGF-α, and currently there are no neutralising antibody to their common receptor in rat. An anti-IGF-1 monoclonal antibody, however, is available that have been reported to neutralise rat IGF-1 *in vitro* (van Wyk et al., 1986; Baxter et al., 1982; B.A. Barres, unpublished data).

For long term delivery of antibodies I gave i.p. injections of hybridoma cells secreting either the SM1,2 anti-IGF-1 antibody (SM1,2) or anti-bromo-deoxyuridine antibody (αBrd-U) after the method of Schnell and Schwab (1990). After 3 days the animals were weighed, perfusion fixed and frozen sections of their kidneys were prepared and stained with propidium iodide. There was no difference in the pyknotic or mitotic indices of kidneys treated with SM1,2 compared with those from αBrd-U-treated or uninjected siblings at any of the doses of hybridoma cells given. Furthermore the body weights of all three groups were the same suggesting that hybridoma cells had not effected the growth rate of the animal.

Since IGF-1 is subject to extensive feedback regulation *in vivo* I also looked at the short term effects of αIGF-1 treatment on the pyknotic index in the nephrogenic zone. To do this I gave a single i.p. injection to P0 rat pups of purified SM1,2 antibody or purified mouse IgG at a dose which is expected to be biologically active for at least 5 days *in vivo* (S. Cobbalt, personal communication) After 8, 12, or 24 hours the pups were perfusion fixed and frozen sections of the kidneys were stained with propidium-iodide. Treatment with purified SM1,2 αIGF-1 antibody

had no effect on the pyknotic or mitotic indices in the nephrogenic zone of the kidney when compared to the effect of random IgG treatment.

These negative results are difficult to interpret as IGF-binding proteins may reduce the neutralizing capacity of the antibody *in vivo* and there is some question whether SM1,2 neutralises rodent IGF-1 (van Wyck, personal communication).

Clearance time of dead cells in the nephrogenic zone

To get an indication of how much cell death occurs in the developing kidney, I compared the pyknotic index in the kidney with that in propidium-iodide-stained cryosections of P7 rat thymus, where a large amount of cell death is known to occur (Shortman et al., 1990; Egerton et al., 1990). The pyknotic index in the cortex of the thymus of P7 rats was 0.2 $\pm$ 0.01% (n=5), confirming the idea that the pyknotic index can be small when the extent of cell death is large.

In order to estimate the clearance time of dead cells I injected EGF (1 intraperitoneal injection) into newborn rat pups and perfusion-fixed the kidneys at 45 minutes, 1 hour, 1.5 hours, and 3 hours after the injection. For each of these time points the pyknotic index in the nephrogenic zone was compared to that of uninjected siblings. For the 11 hour time point, where multiple injections (1 every 3 hours) were given to ensure an elevated level of EGF, the control pups were injected with PBS containing 0.1% BSA. The first time at which a significant reduction of the pyknotic index was seen was 1.5 hours after the first injection (Fig. 3.3), and the mean pyknotic index had reached a basal level by 3 and 11 hours.

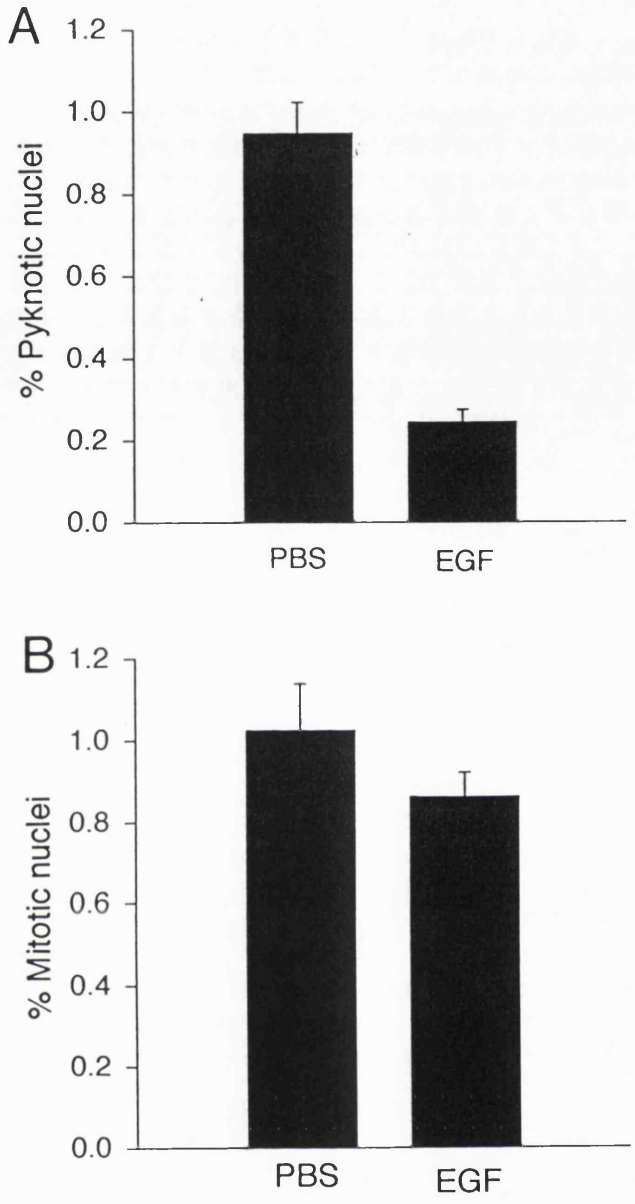


Figure 3.1 Pyknotic (A) and mitotic (B) indices in the nephrogenic zone of P0 rat kidneys that were treated with EGF or PBS. The rats received 4 injections over 9 hours and were perfused 2 hours after the last injection. In this and the following experiments, the slides were read blind, the nuclei in the outer-most 100 μm of the cortex were counted in every second field of 3 non-serial sections for each pup and the results are presented as mean ± s.e.m. of the results from 3-6 sibling pups for each treatment.

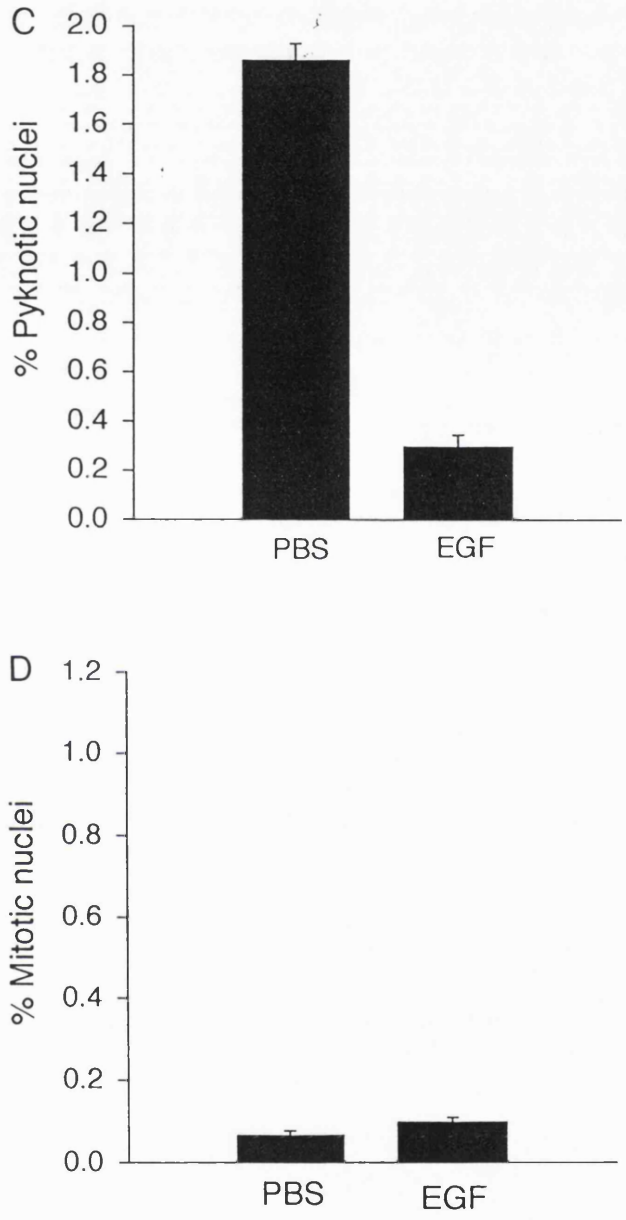


Figure 3.1 Pyknotic (C) and mitotic (D) indices in the medullary papilla in the nephrogenic zone of P0 rat kidneys that were treated with EGF or PBS. The results are presented as mean ± s.e.m. of the results from 3-6 pups for each treatment.

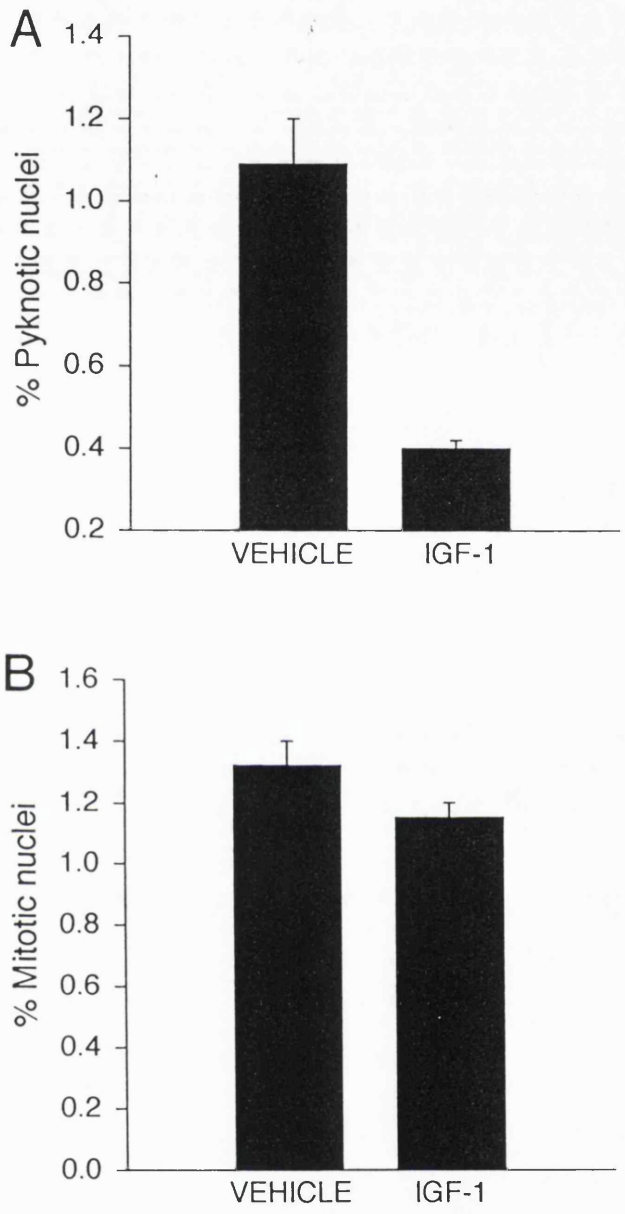


Figure 3.2 Pyknotic (A) and mitotic (B) indices in the nephrogenic zone of P0 rat kidneys that were treated with truncated IGF-1 or PBS. The rats received 1 injection and were perfused 3 hours later. The results are presented as mean ± s.e.m. of the results from 3-6 sibling pups for each treatment.

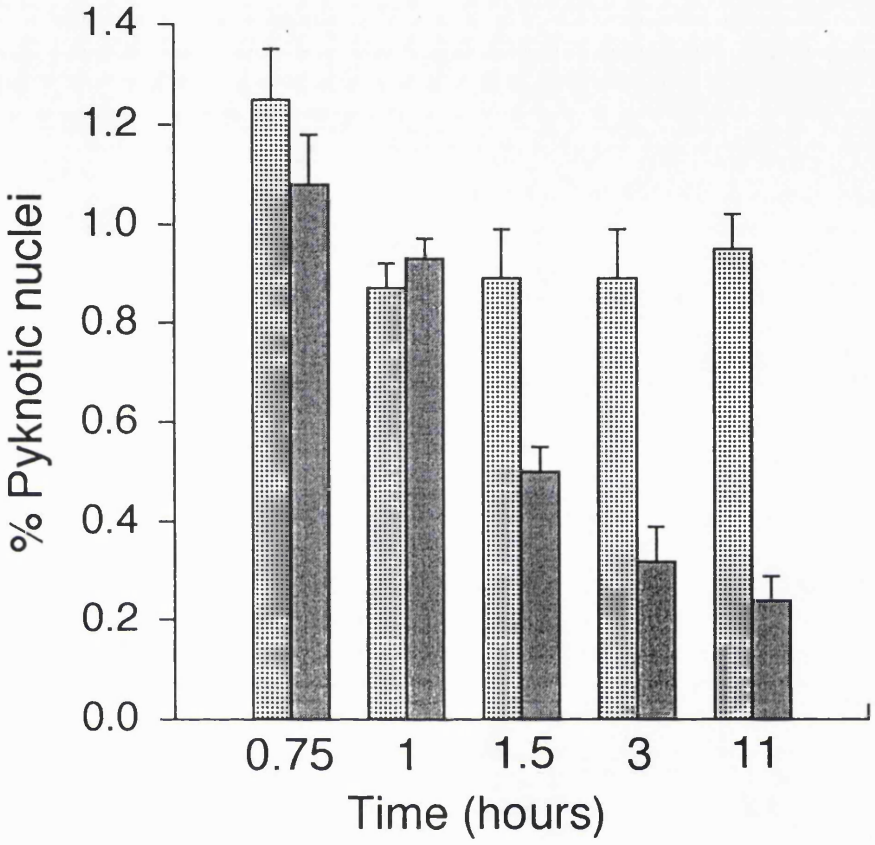


Figure 3.3 Pyknotic indices in the nephrogenic zone of P0 rat kidneys at various times after an intraperitoneal injection of EGF (stippled bars), compared to those of uninjected siblings (black bars). In the 11 hour experiment, the rats were treated as in figure 3.1. * and ** indicate significant differences (P = 0.025 and 0.01, respectively) compared to controls, when analysed by Student's t-test.

Discussion

The fact that cells die in most developing vertebrate tissues has been known for many years (Glucksmann, 1951), although the extent of the cell death is unknown for most tissues. Moreover, with the exception of several neuronal populations in the developing vertebrate CNS and the cell deaths that occur in metamorphosis, the causes of this normal cell death are largely a mystery. The pioneering work of Levi-Montalcini, Hamburger and Cohen on nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1949; Levi-Montalcini, 1987) and its affects on sympathetic and some sensory neurones established a model for the regulation of cell death during neural development – the so-called neurotrophic theory. Neurotrophic factors, which are required for the survival of developing neurones, are produced in limiting amounts by the target cells that the neurones innervate. The neurones are produced in excess and only a proportion receive enough neurotrophic factor to survive and develop further. In this chapter I described experiments that suggest that the neurotrophic mechanism is only one example of a general mechanism that may help to control cell numbers during development of non-neural tissues such as the kidney.

Are there survival factors for cells in the developing kidney?

In contrast to the extensive search for neurotrophic factors over the last two decades (Barde 1989), there have been relatively few attempts to establish the survival requirements of non-neural cell types. For example, EGF is known to be important in kidney development (Fisher et al., 1989), but it has not been considered to be a survival factor. In the

absence of other signalling molecules, however, EGF preserves the structure and histology of kidney explants without stimulating DNA synthesis (Weller et al., 1991), and it also reduces DNA degradation in kidney rudiments *in vitro* (Koseki et al., 1992). Therefore it is likely that EGF acts as a survival factor for metanephric mesenchyme *in vitro*. I found that EGF greatly reduces the pyknotic indices in the nephrogenic zone and the medullary papilla *in vivo* without affecting the mitotic indices, consistent with the idea that EGF can act as a survival factor for cells in the normal developing kidney. EGF receptor mRNA is expressed at high levels in embryonic kidney (Adamson and Meek, 1984), but the ligand for the receptor during normal kidney development is probably TGF-α rather than EGF, since EGF mRNA expression is not detected in rat or mouse kidney until the second postnatal week while TGF-α mRNA is expressed at high levels in the embryo (Lee et al., 1985).

IGFs 1 and 2 are also thought to be required for normal metanephric development (Mendley and Toback, 1988; Rogers et al., 1991), although their role as survival factors has not previously been considered. I found that IGF-1 reduces the pyknotic indices in the nephrogenic zone and the medullary papilla *in vivo* by 60% without affecting the mitotic indices, consistent with the idea that IGF-1 can act as a survival factor for cells in the normal developing kidney. Furthermore, the mRNAs for the IGF-1 receptor (D'Ercole et al., 1976) and IGFs 1 and 2 (Han et al., 1987; Rogers et al., 1991) are all expressed in the embryonic and early postnatal kidney.

Although these results suggest that EGF and IGF-1 can rescue cells that would normally die in the kidney during development, another interpretation is possible: EGF and IGF-1 might reduce the

pyknotic index in the kidney by stimulating the clearance of dead cells, rather than by promoting cell survival. In order to distinguish between these possibilities I did the following experiments.

If EGF prevents cell death then EGF treatment should lead to an accumulation of cells that would otherwise die and therefore to an increase in the amount of DNA in the kidney. However, EGF treatment over 3 days did not increase total kidney DNA. Subsequently I learnt that EGF treatment for the first postnatal week in rats causes an inhibition of DNA synthesis in the kidney, as assessed by ^{3}H-Thymidine incorporation (Gattone et al., 1992). It is, therefore, possible that the effect of EGF on mitosis in the kidney would prevent the detection of increased cell survival by this method. The TUNEL technique was also unsuccessful in establishing whether EGF promotes cell survival or pyknotic cell clearance because in the kidney - unlike in the retina (Chang et al., 1993) - this method does not detect more dead cells than propidium iodide staining.

It therefore remains uncertain whether delivery of extra EGF reduces the kidney pyknotic index by preventing cells from dying or by stimulating the clearance of pyknotic cells. It seems likely, however, that EGF is having a trophic effect in the developing kidney. While it has been found that cytokines can increase recognition of apoptotic leukocytes by macrophages (Savill et al., 1993), these are cell types commonly found in regions of inflammation where phagocytosis of debris by macrophages is high and rapid phagocytosis is important to limit tissue damage from inflammation (Newman et al., 1982; Savill et al., 1993). In regions of normal cell death clearance of apoptotic cells is thought to be often by neighbouring cells (Wyllie et al., 1980; Kálmán

1989). Furthermore, in the developing optic nerve extra doses of the *in vitro* survival factor platelet derived growth factor (PDGF) decreases normal cell death but does not affect clearance *in vivo* (Barres et al.,1992). Similarly, EGF acts to decrease DNA degradation in early metanephric rudiments suggesting that it is a survival factor for metanephric mesenchyme *in vitro* (Koseki et al., 1992). Finally, it is not clear that an increase in the recognition and uptake of dying cells by neighbouring kidney cells would alter the pyknotic index, as almost all the pyknotic kidney cells that are seen in electron micrographs are inside neighbouring parenchymal cells. Therefore the rate limiting step in the clearance of pyknotic cells in the kidney is probably the digestion and not the phagocytosis of cellular debris. It seems likely, then, that EGF and IGF-1 promote cell survival in the developing kidney.

Do kidney cells normally die due to limiting amounts of survival factors?

Why do cells die in the developing kidney? Programmed cell death could be activated in cells that have undergone aberrant cell division. The cells could be positively stimulated to die by extracellular signals, as in the tadpole tail at metamorphosis (Goldsmith, 1966; Kerr et al., 1974). Alternatively, cells could die because they fail to receive adequate survival signals, as in the developing vertebrate nervous system (Hamburger et al., 1981). In the latter case, it has been shown that increasing levels of survival factors greatly reduces the extent of developmental cell death (Hamburger et al., 1981; Hofer and Barde, 1988; Oppenheim et al., 1988).

In the kidney I found that increasing the levels of either EGF or IGF-1 decreases the pyknotic index in the nephrogenic zone and the medullary papilla, consistent with the possibility that the levels of these factors are normally limiting in the kidney *in vivo*. This is supported by the observation that mice transgenic for the human IGF-1 gene have both increased levels of IGF-1 mRNA and protein in their kidneys and larger kidneys with an increased cell number (Mathews et al., 1988). My results raise the possibility that this increase in cell number is due to stimulation of cell survival rather than cell proliferation.

In vertebrate sympathetic ganglia, where NGF is thought to regulate cell death *in vivo*, depletion of NGF by delivery of anti-NGF antiserum leads to almost total deletion of the ganglia within several days (Levi-Montalcini, 1972; Gorin and Johnson, 1979; Goedert et al., 1980). In the kidney the SM1,2 anti-IGF-1 antibody does not alter the pyknotic index over several hours or days. It seems likely that the explanation for this is technical: IGF binding proteins may interfere with the neutralising activity of this antibody *in vivo* (Bartlett personal communication), for example, or the antibody may not recognise rodent IGF-1. It remains possible, however, that IGF-1 does not regulate cell survival during normal kidney development, but nonetheless can enhance kidney cell survival when given in excess.

The extent of cell death in kidney development.

I find that the peak pyknotic index in the developing rat kidney is about 3%. What does this mean in terms of the proportion of kidney cells that die during normal development? Because dead cells are rapidly cleared by phagocytosis and are therefore detectable for only a short time, it is clear

that the pyknotic index in a tissue can be small when the number of dying cells is large. Where the clearance times for normal cells dying by apoptosis have been calculated or directly observed, they are surprisingly short. In the rat retina, for example, where about 100,000 (50%) of the retinal ganglion cells die over several days, the pyknotic index in the retinal ganglion cell layer is about 0.5%, indicating that the clearance time for the dead cells is 2-3 hours (Perry et al., 1983; V. H. Perry, personal communication). In the developing rat optic nerve, where approximately 10,000 (50%) of the newly-formed oligodendrocytes die daily, the maximum pyknotic index is about 0.3%, indicating that the clearance time is about 1 hour (Barres et al., 1992). In the nematode *Caenorhabditis elegans*, a clearance time of 1 hour has been directly observed for cells undergoing normal cell death *in vivo* (Ellis et al., 1991). In hydra a clearance time of 1-2 hours has been calculated for apoptotic epithelial cell (Bosch and David, 1984). In the present study, I find a pyknotic index of 0.2% in the developing rat thymus, and yet it has been estimated that more than 97% of the newly-formed thymocytes die during normal thymus development in the mouse (Shortman et al., 1990). This suggests that dead thymocytes are cleared in minutes.

My findings suggest that the clearance time of dead cells in the nephrogenic zone of the developing rat kidney is comparable to that seen in other developing tissues. I see almost a 50% reduction in the pyknotic index in the nephrogenic zone within 1.5 hours of injecting EGF into newborn rats; assuming that EGF works by blocking cell death and not by increasing the rate of cell clearance, this suggests that from the time a kidney cell becomes committed to die to the time that we can no longer detect it as a dead cell is less than or equal to 1.5 hours for many of

the dying cells. As the pyknotic and mitotic indices in the nephrogenic zone at P0 are both about 1%, and the apparent clearance time for dead cells (1-2 hours) is probably not very different from the duration of mitosis (about 1 hour, Baserga 1985), I estimate that 1 cell dies for every 2-4 cells produced by cell division (in this zone at this time). Thus the proportion of cells dying in the nephrogenic zone during kidney development is likely to be quantitatively similar to the proportion of cells dying in the developing vertebrate nervous system (Oppenheim, 1991).

Methods

Treatment with EGF *in vivo*

To establish whether EGF can block kidney cell death *in vivo*, eight, one day old (P1), sibling rat pups were given 4 intraperitoneal injections of either EGF (0.3 μg/g body weight; Sigma) or the vehicle, PBS containing 0.1% BSA, at 3 hour intervals. Two hours after the last injection, (which was 11 hours after the first injection) the pups were perfused and the kidneys were fixed, processed and stained with propidium iodide as described in Chapter 2. Pyknotic and mitotic nuclei in the nephrogenic zone and the medullary papilla were counted and the numbers obtained were corrected for split cell counts (Abercrombie, 1946) and expressed as a percent of total nuclei.

To estimate the clearance time of pyknotic cells in the developing kidney, newborn rat pups were injected intraperitoneally with EGF (0.3 μg/g body weight; Sigma) and perfused with 4% paraformaldehyde at 45 minutes, 1 hour, 1.5 hours, or 3 hours after the single injection. Pyknotic and mitotic indices in the nephrogenic zone of the kidney were determined as above and compared to those of non-injected siblings. The effect of elevated EGF levels over 11 hours was determined as above. At least 3 sibling pups were used for each time point, in each experiment.

Treatment with IGF-1 *in vivo*

To determine whether IGF-1 can prevent kidney cell death *in vivo*, eight, neonatal, sibling rat pups were given a single intraperitoneal injection of either human recombinant truncated IGF-1 (1.5 μg/g body weight; GroPep) or the vehicle, double distilled water (DDW) containing 1mg/ml

BSA and 10^{-2} M HCL. The dose of IGF-1 given was similar to that previously used to stimulate growth in normal growing rats (Hizuka et al., 1986). Three hours after the injection, the pups were perfused and the kidneys were fixed, processed and stained with propidium iodide as described above. Pyknotic and mitotic nuclei in the nephrogenic zone were counted and the numbers obtained were corrected for split cell counts (Abercrombie, 1946) and expressed as a percent of total nuclei.

Long-term EGF treatment and kidney DNA content

If EGF is a trophic factor for kidney cells, then long-term EGF treatment would be expected to lead to an increase in the total DNA content in the kidney. Ten neonatal rat pups were injected sub-cutaneously with EGF (0.2 μg/g body weight, Sigma, in 150 ml PBS, 0.1% BSA) or vehicle alone every 8 hours for 3 days. 3 hours after the last injection the pups were killed, their kidneys were weighed and then homogenised in a glass homogeniser in 1 ml of TES (10 mM Tris-HCL, 50 mM EDTA, 0.1% SDS). The homogenate was transferred to pre-weighed bijoux containers; the homogeniser was rinsed with 1.97 ml TES, which was added to the sample. Proteinase K (200 μg/ml final concentration, Sigma Type xxviii) was added and the homogenate was incubated at 55°C for 24 hours and the proteinase K refreshed at 12 hours. After digestion of the samples, the bijoux flasks were weighed and the amounts of DNA in the kidneys were measured using the fluorimetric methods of Labarca and Paigen (1980), using the DNA-binding dye bisbenzimidazole (Hoescht 33258). DNA standard curves were obtained using calf thymus DNA (Sigma) in TNE (100mM Tris base, 10 mM EDTA, 2M NaCl pH7.4), with the same percentage SDS as in the sample aliquots. Treatment of

kidney digest with DNase I removed the fluorescence in the presence of Hoescht 33258.

In situ DNA fragmentation assay

To test the sensitivity of the TUNEL technique (Gavrieli et al., 1992) in identifying pyknotic cells in tissue sections, newborn rat pups were injected intraperitoneally with EGF (0.3 μg/g body weight; Sigma) and perfused with 4% paraformaldehyde either immediately, or at 45 minutes after the injection. Cryosections of 6.5 μm were collected on APES-treated slides, air dried and rehydrated with 10 mM Tris.HCL pH8.

Proteins were stripped from the sections with proteinase K (20 μg/ml, Sigma) for 15 minutes at room temperature, and endogenous peroxidases were inactivated with 3% hydrogen peroxide. After extensive rinsing in double distilled water, the slides were treated with biotinylated dUTP (Bio-16-dUTP, 3 nM, both Boehringer Mannheim UK.) in the presence of terminal deoxynucleotidyl transferase (TDT, 10 u/l in TDT buffer, both GIBCO BRL) at 37°C for 1 hour to endlabel any nicked DNA in the sections. The reaction was terminated with 300mM NaCl and 30 mM sodium citrate.

The slides were washed with PBS for 5 minutes at room temperature, blocked with 2% BSA for 10 minutes and then washed again. Biotinylated-dUTP-end-labelled DNA was labelled with and Avidin DH:biotinylated horseradish peroxidase H complex for 30 minutes at room temperature. Labelled nuclei were then visualised by a 7 minute incubation with the peroxidase substrate diaminobenzidine tetrahydrochloride (DAB; Amersham UK.) in the presence of 30% peroxide. The slides were then rinsed, lightly counterstained with Toluidine

Blue, mounted in Citifluor (City University, London) and examined with phase contrast and bright field microscopy. Pretreatment of sections with DNase I (1 μg/ml, 10 minutes at room temperature) caused extensive DNA nicking and all nuclei to be labelled while omission of the dUTP from the end-labeling reaction eliminated all labelling.

Transplantation of αIGF-1 secreting hybridoma cells

The method used was adapted from Schnell and Schwab (1990). Hybridoma cells secreting either the αIGF-1 mouse monoclonal antibody, SM1,2, (UBI, van Wyk et al., 1986) or an anti-Brd-U mouse monoclonal antibody of the same Ig class (IgG1) (Magaud et al., 1988) were grown and harvested at an active phase of growth by spinning the cells in a centrifuge at 1000 rpm for 10 minutes. The cell pellet was re-suspended and then washed in 20 ml of PBS, 0.2% BSA and re-pelleted by centrifuging at 1000 RPM for 5 minutes. The cells were then re-suspended in 1ml PBS, 0.2% BSA and centrifuged for 10 seconds at high speed in an Eppendorf microcentrifuge to form a compact pellet. These cells were re-suspended in PBS, 0.2% BSA at a concentration of approximately 50 million cells per 150 μl of PBS, 0.2% BSA and injected intraperitoneally into PO rat pups that were then returned to their mothers. The dose of cells injected was determined by a pilot experiment that showed that 1 million and 10 million SM1,2-secreting cells had no affect while 50 million cells might have an affect. Three days later the pups were killed, at which time the transplanted cells were visible in the peritoneum.

Delivery of purified aIGF-1 antibody *in vivo*

To look at the short-term effects of αIGF-1 *in vivo*, P0 rat pups were given a single 100μl injection of purified SM1,2 antibody (5 μg/g body weight, in PBS, 1 mg/ml BSA; UBI) or purified random mouse IgG antibody (5 μg/g body weight in PBS, 1mg/ml BSA; Sigma Immunochemicals). This dose was based on the information that a mouse IgG1 of reasonably high affinity given in a 100-fold excess remains at saturating levels in the serum for 5-7 days (S. Cobbalt personal communication; Isaacs et al., 1992), and the fact that SM1,2 is lower affinity (10-30 μg/ml *in vitro* neutralises 20ng/ml human IGF-1 as determined by Balb/C 3t3 cell growth). The animals were perfusion fixed 8, 12 and 24 hours after injection and 6.5 μm cryosections of their kidneys were stained with propidium iodide.

Abercrombie corrections for the thymus

In the cortex of the P6 rat thymus, the average diameter of non-dividing, non-pyknotic nuclei in 5μm sections was 6 μm ±0.1 (n=125), compared to that of pyknotic nuclei which was 3 μm ±0.1(n=26). Calculations of the Abercrombie correction factor were made as in chapter 2.

Chapter 4

The Ubiquity of the Cell Death Programme

Introduction

The observation that cell death occurs selectively and predictably during vertebrate development, and evidence that inhibitors of RNA and protein synthesis prevent normal cell death in certain cells (Wyllie et al., 1980; Martin et al., 1988; Oppenheim et al., 1990) were taken to suggest that PCD in vertebrates, like that in *C. elegans,* has a genetic basis. The requirement for *de novo* mRNA and protein synthesis, however, turned out not to be universal: in many cases protein and mRNA synthesis are not required for PCD (Batistatou and Greene, 1993); indeed protein and RNA-synthesis inhibitors often induce PCD (Martin 1993). More direct evidence for the genetic basis of vertebrate cell death has come from recent studies identifying mammalian homologues of *ced-3* and *ced-9* which seem to be involved in normal cell death (Yuan et al.,1993; Miura et al., 1993). Over-expression of the *bcl-2* gene (Tsujimoto et al., 1985), a mammalian homologue of *ced-9* (Hengartner and Horvitz, 1994), not only prevents many (but not all) vertebrate cell types from undergoing programmed cell death (Vaux et al., 1988; Nuñez et al., 1990; Garcia et al., 1992), but it also prevents the cell deaths normally seen during nematode development (Vaux et al., 1992), and rescues mutant worms (Hengartner and Horvitz, 1994). Therefore, there is striking conservation of the cell death programme and its regulation between nematodes and humans, indicating that PCD is a fundamental property of the cells of multicellular animals.

Many vertebrate cell types are now known to depend on signals from other cell types in order to survive. In addition to the survival-factor-dependence of many neurones and glia (Oppenheim, 1991; Barde, 1989; Barres et al., 1993), epithelial cells in the adult rat ventral prostate

require testosterone in order to survive (Kerr and Searle, 1973; Kyprianou and Isaacs, 1988), the adrenal cortex requires pituitary adrenocorticotropic hormone (Wyllie et al., 1973; Wyllie 1975), T-cell precursors require interleukin-2 (Duke and Cohen, 1986), hemopoetic cell precursors require interleukin-3 (Rodriguez-Tarduchy et al., 1990), hemopoetic cells require colony stimulating factor (Williams et al., 1990), primordial germ cells require stem cell factor (Godin et al., 1991; Dolci et al., 1991), and endothelial cells require EGF (Etoh et al., 1989). If deprived of these factors, these cells will kill themselves. Even anatomically isolated cell types, such as lens epithelial cells and chondrocytes, which can survive in serum-free culture independently of other cell types, require signals from cells of the same type to avoid PCD (Ishizaki et al., 1993; Ishizaki et al., in press). Indeed, it has been recently suggested that dependence on signals from other cells in order to repress PCD may be a general principle in the cell biology of multicellular animals (Raff 1992). Blastomeres are an apparent exception to this model. Mouse blastomeres from the 1-cell stage up until the 16-cell stage, do not require any proteins to survive and divide in culture; nor do they require the presence of other blastomeres (Biggers et al., 1971). Therefore these cells, unlike any others known, can survive in the absence of extracellular signals.

If it is true that all cells except blastomeres require constant survival signalling in order to avoid PCD, then blocking the intracellular signalling pathways activated by survival factors should lead to PCD in these cells. Indeed, blocking protein kinase activity with high doses of staurosporine induces PCD in several cell types in the presence (Bertrand et al., 1993; Ishizaki et al., 1993; Jacobson et al., 1993;

Ishizaki et al., in press; Meikrantz et al., in press) or absence (Jacobson et al., 1994) of protein synthesis. Even enucleated cells apparently undergo PCD when treated with staurosporine or deprived of survival factors (Jacobson et al., 1994). The number of cell types that have been tested for STS-induced PCD is, however, limited.

Continuous survival signals could be required either to repress the transcription of "cell death genes" or the translation of "cell death proteins". Alternatively they could be required to repress the activity of constitutively expressed "cell death proteins". The finding that blocking *de novo* protein synthesis can often stimulate rather than prevent PCD in many cases (Martin, 1993) raises the possibility that many vertebrate cell types may constitutively express the proteins required for PCD, and that survival signals are required to keep the programme suppressed (Jacobson et al., 1994). It is not known whether blastomeres survive in the absence of protein signalling molecules because they do not contain the machinery for PCD, or because they contain the machinery but suppress it autonomously. In this chapter, I ask whether STS-induced PCD, in the presence or absence of protein synthesis, is the exception or the rule for most cell types in the developing and adult rodent.

If cell survival is socially determined (Raff, 1992), then the dependence on other cell types for survival might be expected to arise when the embryo first contains more than one cell type - after the differentiation of blastomeres into cells of the trophectoderm and inner cell mass (ICM). In support of this prediction, cells with the morphological features of PCD (apoptosis) are normally seen after this initial differentiation event - in the inner cell mass cells of preimplantation blastocysts (Potts and Wilson, 1967; El-Shershaby and

Hinchliffe, 1974; Handyside and Hunter, 1986). Moreover, soon after the ICM forms, it differentiates into an inner core of primitive ectoderm cells and an outer layer of endoderm cells, and the cells of the primitive ectoderm have been shown to depend on the endoderm cells for their survival *in vitro* (Rossant and Ofer, 1977; Gardner, 1985). After implantation, as the embryo differentiates further, the incidence of cell death increases (Poelmann and Vermeij-Keers, 1976; Poelmann 1981; Snow, 1988).

Staurosporine (STS) is a bacterial alkaloid that is commonly used as a protein kinase inhibitor (Omura et al., 1977; Tamaoki et al., 1986). The induction of PCD by STS occurs rapidly - often within 8 hours of addition to the culture medium (Ishizaki et al., 1993; Jacobson et al., 1993; Bertrand et al., 1993; Meikrantz et al., in press). In this chapter, I show that almost all cell types tested from early postnatal and adult mice die by apoptosis when treated with both the protein kinase inhibitor staurosporine and the protein synthesis inhibitor, cycloheximide. Blastomeres, however, differ from even their earliest derivatives, inner cell mass cells, in that they fail to undergo PCD when treated with staurosporine. These results suggest that most, and perhaps all, cells other than blastomeres, are capable of undergoing PCD and constitutively express all of the proteins required for PCD.

Results

Signalling and cell survival in postnatal rodent tissues

To test for the presence of the cell death programme I used staurosporine to induce PCD, as it has been reported to be an effective apoptotic agent in several cell types (Jacobson et al., 1993; Meikrantz et al., in press). Preliminary experiments on cultured rat organ explants in the presence or absence of STS indicated that most cells die within 24 hours of STS treatment. In most of the experiments, however, I used mouse tissue so that direct comparisons could be made with studies on early mouse embryos.

To screen many different cell types I cultured explants of postnatal mouse heart, kidney, lung, muscle, pancreas and liver. The explants were cultured for 18 hours in the following conditions: 10% foetal calf serum (FCS) alone; FCS + STS (1 μM); FCS + STS (1 μM) + cycloheximide (CHX, 10 μg/ml). In the third treatment, tissues were pre-incubated with CHX for half an hour before the addition of STS. In all the tissues cultured with STS + CHX, more than 90% of the cells were pyknotic in propidium-iodide stained cryosections, while in all the tissues cultured in 10% FCS alone, less than 10% of the cells were pyknotic (Fig. 4.1). Pyknotic nuclei were clearly visible in propidium iodide stained cryosections of the explants: they were shrunken, brightly stained and often fragmented (Fig. 4.2A, B). Furthermore, end-labelling of fragmented DNA *in situ* showed that the pyknotic nuclei contained fragmented DNA, suggesting that the cells had died by apoptosis (Fig.4.3A,B).

Signalling and cell survival in blastomeres

To determine whether STS and CHX can induce PCD in blastomeres, cells from 4-cell stage embryos were treated with STS and CHX. 100% (n=22) of blastomeres were alive after being cultured for 18 hours at low density in M-16 medium alone; 90% (n=22) of blastomeres were alive after being cultured for 18 hours in the presence of 1 μM staurosporine; and 100% (n=22) of blastomeres were alive after being cultured for 18 hours in the presence of both CHX and STS (Fig.4.4). Cells were counted as alive if they had intact plasma membranes (as judged by exclusion of ethidium homodimer), were phase bright, and had a large oval nucleus with chromatin that appeared textured with Hoescht staining. The MTT assay for mitochondrial activity was not suitable because the MTT metabolising activity of blastomeres under all conditions was low. The single blastomere that died in STS had a diffuse and dimly fluorescent nucleus with Hoescht staining typical of cells dying by necrosis (M. D. Jacobson personal communication); it was phase dark but not shrunken, making it unlikely that it died by apoptosis. Furthermore, using the TUNEL technique to end-label nicked DNA *in situ*, I found that fragmented DNA was not present in any blastomeres in any of the treatments (Fig.4.5). Therefore 2-4-cell stage blastomeres do not undergo PCD when treated with STS and CHX.

When 8-16 cell stage blastomeres were cultures for 24 hours, 100% (n=25) of the cells cultured at low density in M-16 medium alone were alive, whereas 80% (n=20) of the cells cultured with 1 μM STS were alive, 77% (n=31) of the cells cultured with 10 μg/ml CHX were alive, and 70% (n=20) of the cells cultured with 10 μg/ml CHX and 1 mM STS were

alive (Fig. 4.6); 8/17 of the cells that were not alive were pyknotic, the remainder were not found. Thus similar to early blastomeres, most 8-16 cell stage blastomeres do not undergo PCD when treated with STS and CHX. The small number of cells that died by apoptosis in STS and CHX may reflect an underlying change in cell behaviour during early development.

Signalling and cell survival in blastocysts

To further determine when cells in the developing embryo first become susceptible to STS and CHX-induced PCD, I looked at inner cell mass and trophectoderm cells - the earliest derivatives of blastomeres. Without STS or CHX, 82% (n=76) of ICM cells were alive after 18 hours; in 1 μM STS 67% (n=77) cells were alive; in 10 μg/ml CHX 39% (n=74) cells were alive; and in 1 μM STS and 10 μg/ml CHX 0% (n=56) were alive (Fig. 4.7). Similarly, whereas 90% of ES cells (a cell line derived from ICMs) were alive after 18 hours cultured without STS, only 3% of ES cells were alive after 18 hours cultured in 1 μM STS (n>1000 in both cases). Most of the ICM cells that died in STS or STS + CHX and all of the ES cells that died in STS had the characteristic features of apoptosis.

To test the behaviour of trophectoderm cells, intact blastocysts were cultured for 18 hours under the various conditions after removal of the zona pellucida. Without STS or CHX, 94% (n=154) of trophectoderm cells were alive; in 10 μg/ml CHX 97% (n=79) cells were alive; in 1 μM STS 80% (n=95) cells were alive; and in 1 μM STS and 10 μg/ml CHX only 9% (n=75) were alive (Fig. 4.8). In the presence of STS ± CHX the structure of the blastocyst had broken down, in no case was a blastocoelic cavity visible, and the dead cells had the characteristic features of apoptosis.

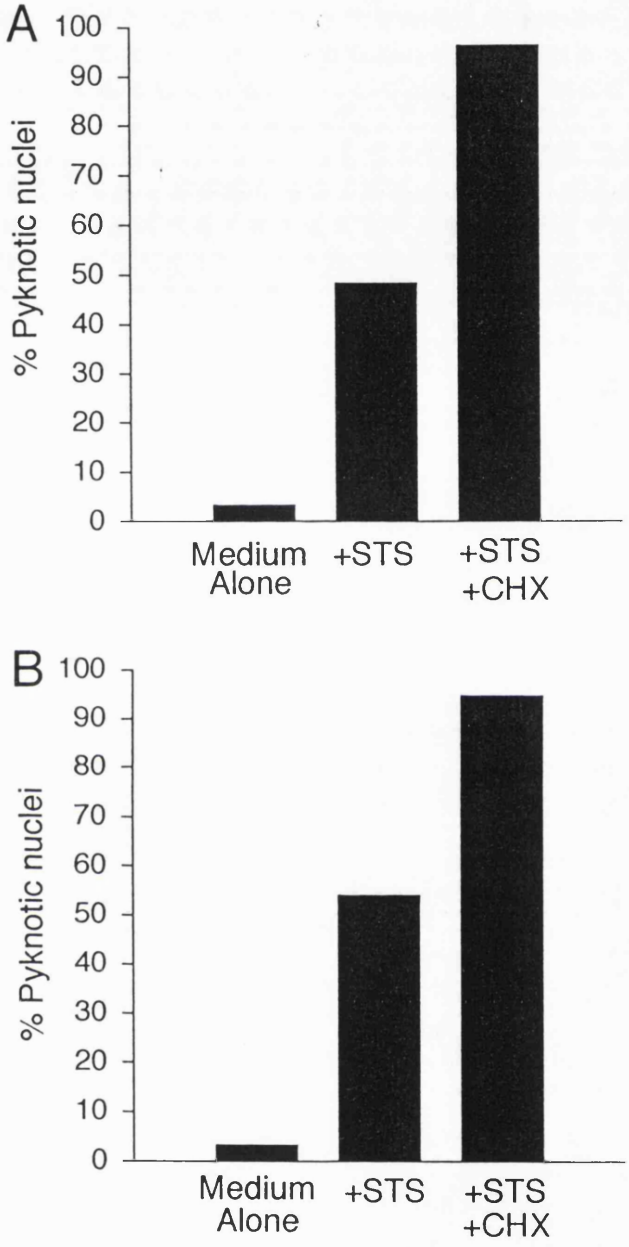


Figure 4.1 (and overleaf) Pyknotic indices in explants of P1 mouse kidney (A) and muscle (B) treated with STS (1 μM), STS and CHX (10 μg/ml) or medium alone. One thousand nuclei were counted from each tissue in fields at the edge of the sections.

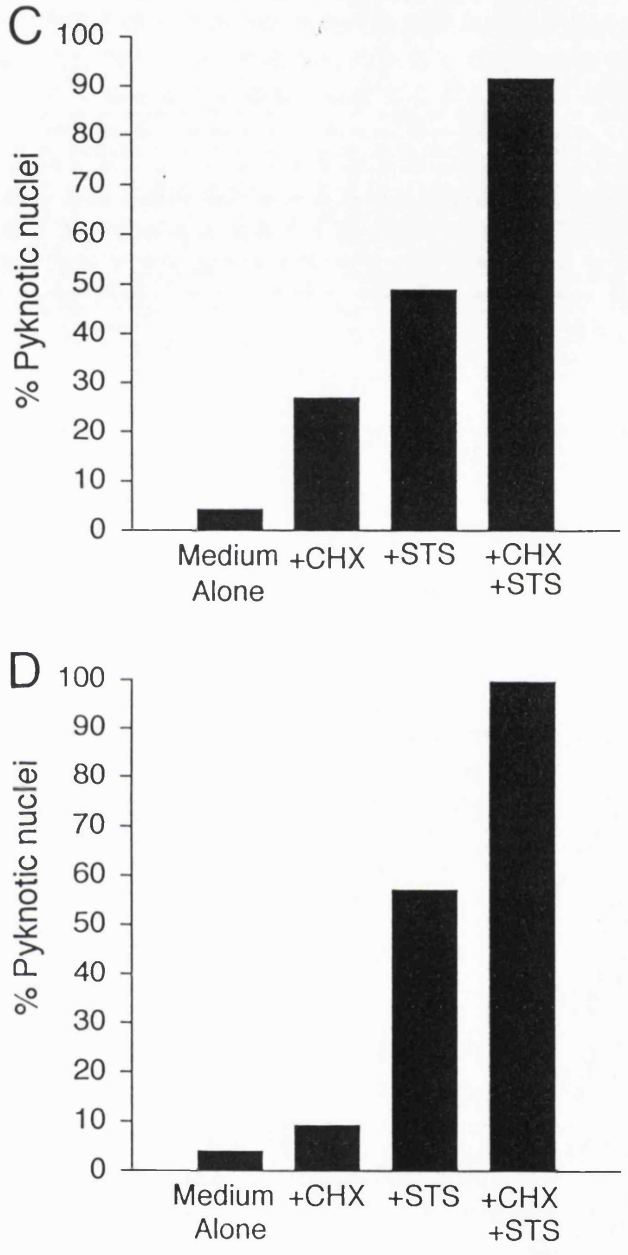


Figure 4.1 Pyknotic indices in explants of P1 mouse lung (C) and pancreas (D) treated with STS (5 μM), CHX (10 μg/ml), STS and CHX or medium alone.

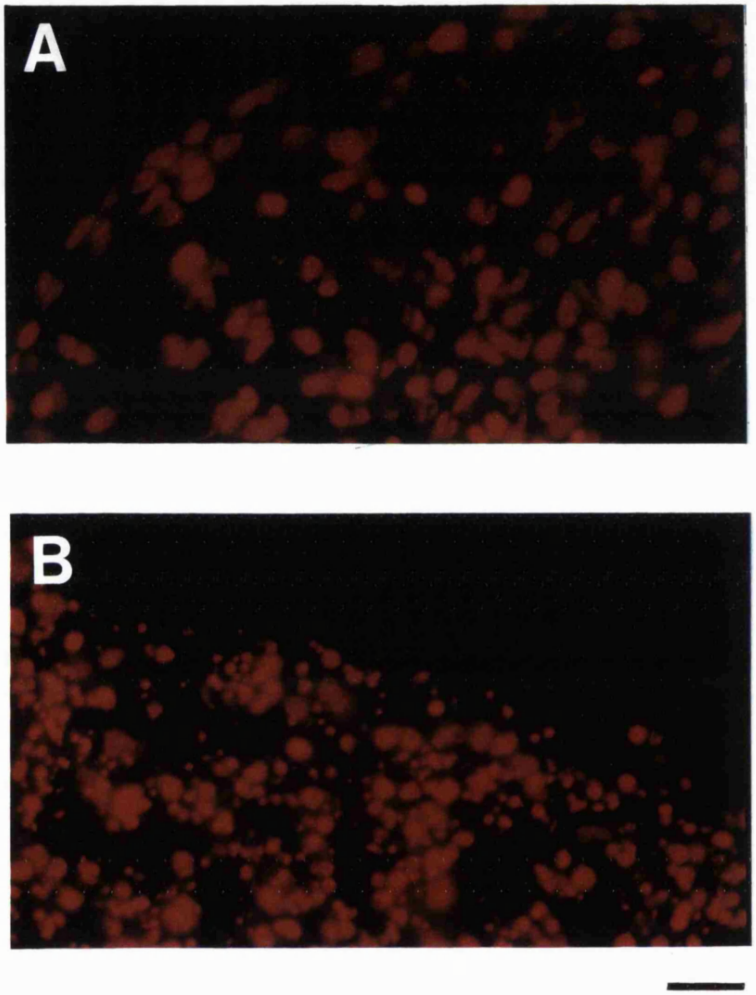


Figure 4.2 Fluorescence micrographs of propidium-iodide-stained nuclei in P1 mouse explants cultured for 18 hours in (A) the absence or (B) the prescience of staurosporine. In (B) the majority of nuclei are pyknotic, while in (A) they are not. Bar = 25μm.

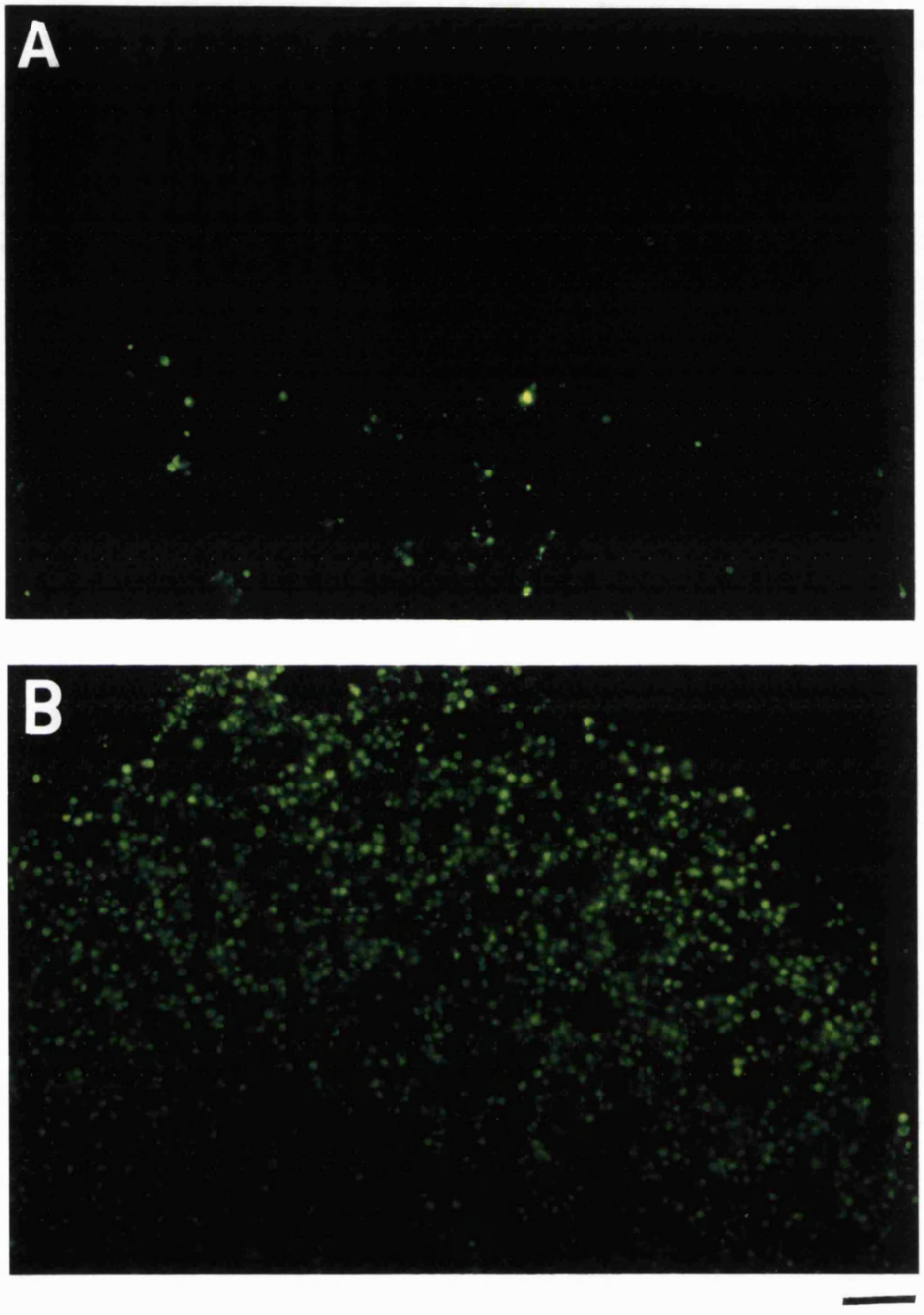


Figure 4.3 End-labelling of nicked DNA *in situ* in explants of P1 mouse kidney. The explants were cultured in the absence (A) or presence (B) of staurosporine and cycloheximide. The labelled nuclei contain fragmented DNA. Bar= 28μm.

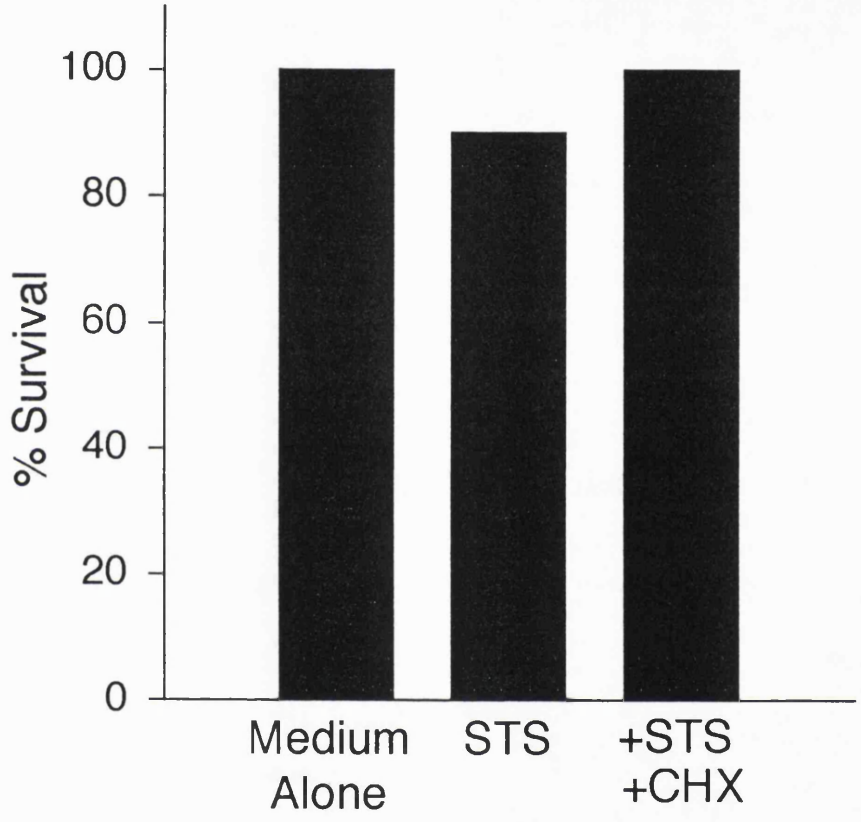
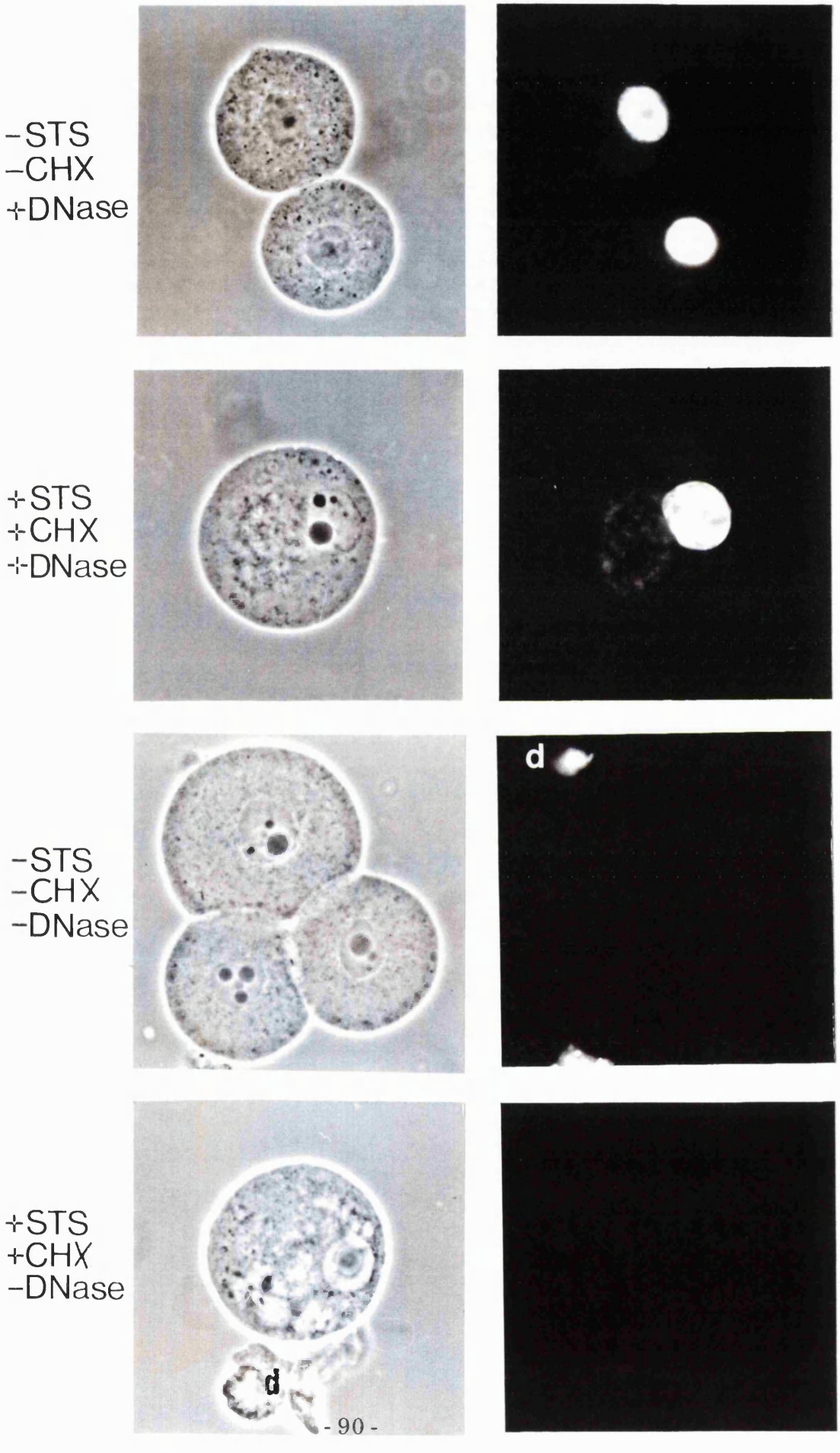


Figure 4.4 Survival of 2-4 cell-stage blastomeres cultured for 18 hours in medium alone, medium with STS, or medium with both CHX and STS. The results are expressed as a percent of the total number of cells (n=22) in each treatment.

Figure 4.5 (overleaf) End-labelling of nicked DNA *in situ* in 2-4 cell-stage blastomeres cultured with or without STS and CHX for 18 hours. Controls were treated with DNase to introduce nicks in the DNA. Phase contrast and fluorescence micrographs showing that none of the blastomeres appear pyknotic and that only those treated with DNase contain nicked DNA. Debris is marked **d**. Bar = 15μm


-STS
-CHX
+DNase
+STS
+CHX
+DNase
-STS
-CHX
-DNase
+STS
+CHX
-DNase
d
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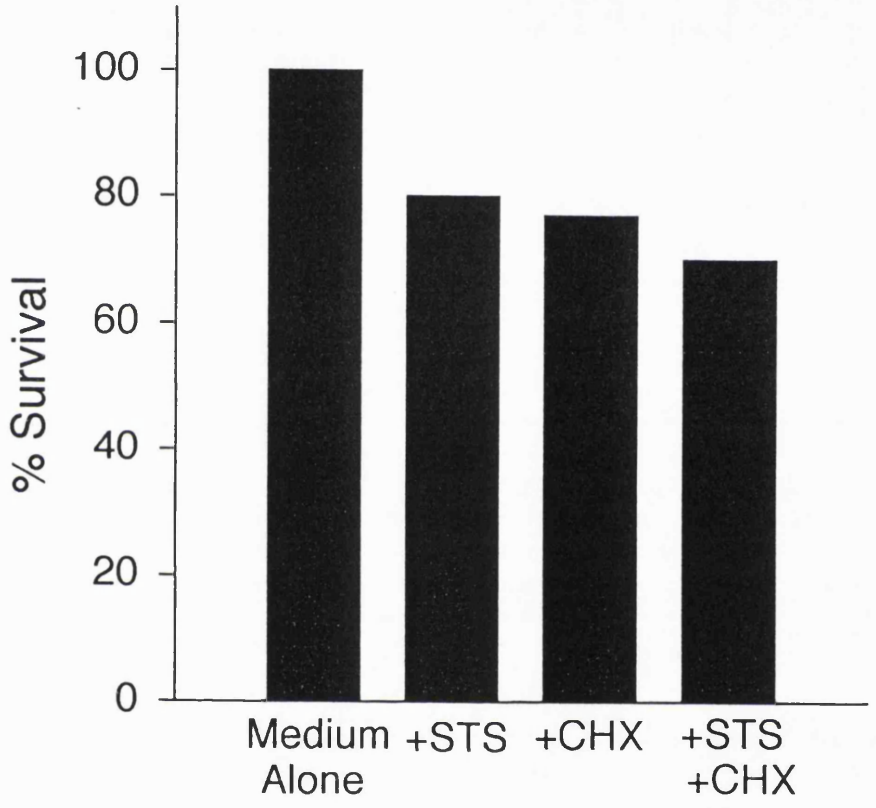

Figure 4.6 Survival of 8-16 cell stage blastomeres cultured for 24 hours in medium alone, with STS, with CHX, or with both CHX and STS. The results are expressed as a percent of the total number of cells ($n \geq 20$) in each treatment.

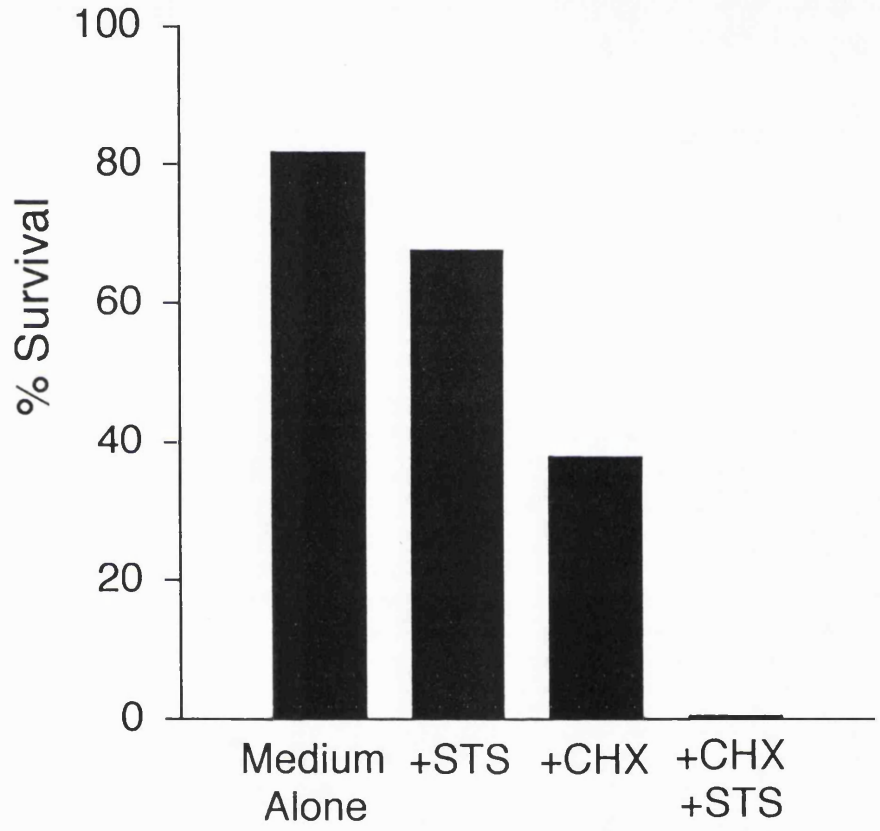

Figure 4.7 Survival of inner cell mass cells cultured for 18 hours in medium alone, with STS, with CHX, or with both CHX and STS. The results are expressed as a percent of the total number of cells (n>55) in each treatment.

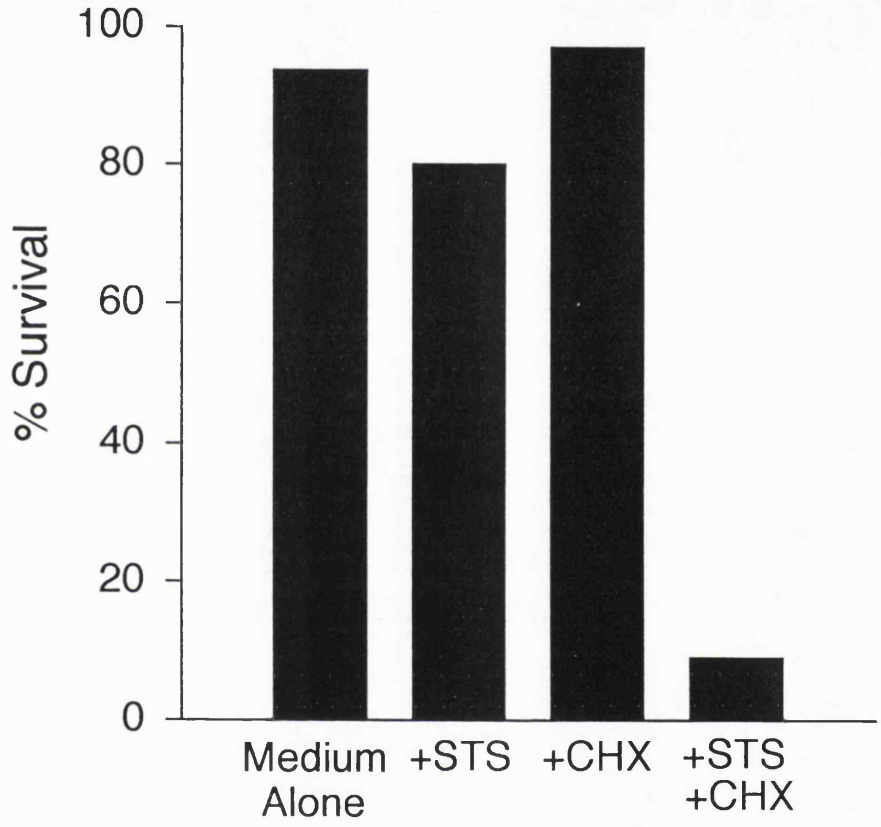

Figure 4.8 Survival of trophectoderm cells cultured for 18 hours in medium alone, medium with STS, or medium with both CHX and STS. The results are expressed as a percent of the total number of cells ($n \geq 75$) in each treatment.

Discussion

There is increasing evidence that many vertebrate cell types require signals from other cells to survive, as the culture requirements of purified primary cells are analysed in detail. Even cells in tissues of a single cell type that do not contain blood or lymph vessels, such as lens cells and chondrocytes, require extracellular survival signals in order to survive, but in this case the signals can be provided by cells of the same type (Ishizaki et al., 1993; Ishizaki et al., in press).

In the present study the protein kinase inhibitor STS (Omura et al., 1977; Tamaoki et al., 1986) was used as a potent and predictable inducer of PCD. Several vertebrate cells die by apoptosis in the presence of high concentrations of staurosporine, including thymocytes (Bertrand et al., 1993), lens epithelial cells (Ishizaki et al.,1993), chondrocytes (Ishizaki, 1994, human fibroblast cell lines (Jacobson et al., 1994) oligodendrocytes and their precursors (Jacobson and Barres, unpublished observations). In the present study, we show that almost all of the cells in 6/6 organs can die by apoptosis: STS and CHX induce more than 90% pyknosis in postnatal explants of mouse heart, liver, lung, kidney, muscle, and pancreas, and rat gut, skin and cartilage (data not shown) within 18 hours. In no case, however, did 100% of the cells die. This may be due to technical reasons, since increasing the concentration of STS increased that pyknotic index in postnatal lung and pancreas. Furthermore, in dissociated-cell cultures 100% pyknosis is not seen until 48 hours after STS treatment, even though over 90% pyknosis is observed within 14 hours (Jacobson et al., 1993). A stochastic element in the timing of PCD in Rat-1 fibroblasts has been noted (Evan et al., 1992). It is also possible, but unlikely that a minority of cells in most organs cannot be

induced to undergo PCD by STS and CHX. Even cells from adult tissues undergo PCD in response to STS and CHX, although they required a longer incubation with STS before high levels of pyknosis was apparent (data not shown). These results support the idea that most cell types constitutively express the proteins required to undergo PCD. Consistent with this interpretation, the nematode cell death repressor protein - Ced-9 - regulates the cell death proteins - Ced-3 and Ced-4 - post-translationally: ced-9 expression does not alter ced-3 and ced-4 mRNA expression (Yuan, unpublished data).

In some cases, however, protein synthesis inhibitors can prevent apoptosis (Martin et al., 1988; McConkey et al., 1988; Oppenheim et al., 1990), although in other cases protein synthesis inhibitors induce apoptosis (Martin, 1993). Indeed, a single cell type in the presence of these inhibitors may be saved from the killing effects of one stimulus, and yet be induced to die by PCD by a second stimulus, suggesting that it is the activation of PCD, and not the cell death programme itself, that in some cases requires protein synthesis (Cotter et al., 1992).

It has been proposed recently that death is the default state of a cell, and therefore that survival signals are constantly required by almost all cells (Raff, 1992). Blastomeres, the first cell type of the vertebrate embryo, are an exception: they survive and divide in the absence of proteins in the culture medium and in single cell cultures (Biggers et al., 1971). If STS acts to block survival signals then I would predict that blastomeres do not undergo STS-induced PCD. I show that blastomeres, unlike all other cells tested, do not die by apoptosis in the presence of high concentrations of staurosporine, with or without CHX.

Recent studies (Vaux et al., 1992; Yuan and Horvitz, 1992; Miura et al., 1993) indicate that normal cell death in vertebrates, like in the nematode, has a genetic basis and may therefore be viewed as part of the behavioural repertoire of cells of multicellular animals, along with division and differentiation. If blastomeres do not undergo PCD, at least in response to STS and CHX, at what stage in development do cells first express this potential? If cell survival is socially controlled (Raff, 1992), we would expect to see survival regulated by cell-cell interactions from the time that multiple cell types first appear in the embryo. Indeed, dying cells are first seen in the blastocyst at around 86 hours post-coitum (p.c.), the time at which blastomeres become either inner cell mass (ICM) or trophectoderm (Potts and Wilson, 1967; Wilson and Smith, 1970; El-Shershaby and Hinchliffe, 1974; Handyside and Hunter, 1986). One cell division after the ICM forms (95 hours p.c.), the ICM consists of an inner core of primitive ectoderm cells, which are dependent on the outer rind of endoderm cells for survival *in vitro* (Rossant and Offer, 1977; Gardner 1985), suggesting that the cell death seen *in vivo* in the blastocyst is socially controlled. Consistent with the idea that ICM cell survival *in vitro* is a signalled event, I have found that blocking protein kinase activity rapidly induces ICM cells to undergo PCD. Furthermore, ICM cell death occurs in the absence of protein synthesis, again suggesting that the proteins required for active cell death are constitutively expressed.

Not only can the earliest derivatives of blastomeres die by PCD, but o can the cells from which blastomeres are themselves derived. Oocytes die by apoptosis in response to STS *in vitro* (R. Garsden, personal communication), and normal cell death is seen during oogenesis

in vivo (H. Charlton, personal communication). Sperm also die by apoptosis *in vivo* (Allan et al., 1987). Together, these results suggest that at some time during development the fertilised egg or its most recent precursors undergo a transition to independence of survival factors, and that this stage lasts until blastomeres differentiate to form either TE or ICM.

It is possible that the inner cell masses used in these experiments were already a mixture of primitive ectoderm and endoderm because survival was assayed after 18 hours in culture and ICM becomes either primitive ectoderm or endoderm after one cell division *in vivo*. The cells of the trophectoderm stop dividing and become giant cells in the absence of ICM, and, since early ICM can convert to trophectoderm experimentally (Gardner 1972), it may be that early ICM cells do not require other cell types for their survival. Consistent with this, there is a sharp peak of cell death in the ICM at the time (95 hours) when ICM becomes either primitive ectoderm or endoderm (El-Shershaby and Hinchliffe, 1974). Conversely, isolated apoptotic bodies have been reported in ICMs from 86- and 90-hour mouse blastocysts (Wilson and Smith, 1970; Potts and Wilson, 1967, respectively). Therefore it remains unclear whether or not the survival of early ICM is socially controlled.

Between 6-8 dead cells are found in the ICM at a time when it consists of around 15 live cells (El-Shershaby and Hinchliffe, 1974). What is the function of this proportionately extensive PCD? During the last 15 hours before implantation the ICM does not increase in cell number in spite of continuing cell division, leading several researchers to suggest that the blastocyst is marking time before implantation and continued development (Handyside and Hunter, 1986). Another

possibility is that socially controlled cell death may play an important part in restricting the location of cell types within the body (Raff 1992). Primitive ectoderm cells are the pluripotent precursors of all embryonic tissues, therefore their dependence on endoderm for survival signals may limit their viability and prevent multiple embryos from colonising the blastocyst. The restriction of cell localisation may underlie other cases of normal cell death such as chondrocytes and lens cells, and suggests that mutations in genes that make cells autonomous for survival could promote metastasis in cancer. The function of this proportionately massive cell death is unlikely to be fully understood until ICM cell death can be experimentally manipulated.

Blastomeres are distinctive cells: they are relatively large and do not adhere to the substratum *in vitro*; they spend the majority of their short cell cycle in S-phase; they lack any feedback mechanisms preventing cell cleavage before the completion of S-phase; unlike all other cell types tested they can not express heat shock proteins in response to physiological stress. It would be interesting to know whether blastomeres' incapacity to kill themselves is related to any of these characteristics. In particular, the unique cell cycle machinery of blastomeres may explain the unique survival properties of these cells because accumulating evidence suggests a close link between the control of cell division and cell death (Ucker 1991; Rubin et al., 1993; Shi et al., 1994; Meikrantz et al., in press). It makes some teleological sense that blastomeres do not need signals from other cell types: it enables them to survive in the changing environment of the fallopian tube and uterus from the 1-cell to around the 16-cell stage. However, unlike other cells - even anatomically isolated cell types such as lens cells and chondrocytes

(Ishizaki et al., 1993; Ishizaki et al., in press) - blastomeres do not require other cells - not even blastomeres - to survive. Until recently, this was assumed to be the case for most cell types; now, it appears that blastomeres may be the exception and not the rule.

Methods

Explant culture

Sprague-Dawley rats and mice were bred in the UCL animal facility. The animals were killed and explants of several hundred microns cubed were prepared in cold L-15 air medium from dissected organs using a haemostat-held blade; and transferred to sterile filters (Nucleopore; 0.8μm pore-size) floating in the well of 24-well plates (Falcon), each well containing 700μl of Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with 10% FCS and gentamicin. After 48 hours of culture, STS and CHX were added to the appropriate wells, and the explants were cultured for a further 18 hours. The tissues were fixed in 4% paraformaldehyde in 0.1 M $NaPO_4$, pH 7.4 at 4°C overnight and then cryoprotected in 30% sucrose, PBS at 4°C for at least 2 hours. The tissue was then frozen, 6 μm cryosections were cut and collected on APES-coated glass slides, and the sections were stained with propidium iodide and the pyknotic index determined as previously described. Abercrombie correction factors (Abercrombie, 1946) were determined, using measurements of particle diameters made for each tissue (see methods, chapter 2).

Recovery of embryos

Reproductive tracts were isolated from female PO (Pathology, Oxford) albino random bred mice of timed pregnancies. The mice were either exposed to a standard lighting regime of light between 07.00 and 19.00 every day, or daylight reversed depending on the time of day that each stage of embryo was required. The morulae and blastocysts were flushed

with medium from the oviduct or uterus and the zona pellucida dissolved with acidified Tyrode's saline (pH 2.5 with HCL, Nichols and Gardner, 1984). Morulae were dissociated by incubation in calcium-free M-2 medium for 10 minutes at 37 °C, followed by gentle pipetting in a siliconized micropipette.

Immunosurgery

Inner cell masses were recovered from blastocysts by immunosurgery (Solter and Knowles, 1975; Nichols and Gardner, 1984). In brief, after removal of the zona pellucida, the blastocysts recovered in α-medium containing 10% FCS for 1 hour, they were rinsed in PBS and phosphate buffer (PB) + FCS and then incubated in heat-inactivated serum (rabbit anti-mouse, 1:9 in PB) for 45 minutes at 37°C. After three, 1 minute rinses in PB + FCS, filtered rat complement (1:9 in PB) was added and the blastocysts were incubated for 8 minutes at 37°C. The blastocysts were then transferred to α-medium containing 10% FCS for at least 45 minutes recovery at 37°C, and then gently pipetted in a micorpipette to remove the lysed trophectoderm from the intact inner cell masses.

Embryo cell culture

The cells were placed in 300 μl drops of MTF medium in the case of blastomeres and α-medium in the case of inner cell masses, either alone or containing STS, 1μM (Sigma) or CHX, 1 μg/ml (Sigma). The cultures were incubated at 37°C in bacteriological grade plastic dishes (Sterilin, UK.) in a humidified atmosphere of 5% CO_2 in air. When cells were cultured in the presence of both STS and CHX, the cells were pre-incubated in CHX for 30 minutes before the addition of STS.

Cell survival assays

Vital nuclear stain Nuclear morphology was determined using the bisbenzimide dye Hoescht 33342, which is membrane permeable and therefore stains both live and dead nuclei. Hoescht 33342 (2mg/ml in H_2O, Sigma) was serially diluted and added to the culture medium at a final concentration of 4 μg/ml, gently mixed and then the cultures were incubated for 15 minutes at 37°C. The cells were viewed in an inverted fluorescence microscope.

Plasma membrane permeability. Plasma membrane integrity was determined using the DNA-intercalating dye ethidium homodimer, which is membrane-impermeant. Ethidium homodimer (Molecular Probes, Inc.) was added to the culture medium at a final concentration of 4 μM, gently mixed and then the cultures were incubated for 15 minutes at 37°C. The cells were viewed in an inverted fluorescence microscope.

Mitochondrial Activity. Mitochondrial dehydrogenase activity was measured using the MTT assay (Mosmann, 1983). 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT; Sigma), a substrate for mitochondrial dehydrogenase enzymes, was dissolved in DMEM at 5 mg/ml and sterilised through a filter (0.22 μm; Millipore Corp., Bedford, MA). 1 μl of this stock solution was added to every 10μl of culture medium and the cells incubated for 1 hour at 37°C. Viable cells with active mitochondria cleave the tetrazolium ring to form a blue formazin reaction product.

Chapter 5

General Discussion

In this chapter I discuss the implications and future directions of the studies presented in this thesis.

PCD during the development of the metanephric kidney

In chapters 2 and 3 , I described PCD during kidney development in the rat. PCD had not been previously reported in the kidney, and yet I showed that the proportion of dead cells in regions of the developing kidney was similar to, or greater than, that in the developing thymus or CNS where large numbers of cells are known to die. Developmental cell death in the kidney occurs in two regions, where it follows distinct time courses. The dead cells, which have the characteristic morphological features of apoptosis, are rapidly cleared, by neighbouring parenchymal cells. I showed that at times of peak cell death as many as 3% of cells are dead in tissue sections and I estimated that at least 25% or more of cells in the nephrogenic zone (NZ) die by PCD during development.

It is surprising that PCD in the developing kidney was missed for so long, given the intense study of kidney development. One reason is that the research community has been slow to accept PCD as a fundamental behaviour of cells in multicellular animals. With increasing evidence for a genetically encoded cell death programme in vertebrates, this is no longer the case (Tsujimoto et al., 1985; Yuan and Horvitz 1992; Yuan et al., 1993; Boise et al., 1993; Oltavi et al., 1993). A second reason is that most of the recent advances in developmental renal biology have been made using the organ culture technique (Grobstein 1953; Saxén and Lehtonen, 1987). Although early nephron formation has been described using this transfilter technique, PCD was probably discounted as an artefact of the culture conditions. Given that PCD in metanephric

development has been missed for so long, it will be interesting to see whether PCD is important in the development of other organs that develop from interactions between a branching epithelial duct and a group of mesenchymal cells, such as the submandibular gland, lung, and mammary gland.

What are the functions of PCD in the kidney?

PCD contributes to the re-modelling of many epithelial sheets - such as the palate (Shah, 1979) and neural tube (Glucksmann 1951). In chapter 3, I suggest that the epithelial cell deaths in the nephrogenic zone may contribute to the morphogenesis of the nephron: glomerular crevice formation, for example, may involve the PCD of cells in the nascent Bowman's space, as well as changes in adhesion (Saxèn 1987), and the clearance of other renal lumens and the fusion of the epithelial cell layers of the developing nephron and the collecting duct may also involve PCD. In each case, cells in the interior of the epithelial structure, with no contact with either the epithelial basement membrane or interstitial fluid, may be cut off from trophic support and therefore die by PCD.

Little is known about the morphogenesis of the distal collecting duct system, and it is possible that PCD in the developing papilla contributes to this process. Variations in the pattern and extent of PCD between species determine the degree of webbing of bird feet (Hurle and Colvée 1982; Hurle et al., 1985). Similarly, variations in the pattern and extent of PCD between species may explain the variation in morphologies of the papilla: for example, rats are uni-papillate while humans are multi-papillate.

The mesenchymal PCD occurs in the developing kidney at a time of inductive interactions between two cell lineages - the metanephric mesenchyme (MM) and the ureteric bud (UB) - during which groups of MM cells are induced to form nephrogenic structures at the tips of the branching UB. PCD may limit the number of induced MM cells that contribute to each nephron. Alternatively, PCD may limit the number or proportion of uninduced MM cells that will form the stroma. In either case, PCD would help to match the number of cells in two interacting populations, similar to the PCD in the developing vertebrate nervous system (Cowan et al., 1984). It is likely that the functions of PCD during renal development will ultimately be defined by experimental manipulations that inhibit the PCD.

The control of kidney programmed cell death

The regulation of neuronal survival in the developing vertebrate nervous system has been shown, in some cases, to be due to the limiting availability of survival factors (Korsching and Thoenen, 1983; Purves 1988). In general, increasing the level of survival factor, *in vivo* or *in vitro,* increases the number of cells that survive (Hamburger and Yip, 1984; Barres et al., 1992), while decreasing the level of survival factors, *in vivo* or *in vitro,* decreases the number of surviving cells (Levi-Montalcini, 1972; Gorin and Johnson, 1979; Goedert at al., 1980). It has been suggested that almost all cells require survival signals from other cells in order to avoid PCD (Raff 1992); in which case, regulating cell survival by regulating the availability of survival factors may be common. Consistent with this idea, I found that increasing the levels of EGF or IGF-1 decreases the pyknotic index in the developing kidney. To

establish whether control of renal PCD is due to limiting supplies of survival factors or not, two questions need to be addressed:

1) Do EGF and IGF-1 lead to an increase in cell number?

2) Does a decrease in normal levels of these factors lead to an increase in PCD?

The first experiment would help to determine whether EGF and IGF-1 treatment decrease the pyknotic index by decreasing the clearance time of dying cells or by saving cells that would otherwise die, while the second experiment would establish whether these factors act during normal development to promote cell survival in the developing kidney.

Requirement for multiple survival factors

There is increasing evidence that cells require more than one survival factor for long-term survival *in vitro*. Fibroblast growth factor (FGF) or ciliary neurotrophic factor (CNTF) alone promote 50% survival of chick embryonic motor neurones over several days in culture, but together they promote 100% motor neurone survival (Arakawa et al., 1990). Neurotrophin-3 (NT-3), CNTF or IGF-1 alone promote 50-75% survival of oligodendrocytes over 3 days in culture, but together they promote 100% oligodendrocyte survival for at least 15 days (Barres et al., 1993). Insulin, progesterone and serum, in addition to NGF are required for the survival of some DRG neurones in vitro (Bottenstein et al., 1980). Retinal ganglion cells *in vitro* require a combination of 4 survival factors for maximal survival (B. Barres, unpublished data), and haemopoietic stem cells require multiple factors for maximal survival in culture (M. Dexter, personal communication).

In chapter 3 , I show that EGF and IGF-1 treatment apparently increase the survival of cells in the nephrogenic zone of developing kidney *in vivo*. While IGF-1 promotes the survival of both mesenchymal / stromal and epithelial cells equally, EGF predominantly promotes the survival of mesenchymal / stromal cells. It would be interesting to know whether EGF and IGF-1 are affecting the same cell types in the mesenchymal / stromal compartment and if so, whether they have an additive affect on survival similar to that seen in other systems. The most powerful demonstration of this would be *in vitro* studies of survival requirements of purified cell types from the kidney. At the doses given, neither factor alone saves 100% of the dying cells in either population, and so a dose response curve would have to be obtained to determine whether incomplete rescue is because the doses given were not saturating. Also, it is likely that there are many other renal survival factors which have yet to be identified; one candidate is NT-3, since both NT-3 and its receptor (Trk C) are expressed in the developing kidney (Maisonpierre et al., 1990; Durbeej et al., 1993).

Although the dependence of cells on multiple trophic factors for their survival has not been demonstrated *in vivo*, there could be several advantages for combinatorial control of cell survival. If each cell type depends on a specific combination of survival factors for its long term survival, relatively few factors could be used to regulate specifically the survival of many cell types. Furthermore, some survival factors could be produced locally to regulate cell number within a tissue or organ, while others could be supplied systemically to match organ size to whole body size. It has also been suggested that combinatorial control may help to

avoid ambiguity in the signalling process (M. Pagal, personal communication).

Insulin-like growth factors: a special case?

The insulin-like growth factors (IGFs) are important regulators of growth. Most cell types seem to respond to IGFs *in vitro* and the IGFs, their binding proteins, and the IGF- I receptor are expressed in most tissues throughout development (Sara and Hall 1990); although IGF-II acts mainly pre-natally while IGF-I acts both pre- and post-natally (Baker et al., 1993). Growth deficiency is a feature of all animals with loss of function mutations in the genes encoding IGFs or the IGF-I receptor (Baker et al., 1993; Liu et al., 1993), and growth enhancement occurs in transgenic mice which over-express IGF-1 (Mathews et al., 1988). Furthermore, IGF-1 knockout mice, though dwarfed, are roughly proportionate (Baker et al., 1993). Taken together, these results suggest that IGFs are general growth factors affecting a large number of cell types.

Although it has been generally believed that IGFs control growth by controlling cell division, there is increasing evidence that they may do so by promoting cell survival. It will be important to reassess much of the functional data on IGFs given the increasing awareness of PCD as an important feature of cell biology in multicellular animals: without careful analysis, IGFs may be appearing to promote cell division or differentiation when, in fact, they are promoting cell survival. For example, IGF-1 was reported to be a mitogen for rat proximal tubule epithelial cells (RPTE) cultured in serum-free medium (Zhang et al., 1991). This effect, however, is only seen at high cell density. At low cell

density, IGF-1 alone led to only a slight increase in ^{3}H-thymidine incorporation over 24 hours. IGF-1 could be leading to a significant increase in ^{3}H-thymidine incorporation in high density cultures, either by acting as a co-mitogen for an autocrine factor produced by the RPTE cells, or by promoting the survival of newly produced RPTE cells. Such studies, therefore, remain inconclusive. Indeed, in studies where cell survival and cell division are determined individually, IGFs are often found to be survival factors and not mitogens. This is the case for Balb/c 3T3 murine fibroblasts (Tamm and Kikuchi, 1990), neuroepithelial cells (Drago et al., 1991), and oligodendrocytes and their precursor cells (Barres et al., 1992). Consistent with this re-assessment of the function of IGF-1, I find that IGF-1 delivered over a short period *in vivo*, decreases the pyknotic index in the nephrogenic zone without affecting the mitotic index. Furthermore, IGF-1 affects the pyknotic index in both epithelial and mesenchymal cells equally, suggesting that, unlike EGF, IGF-1 is acting as a general survival factor in the kidney.

There is also need for caution in interpreting experiments on the role of IGFs in cell differentiation, since an increase in the proportion of one cell type may be due to enhanced survival rather than enhanced differentiation of this cell type. For example, IGF-1 was reported to promote the differentiation of oligodendrocytes from O-2A progenitor cells (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1990). A subsequent study on purified cultures of O2-A cells *in vitro*, and the development of the optic nerve *in vivo*, showed that IGF-1 does not promote differentiation of oligodendrocytes, but rather it is a survival factor for newly formed oligodendrocytes (Barres et al., 1993).

In keeping with the traditional view of IGFs primarily as mitogens, it has recently been hypothesised that IGFs may control growth of organisms by controlling the length of the cell cycle. Thus, IGF-I (-/-), IGF-I receptor (-/-), and IGF II (p-) transgenic mice are dwarfed because there are "fewer than normal proliferative events occurring in the various tissues" during the normal period of development (Baker et al., 1993). Consistent with this interpretation, embryonic fibroblasts from IGF-I receptor (-/-) transgenic mice in culture have a cell cycle in which all phases are doubled in length (A. Efstratiadis, unpublished data). An alternative hypothesis is that IGFs control growth of organisms primarily by regulating the extent of PCD that occurs during organogenesis. In several species of hydra, for example, changes in the cell cycle are insufficient to explain reduction in growth when food is limiting, while changes in the pyknotic index can probably account for the reduction in growth observed (Bosch and David, 1984). Furthermore, insulin or an insulin-like molecule has been implicated in this regulation of body size (C. N. David, unpublished data). As it seems that IGF-I can act as a mitogen, an anabolic agent, and a survival factor, it will be important to determine the relative importance of each of these functions for each vertebrate cell type.

De-regulation of PCD

As discussed in chapter 2, de-regulation of genes that control PCD may contribute to the pathogenesis of some diseases, including cancer (Tsujimoto et al., 1985; Vaux et al., 1988; Strasser et al., 1990) and autoimmune diseases (Strasser et al., 1991b). Similarly, disruptions in the normal control of PCD in the kidney could lead to developmental

kidney disorders (Coles et al., 1993). Recently, transgenic mice homozygous for loss-of-function mutations in the cell-death-repressor gene *bcl-2* were found to have poly-cystic kidneys (Veis et al., 1993); moreover, detectable levels of DNA laddering - a common feature of apoptosis - were found in mice with autosomal dominant polycystic kidney disease (Woo, 1993). These results suggest a role for apoptosis in the pathogenesis of some forms of polycystic kidney disease. Consistent with this, preliminary studies (data not shown) suggest that PCD is 3-fold higher in the medulla of kidneys of the CPK mouse, which suffer from autosomal recessive polycystic kidney disease (Russell and McFarland, 1977; Preminger et al., 1982). This increased PCD appears to be mainly in the interstitium (data not shown), as is the PCD seen in bcl-2 knockout mice (Veis et al., 1993). In autosomal dominant polycystic kidneys, however, increased PCD is seen mainly in the hyperplastic epithelial cell population lining the cysts (S. Flemming, personal communication). Further work is necessary to characterise the PCD in these models of polycystic kidney disease. In particular, it will be interesting to see if delivery of survival factors such as EGF and IGF-1 will alter the extent of PCD and the severity of the lesions in these kidneys.

How widespread is the programme for cell death?

It has been suggested that PCD may be the default state of all cells except blastomeres (Raff, 1992). Indeed, an increasing number of vertebrate cells are being shown to depend on survival factors for their survival *in vitro* and *in vivo* (Kerr and Searle, 1973; Wyllie et al., 1973; Duke and Cohen, 1986; Kyprianou and Isaacs, 1988; Godin et al., 1991; Barres et al., 1993; Ishizaki et al., 1993; Ishizaki et al., in press). If these

survival signals prevent cells from undergoing PCD, then one would predict that blocking the signalling pathways activated by survival factors would lead to PCD. Although these pathways are not completely understood, a common feature of most survival and growth factors is that they signal to cells by binding to receptor tyrosine kinases which then activate multiple protein kinase cascades (Alberts et al., 1994). Previous experiments have shown that blocking of protein kinase activity with staurosporine induces several cell types to undergo PCD (Bertrand et al., 1993; Ishizaki et al., 1993; Ishizaki et al., in press Jacobson et al., 1993 and 1994; Meikrantz et al., in press). The results in chapter 4 extend these observations to many cell types in developing rodents, consistent with the idea that constant survival signalling is required for cells to avoid PCD. I did not, however, find 100% cell death in response to STS in any tissues. This may be because of technical limitations of the experiments, such as insufficient concentration or penetration of the drug; or it may mean that some cells are resistant to STS-induced cell death. I also found an increased time to die in adult as opposed to postnatal tissues (data not shown), suggesting that cells vary in the rate of response to apoptotic stimuli.

Enucleated cells (cytoplasts) can undergo PCD in response to STS and survival factor deprivation, suggesting that the nucleus is not required for PCD - at least in the two cell lines tested (Jacobson et al., 1994). Consistent with this, I show in chapter 4 that the protein synthesis inhibitor cycloheximide increases the amount of STS-induced PCD in explants of many tissues, suggesting that all of the protein components required for a cell to die by PCD are constitutively expressed in most rodent cell types. Whether all cells have the cell death

programme remains to be determined and will be answered once unambiguous molecular markers for the programme are identified. Varying susceptibilities of cells to the induction of PCD could reflect differences in the ways the cell death machinery is regulated in different cell types.

Blastomeres and PCD

Ishizaki and co-workers (1993 and in press) looked at two cell types - lens epithelial cells and cartilage chondrocytes - in order to determine whether some cell types do not require survival factors. They predicted that these, if any, cell types may not require survival signals, as they are anatomically isolated *in vivo*: lens and cartilage contain no vasculature, no lymph vessels, no innervation, and only one cell type. While it was found that neither cell type requires survival factors from other cell types to survive *in vitro*, culturing the cells at various densities showed that the cells do require signals from other cells of the same type for survival (Ishizaki et al., 1993; Ishizaki et al., in press). Furthermore, both lens and cartilage cells undergo STS-induced PCD. These results showed that both cell types undergo PCD in the absence of survival factors.

Since blastomeres are known not to require survival factors *in vitro* (Biggers et al., 1971), we predicted that blocking intercellular signalling with STS would not induce PCD. In Chapter 4, I show that blastomeres differ from even their earliest derivatives - inner cell mass cells and trophectodermal cells - because they do not undergo PCD when treated with STS and CHX. Blastomeres themselves derive from oocytes and sperm, both of which are known to undergo PCD *in vivo* (H. Charlton, personal communication; Allan et al., 1987), therefore there is

apparently a developmental window - from the fertilised mammalian egg until around the 16-cell stage - during which PCD has not been observed to occur - either normally *in vivo,* or experimentally in response to withdrawal of survival signals or treatment with high concentrations of STS and CHX. This resistance to PCD may allow early embryos to survive in the rapidly changing environment of the fallopian tubes and uterus during early pregnancy.

The cell cycle and PCD

The idea that cells constitutively express a cell death programme which requires constant signalling to be repressed suggests that competition for limiting supplies of survival factors may be more common than previously thought (Raff, 1992). Competition for survival factors has been shown to regulate the extent of PCD in post-mitotic populations of neurones (for reviews see Cowan et al., 1988; Oppenheim, 1981; Purves, 1988) and oligodendrocytes (Barres et al., 1993). The same mechanism may also regulate PCD in proliferating cells: in the nephrogenic zone of the metanephric kidney (Chapter 3; Coles et al., 1993) and haemopoietic progenitor cells in bone marrow (M. Dexter, personal communication). Under these circumstances, however, limiting supplies of survival factors could rapidly select for cells that are independent of survival signals and therefore favour cells carrying oncogenic mutations. One safeguard against this would be for cells to require multiple factors for long-term survival. A second safeguard could be to link the control of PCD to the control of other vital cellular functions, such as the cell cycle.

Similarities between mitosis and cell death, including the loss of cell adherence, chromatin de-condensation, breakdown of the nuclear envelope, and plasma membrane blebbing, have lead to the speculation that PCD may be an abortive mitosis (Ucker et al., 1991; Rubin et al., 1993; Shi et al., 1994; Meikrantz et al., in press). In support of this, rat prostate epithelial cells dying by apoptosis incorporate Brd-U into high molecular weight DNA, and express increased levels of proliferating cell nuclear antigen (Colombel et al., 1992). There is also accumulating evidence that PCD and mitosis may share molecular components. The activity of the cyclin-dependent Cdc2 kinase, which is responsible for entry into mitosis, is increased in HeLa cells by several apoptotic-inducing stimuli, including STS (Tamm and Schlegel, 1992). Ectopic expression of cdc2 induces apoptosis in CTLs (Shi et al., 1994); while over-expression of the apoptosis-suppressor protein Bcl-2 blocks this PCD and leads to attenuation of Cdc2 and Cdk2 in the cytoplasm, suggesting a mechanism by which Bcl-2 could suppress PCD (Shi et al., 1994; Meikrantz et al., in press). Furthermore, expression of the proto-oncogene *c-myc* is required for cell division in rat-1 fibroblasts but it also increases the susceptibility of these cells to die by PCD since ectopic expression of c-myc in the absence of growth factors induces PCD (Evan et al., 1992).

Whether or not apoptosis is a molecular catastrophe, these findings are consistent with a close association between the control of cell death and cell division. This association could ensure that cells that are incapable of PCD are also incapable of cell division, thereby preventing such aberrant cells from threatening the survival of multicellular organisms with a long life expectancy. It could also explain why attempts

to isolate somatic cell mutants of the cell death programme in vertebrates have not proved very fruitful.

Adult tissues and PCD

In adult tissues there is a steady turnover of many cell types, although a steady state tissue mass is maintained (Goss 1966; Wyllie et al., 1980). It is usually assumed that control of cell proliferation is responsible for this balance (Goss 1966 and 1978). Control of cell death, however, could also play an important role (Wyllie et al., 1980; Raff 1992). The importance of PCD in regulating cell number in the adult is suggested by studies on the regression of experimentally induced hyperplasia in adrenal cortex, liver and kidney, which is rapid and is caused by high levels of apoptosis in each case (Wyllie et al., 1980; Bursch et al., 1985 and 1986; Ledda-Columbano et al., 1989). Studies in adult hydra also suggest that the regulation of PCD is more important than the regulation of cell division in the maintenance of steady state tissue mass (Bosch and David, 1984). Thus, although the control of cell number in adult tissues is usually seen as control of cell proliferation, control of PCD is also important and the relative significance of each has yet to be established.

PCD during regulation of tissue cell number could be regulated by "cell death factors" as postulated in endometrial explants (Lynch et al., 1986) and liver (Steiner 1978), or PCD could be regulated by limiting supplies of survival factors (Raff 1992; Barres et al., 1993). In the second model a set level for the number of cells in adult tissues would be determined by the limited availability of survival factors: if the cell number increases above this level, then the number of cells dying by apoptosis increases; if the cell number decreases below this level, then

the number of dying cells decreases. In fact both mechanisms of controlling cell survival and PCD are known to occur during the involution of endocrine responsive tissues: increases in gluccocorticoids induce apoptosis and atrophy in lymphoid tissues (Claman, 1972) while decreases in ACTH and oestrogen induce apoptosis and atrophy in the adrenal cortex (Wyllie et al., 1973) and mouse endometrium (Martin et al., 1973), respectively.

Why cell death by default?

The inspiration behind this thesis has been the hypothesis that cell death is the default state for all mammalian cells (except blastomeres; Raff, 1992), and my results support this counter-intuitive idea. I have shown that the cell death programme is ubiquitously expressed in mammalian cells. A constitutively expressed cell death programme that is repressed by survival factors produced by other cells would ensure that cells only survive when and where they are needed: misplaced cells would automatically kill themselves because they would fail to receive the specific signals they need to survive. Death-by-default and competition for limiting amounts of survival signals could play an important part in controlling cell number, both in development and in mature tissues that undergo cell turnover; in the developing nervous system, for example, this mechanism allows for the numerical and spatial matching of interacting nerve cells and it seems possible that it plays a similar role in non-neural tissues. It seems probable that programmed cell death was essential for the evolution of complex multicellular organisms.

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