Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor

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SUMMARY

Although normal cell death is known to occur in many developing vertebrate organs, it has not been thought to play an important part in the development of the mammalian kidney. We show here that normal cell death is found in both the nephrogenic region and medullary papilla of the developing rat kidney and, in each of these areas, it follows a distinct developmental time course. As many as 3% of the cells in these areas have a typical apoptotic morphology and the dead cells seem to be cleared rapidly (within 1-2 hours) by phagocytosis by neighbouring parenchymal cells. 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. We also show that in vivo treatment with epidermal growth factor inhibits cell death in the developing kidney, consistent with the possibility that the cells normally die because they lack sufficient survival factors. Our findings suggest that the extent of normal cell death in developing animals is still greatly underestimated and they raise the possibility that many of these cell deaths may reflect limiting amounts of survival factors.

Key words: apoptosis, programmed cell death, epidermal growth factor, kidney development

INTRODUCTION

Normal, or ‘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). The cells usually die by a process called apoptosis, which is morphologically different from most cell deaths that result from acute injury, a process called cell necrosis (Kerr et al., 1972; Wyllie et al., 1980). In cell necrosis, the cell usually swells and lyses, spilling its cytosolic components into the extracellular space, which elicits an inflammatory response. In apoptosis, the cell and its nucleus shrink and often fragment, and the cell or its fragments (called apoptotic parenchymal cells). 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. We also show that in vivo treatment with epidermal growth factor inhibits cell death in the developing kidney, consistent with the possibility that the cells normally die because they lack sufficient survival factors. Our findings suggest that the extent of normal cell death in developing animals is still greatly underestimated and they raise the possibility that many of these cell deaths may reflect limiting amounts of survival factors.

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Although normal cell death is known to occur in many developing vertebrate organs, it has not been thought to play an important part in the development of the mammalian kidney. We show here that normal cell death is found in both the nephrogenic region and medullary papilla of the developing rat kidney and, in each of these areas, it follows a distinct developmental time course. As many as 3% of the cells in these areas have a typical apoptotic morphology and the dead cells seem to be cleared rapidly (within 1-2 hours) by phagocytosis by neighbouring parenchymal cells. 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. We also show that in vivo treatment with epidermal growth factor inhibits cell death in the developing kidney, consistent with the possibility that the cells normally die because they lack sufficient survival factors. Our findings suggest that the extent of normal cell death in developing animals is still greatly underestimated and they raise the possibility that many of these cell deaths may reflect limiting amounts of survival factors.

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normal developing rat kidney. We chose to study the kidney because normal cell death has not been thought to be a feature of mammalian renal development, despite intense study of kidney development. The development of the mammalian kidney requires interactions between 4 cell lineages: the epithelial cells of the ureteric bud, the mesenchymal cells of the metanephric blastema, endothelial cells and nerve cells (Bard, 1992; Ekbлом et al., 1987; Saxén, 1987; Saxén and Sariola, 1987). Early in development the ureteric bud invades the metanephric mesenchyme, which induces the ureteric bud to branch repeatedly. The branching ureteric bud, in turn, induces the mesenchymal cells to differentiate into epithelial cells, which assemble into nephrons.

Here we show that (1) up to 3% of the cells in some regions of the developing rat kidney are apoptotic; (2) the dead cells seem to be cleared within 1-2 hours and (3) the number of dead cells is greatly reduced by treatment with epidermal growth factor (EGF) in vivo. The proportion and clearance rate of dead cells in the kidney are similar to those in neural tissues where 50% or more of the cells die during normal development, suggesting that large-scale cell death is a fundamental feature of normal kidney development. These results support our suspicion that the extent of normal cell death in developing animals is still greatly underestimated and that many of these cell deaths may be due to limiting amounts of survival factors.

MATERIALS AND 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 (PB), pH 7.4. The kidneys and thymus glands were removed and fixed overnight at 4°C and cryoprotected with 30% sucrose in PBS until equilibrated. Embryonic kidneys were dissected from newborn rat pups were given four intraperitoneal injections of BrdU (0.1 mg/g body weight; Boehringer Mannheim) at 8 hour intervals. 2 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 postfixed with 70% ethanol for 10 minutes at −20°C. The DNA was denatured by incubation in 2 M HCl for 10 minutes and then neutralized 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-BrdU antibody (Gonchoroff et al., 1985; Greipp et al., 1985; concentrated supernatant diluted, 1:10), followed by biotinylated anti-mouse IgG (Amersham; diluted 1:50) and then fluorescein-coupled streptavidin (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 BrdU-treated rats, suggesting that the BrdU treatment was not toxic to kidney cells at the concentrations used.

Treatment with EGF in vivo

To establish whether EGF can block kidney cell death in vivo, eight 1-day-old (P1) sibling rat pups were given four intraperitoneal injections of EGF (0.3 µg/g body weight; Sigma) or the vehicle, PBS containing 0.1% BSA, at 3 hour intervals. 2 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 above. 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 percentage of total nuclei.

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, counterstained with uranyl acetate and then lead citrate and examined in a JEOL 100-CX II electron microscope at 80 kV.
RESULTS

Apoptotic cells in the developing kidney

To identify apoptotic cells in the developing kidney, we stained frozen sections of embryonic and postnatal perfusion-fixed rat kidneys with propidium iodide to label nuclei and examined the sections in a fluorescence microscope. Pyknotic nuclei, which often appeared as a cluster of two or more brightly stained fragments, were readily recognized in such sections (Fig. 1A,B). Whereas normal nuclei were generally oval in shape and had a mean diameter of 8±0.2 µm (n=83), pyknotic nuclei, or their fragments, were roughly spherical, had a mean diameter of 3±0.2 µm (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. 1C). In embryonic non-perfused kidneys, nucleated red blood cells were easily distinguishable from apoptotic cells: the red blood cell nuclei were more spherical, larger, more textured and less brightly stained than apoptotic nuclei, and they were never fragmented (Fig. 1D).

Apoptosis is characterised by profound ultrastructural changes, including condensation of the chromatin and fragmentation of the cell to form small, membrane-bounded apoptotic bodies (Wyllie et al., 1980; Clarke, 1990). To confirm that the cells that die in normal kidney development do so by apoptosis, we examined sections from newborn rat kidneys by electron microscopy. All the dead cells seen had the characteristic features of apoptosis. Of the 22 apoptotic cells examined, 20 had the form of typical apoptotic bodies contained within normal cells (Fig. 2A,B), while 2 were in the lumen of developing nephrons (Fig. 2C). The cells that contained apoptotic bodies were morphologically indistinguishable from their neighbours. Thus most of the apoptotic cells in the developing kidney apparently fragment into apoptotic bodies, which are very quickly phagocytosed by neighbouring parenchymal cells (rather than by macrophages).

Location and timing of cell death in the developing kidney

We 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. 3A). 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. 3B). 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. 3C). In the most distal regions of the papilla, cell death was especially high in the embryo (Fig. 4), but we did not quantitate the death in this region.

At all ages examined, we found mitotic figures mainly in the nephrogenic zone, where their proportion paralleled that of pyknotic nuclei in this zone (Fig. 3B). By contrast, mitotic figures were rare in the papilla at all ages examined (Fig. 3C). The close relationship between mitosis and apoptosis in the nephrogenic zone (Fig. 3B) 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, we injected the thymidine analogue bromodeoxyuridine (BrdU, four intraperitoneal injections given 8 hours apart) into P0 animals. 2 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-BrdU 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 BrdU labelled (not shown), indicating that at least one quarter of the dead cells had synthesized DNA sometime in the 26 hour period before their death.

The nephrogenic zone contains epithelial cells of the ureteric bud, epithelial cells derived from the metanephric mesenchyme, uninduced mesenchymal cells and endothelial cells. To get an idea of which cell types are mainly dying in the nephrogenic zone, we examined propidium-iodide-stained cryosections of kidneys from E19 rats and recorded the location of over 400 pyknotic nuclei in this region. Most of the pyknotic nuclei (60%) were found among metanephric mesenchymal cells that were close to, but not in, nephrogenic structures (Fig. 5A); they were not seen among the densely packed metanephric mesenchymal cells at the outer edge of the embryonic kidney. The remaining

Fig. 1. Propidium iodide staining of nuclei in developing rat kidney. (A,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 in D.
40% of pyknotic nuclei were mainly found in tubular epithelial structures that could be readily identified as developing nephrons. They were either in the walls of the developing nephrons or in their lumen (Fig. 5B, see also Fig. 2C). It was common to see dead cells among the proximal-most cells of s-shaped bodies (Fig. 5C), easily recognisable structures that will eventually form glomeruli. Pyknotic nuclei were rarely seen in the branches of the ureteric bud in the nephrogenic zone.

The papilla of the developing kidney contains epithelial cells of both the ureteric bud and the loops of Henle of the nephrons, 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 wall or lumen of tubular structures, at least some of which seemed to be branches of the ureteric bud (Fig. 4). 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 (not shown).

**Effect of increased EGF on kidney cell death in vivo**

If normal cell death in the kidney is due to limiting amounts of survival factors, then increasing the amount of available survival factors should decrease the death. EGF is thought to be an important growth factor in kidney development (Fisher et al., 1989) and has recently been shown to prevent degradation of DNA in kidney explants (Koseki et al., 1992). To determine if an increase in the amount of EGF available to developing kidney cells would decrease the number of these cells that die, we injected EGF into P1 rat pups (four intraperitoneal injections given 3 hours apart). 2 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 did not significantly affect the mitotic index in the nephrogenic zone (Students' t-test, P>0.05; Fig. 6B), it reduced the pyknotic index in this region by 75% (Fig. 6A). Similarly, EGF treatment did not significantly affect the mitotic index in the medullary papilla (Student's t-test, P>0.05; Fig. 6D), but it reduced the pyknotic index in this region by 85% (Fig. 6C).

**Clearance time of dead cells**

In order to estimate the clearance time of dead cells, we injected EGF (one intraperitoneal injection) into newborn rat pups and perfusion-fixed the kidneys at 45 minutes, 1.5 hours and 3 hours after the injection. For these time points, the pyknotic index in the nephrogenic zone was compared...
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to that of uninjected siblings. For the 11 hour time point, where multiple injections (one 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. 7). At 3 and 11 hours, the mean pyknotic index had reached a basal level.

Apoptotic cells in the developing thymus

To get an indication of how much cell death occurs in the developing kidney, we 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±0.01% (n=5).

DISCUSSION

Cell death has not been thought to play an important role in kidney development. In his comprehensive monograph on kidney development, for example, Saxén (1987) does not mention cell death. We 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, cell shrinkage and phagocytosis of the dead cells or their fragments by neighbouring cells.

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) histiogenic death, associated with cell differentiation. Most of the deaths that we see in the nephrogenic zone in the developing kidney fit best into the category of histiogenic deaths, as they are scattered and seem to occur mainly in metanephric mesenchymal cells at a time when these cells are being induced

Fig. 3. 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 midsaggittal section through a developing kidney is shown in A to indicate the regions where normal cell death was assessed. (B) The nuclei in the outermost 100 \( \mu \)m of cortex were counted in every second field (total of 10-30 fields) of three non-serial sections for each pup. (C) The nuclei in a 300×300 \( \mu \)m grid at each side of the proximal papilla (shown in A) were counted in four 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.

Fig. 4. Confocal fluorescence micrograph of propidium-iodide-stained pyknotic nuclei in the most distal part of the papilla of an E19 kidney. The tubular epithelial structure shown is a collecting duct. Bar, 15 \( \mu \)m.
Fig. 5. Localization of propidium-iodide-stained pyknotic nuclei in the nephrogenic zone of a P1 kidney. (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.

Fig. 6. Pyknotic (A) and mitotic (B) indices in the nephrogenic zone and pyknotic (C) and mitotic (D) indices in the medullary papilla of P0 rat kidneys that were treated with either EGF or PBS. The rats received four injections over 9 hours and were perfused 2 hours after the last injection. In this and the following experiment, the slides were read blind, the nuclei in the outermost 100 µm of the cortex were counted in every second field of three non-serial sections for each pup and the results are presented as mean±s.e.m. of the results from 3-6 pups for each treatment.
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. 3B), and BrdU 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. It is less clear how to categorise the cell deaths in the developing nephrons or collecting ducts; they could play a part in forming the lumen and/or in shaping these structures, in which case they would be examples of morphogenetic deaths.

Why do cells die in the developing kidney? In the cases of developing vertebrate neurones (Hamburger et al., 1981; Hofer and Barde, 1988; Oppenheim et al., 1988) and oligodendrocytes (Barres et al., 1992), it has been shown that increasing the levels of survival factors can greatly reduce the amount of normal cell death, suggesting that the cells die because the supply of survival factors is normally limiting. Even the morphogenetic cell deaths that occur in the region where the two halves of the developing palatal fuse can be suppressed in explant cultures by adding EGF to the culture medium (Hassell and Pratt, 1977). In the present study, we find that increasing the levels of EGF in vivo greatly decreases the pyknotic index in both the nephrogenic zone and in the medullary papilla, without significantly affecting the mitotic index. This is consistent with the possibility that cells in these regions normally die because the levels of survival factors there are limiting. This finding raises the possibility that many normal cell deaths in other non-neural organs also reflect a limiting supply of survival factors (Raff, 1992).

After our studies were completed, we learned that Koseki et al. (1992) independently found apoptotic cells in the embryonic rat kidney. They found that some metanephric mesenchymal cells in explant cultures of embryonic kidney undergo the morphological and biochemical changes of apoptosis and that these cells can be rescued by EGF, activators of protein kinase C, or tissues with nephrogenic-inducing activity. These findings suggest that metanephric mesenchymal cells may be programmed to die unless signalled to differentiate by invading ureteric bud cells. The normal cell deaths occurring among cells around nephrogenic condensates may therefore represent mesenchymal cells that failed to receive adequate inducing signals from ureteric bud cells. This mechanism, however, could not account for the cell deaths within developing nephrons or in the medullary papilla.

Cell death in the developing nervous system is thought to be important in matching the numbers of interacting cells: neurones with their target cells (Cowan et al., 1984) and oligodendrocytes with the axons that require myelination (Barres et al., 1992; B. Barres and M. C. R., unpublished data). 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.

We 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 clearence 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 one hour has been directly observed for cells undergoing normal cell death in vivo (Ellis and Horvitz, 1991). In the present study, we 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.

Two of our findings suggest that the clearance time of dead cells in the nephrogenic zone of the developing rat kidney is short. First, almost all of the apoptotic cells seen in electron micrographs are present as apoptotic bodies inside neighbouring parenchymal cells. Second, we 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 that 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), we estimate that 1 cell dies for every 2-4 cells produced by cell division (in this zone at this time). Thus the proportion of cell death in kidney development is likely to be quantitatively similar to the proportion of cell death in the developing vertebrate nervous system (Oppenheim, 1991).

Our finding that cell death is an important feature of normal kidney development raises the possibility that defects in cell death might play a part in some developmental abnormalities of the kidney. Most important, our findings suggest that the amount of normal cell death that occurs in other developing non-neural tissues (Glucksmann, 1951) may be very much greater than is generally thought and that at least some of this death may reflect limiting amounts of survival signals.

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