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Apoptosis and mitosis during metanephrogenesis and in congenital renal disease

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SUMMARY

Apoptosis and mitosis are carefully balanced to allow metanephrogenesis to occur at an exponential rate. Congenital renal abnormalities can occur due to alterations in this fine balance. It has been suggested previously that the nephrogenic zone has a mitotic index of 50% with an equivalent apoptotic index. Our study therefore evaluated the rates of apoptosis and mitosis in the developing murine metanephros.

Embryonic murine kidneys (E12.5-E16.5) were stained with propidium iodide, and confocal microscopy was used to count the number of apoptotic and mitotic cells. The pyknotic index and mitotic index were calculated for each compartment of the developing metanephros.

The mitotic index in the cortex was 3.4 times greater than the pyknotic index. Apoptosis occurred uniformly throughout the developing kidney, at a lower rate than previously thought. The mitotic index was found to increase in each compartment with age, except in the stem cell compartment, where it decreased.

In sum, apoptosis plays a less significant role in normal metanephrogenesis than is currently believed. However, we suggest that apoptosis may be upregulated during abnormal metanephrogenesis, leading to the development of congenital renal abnormalities.

Key words: Apoptosis – Mitosis – Metanephrogenesis – Congenital renal disease

INTRODUCTION

Nephrogenesis is carefully regulated through the fine balance of cell death (apoptosis) and cell growth (mitosis). Apoptosis is essential for eliminating injured cells, adjusting cell numbers, and sculpting organs. An imbalance between apoptosis and mitosis may cause a predisposition to congenital renal abnormalities such as renal agenesis, dysplasia and hypoplasia. The majority of these congenital abnormalities are due to mutations of multiple specific genes that are involved in normal nephrogenesis. Young children with these congenital renal abnormalities may develop chronic renal failure (Pope et al., 1999; Woolf and Welham, 2002)

Metanephrogenesis involves initial reciprocal induction interactions between the ureteric bud and the metanephric mesenchyme (MM), by the activation of genes encoding growth factors and transcription fac-

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tors (Lechner and Dressler, 1997). These activate specific differentiation programs, which result in the basic pattern of formation in the developing kidney (Kuure, 2000). Uninduced mesenchymal cells will undergo apoptosis. This matches the number of cells available in the bud to those available in the MM and helps to construct the normal architecture of the kidney. Programmed cell death is considered a normal and prevalent feature of embryonic development (Raff, 1992).

After induction the metanephros develops at an exponential rate, its volume doubling every 8hrs between E11 and E16. This rapid growth is enabled by the high incidence of mitosis found in the nephrogenic zone (50%). However, there is apparently an equivalent rate of apoptosis (Coles et al., 1993). Considering that the metanephros exhibits exponential growth, the high apoptotic rate found in kidney development may seem counter-productive.

This study re-examined the relative rates of apoptosis and mitosis within each of the compartments of the murine embryonic kidney and reviewing the literature regarding the influence of apoptosis in congenital renal disease.

Materials and Methods

Experimental Design

All work was done in accordance with the United Kingdom Home Office Animals Act (Scientific Procedures) of 1986. All chemicals were from Sigma (Poole, Dorset, UK) unless otherwise stated. Pregnant (CD57BL× CBA/Ca)F_1 mice were killed at the appropriate embryonic age. Embryonic kidneys were dissected and fixed in 4% paraformaldehyde in 0.01M phosphate-buffered saline solution (PBS) and stored in PBS at 4°C.

Immunohistochemistry

The embryonic kidneys were then incubated with 5μls of anti-laminin rabbit antibody on a shaker at room temperature for 48 hours. Controls were incubated without the antibody. Both samples were washed with 1% PBS every 30 minutes for several hours to remove any unbound antibody. Both samples were incubated with 5μls of anti-rabbit IgG FITC antibody, on a shaker at room temperature for 48 hours. The samples were again washed with fresh 1% PBS to remove any unbound antibody and then incubated with 10 μg Ribonuclease A for 2 hours at 37°C. After this, they were left overnight at 4°C with 2.4 μg propidium iodide, washed with PBS, and mounted on Tespa slides with 1:1 PBS/glycerol.

Confocal Microscopy

Tissue specimens were viewed with the Argon/Krypton mixed gas laser of a Leica TCS NT confocal system (Leica Microsystems, Heidelberg, Germany, GmbH), using a x 63 water immersion lens. The FITC labelled basal laminae appeared green and the propidium iodide-labelled nuclei appeared red. Serial sections were taken through each embryonic kidney; each set was started from the top of the kidney in a field filled by stem cells. Serial sections were taken at 10 μm intervals so as to avoid filming the same cells in successive sections.

The confocal images were analysed using Adobe Photoshop and apoptotic and mitotic cells were first identified and counted for each compartment. Then, the remaining nuclei were individually marked. Counting of apoptotic and mitotic cells was rigorously validated and bias was eliminated by verification of all mitotic and apoptotic cells by a second viewer, thus standardising all cell counts.

Counts of Apoptotic and Mitotic Cells

Pyknotic and mitotic cell nuclei stained with propidium iodide were easily analysable using confocal miscroscopy. The identification of apoptotic nuclei involved the same criteria as those reported by Wyllie and Coles: 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 a single pyknotic nucleus (Wyllie et al., 1980; Coles et al., 1993). Mitotic figures were readily identifiable between prometaphase and late telophase because of their characteristic morphology. Using FITC antibodies to label laminin, it was easy to distinguish epithelial structures (collecting duct, tubules, glomeruli), which were surrounded by a basal lamina as previously outlined by Davies and Bard (1998) (Fig. 1).

Stem cells were identified by their location on the periphery of the kidney. In the first serial section, the supporting stromal cells constituted the remainder of the tissue between epithelial structures.

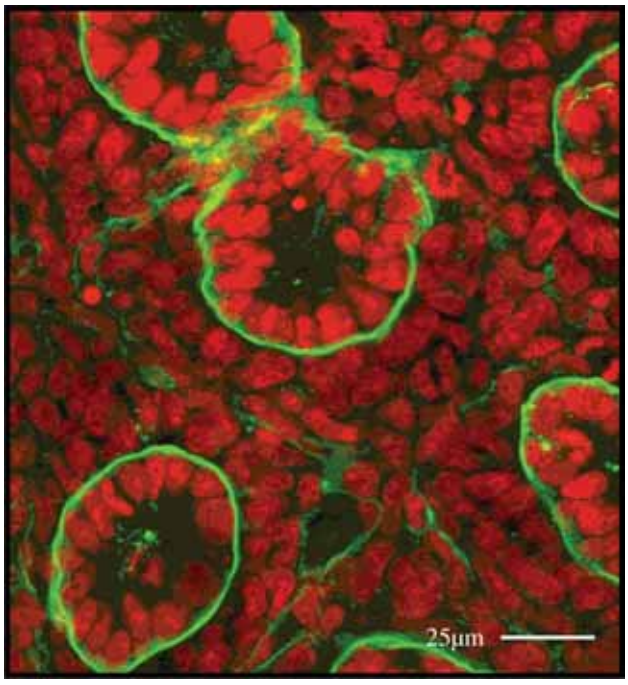

Fig. 1. A typical section through a kidney, showing red propidium iodide-stained nuclei, green FITC-labelled basal laminae and a cell undergoing mitosis in the collecting duct.

Statistics

Results are expressed as means and ± standard deviations. One-way analysis of variance (ANOVA) was used to examine differences between the mean MI and PI of each compartment. Linear regression analysis was applied to determine the correlation values (r) and goodness of fit (r^2) between parameters. Student's t-tests were used to examine differences in apoptosis and mitosis in the different compartments. A value of $p<0.05$ was considered significant.

Results

To determine the pyknotic index (PI) and mitotic index (MI) in the developing kidney as a whole, the data were pooled. 74,358 cells were counted: 661 were mitotic and 191 were apoptotic, affording a mean MI of 0.89% and PI 0.26%. The MI in the cortex was 3.4 times greater than the PI ($p<0.05$). A study by Coles showed that the PI is 3% in the cortex of the embryonic kidney and that the PI was equivalent to the MI (Coles et al., 1993). Our study shows that the PI is approximately one tenth of Coles' value.

To establish if there is a relationship between embryonic age and the MI and PI, data were pooled from all the compartments for each age (Table 1 and Fig. 2).

The trend lines suggest that both the MI and PI increase with age throughout the metanephros. Initially negligible apoptosis occurred at E12.5, as indicated by the fact that only 3 apoptotic cells were counted at this age and at E13.5 it plateaud ($p<0.05$); this is of course when the metanephros undergoes exponential growth. Regression analysis revealed that the increase in PI with age was statistically significant ($p<0.05$) while for MI it was not significant.

A two-way ANOVA test was used to compare the mean MI and PI of each compartment (Table 2).

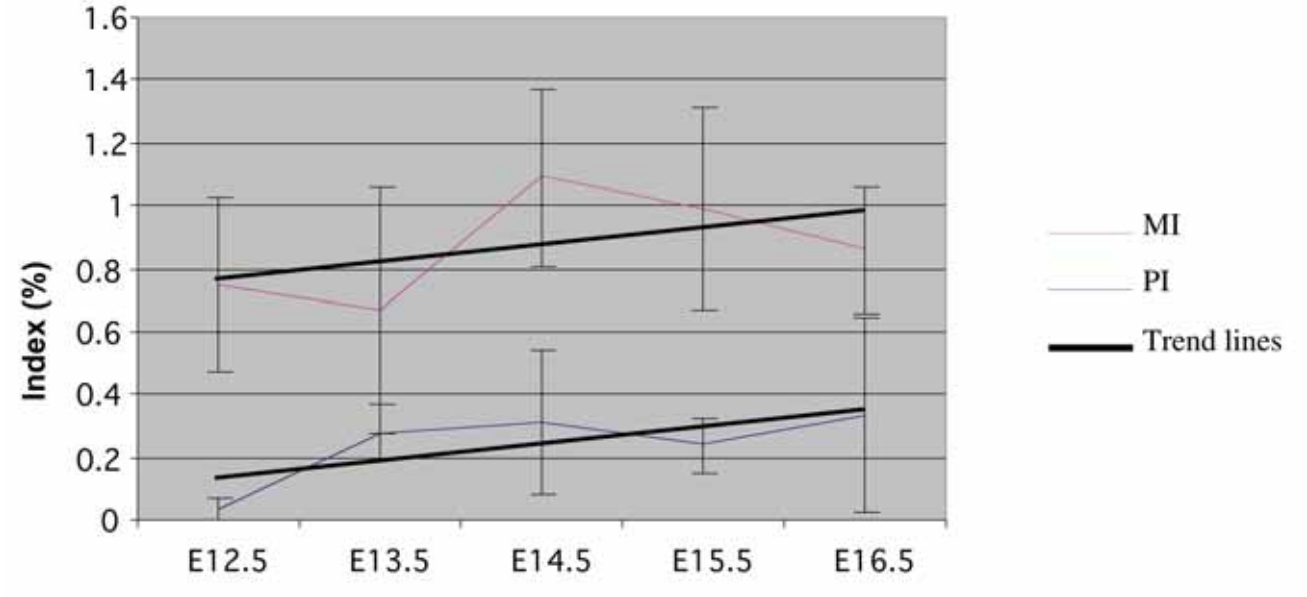

Fig. 2. Variation of the MI and PI with embryonic age.

Table 1. PI and MI for each age in all compartments.

		E12.5 (n=6)	E13.5 (n=3)	E14.5 (n=2)	E15.5 (n=3)	E16.5 (n=3)
Stem cells	Mean PI (%)	0.00 ±0.00	0.26 ± 0.29	0.30 ± 0.10	0.09 ± 0.11	0.00 ± 0.00
	Mean PI (%)	1.39 ± 0.86	0.43 ± 0.41	0.96 ± 0.38	0.23 ± 0.06	0.33 ± 0.20
Collecting Duct	Mean PI (%)	0.00 ± 0.00	0.26 ± 0.19	0.08 ± 0.11	0.37 ± 0.45	0.22 ± 0.73
	Mean PI (%)	1.78 ± 1.01	0.92 ± 0.24	1.15 ± 0.23	1.78 ± 0.21	1.85 ± 1.04
Stroma	Mean PI (%)	0.04 ± 0.06	0.34 ± 0.26	0.34 ± 0.21	0.29 ± 0.12	0.34 ± 0.20
	Mean PI (%)	0.50 ± 0.16	0.68 ± 0.57	0.90 ± 0.25	1.00 ± 0.58	0.75 ± 0.18
Tubules	Mean PI (%)	-	0.07 ± 0.05	0.39 ± 0.01	0.17 ± 0.10	0.45 ± 0.49
	Mean PI (%)	-	0.59 ± 0.36	1.80 ± 0.16	1.06 ± 0.40	0.96 ± 0.15
Glomeruli	Mean PI (%)	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.29 ± 0.61
	Mean PI (%)	-	0.46 ± 0.27	1.7 ± 1.32	1.39 ± 3.03	1.17 ± 2.12

There were no significant differences in the PI, suggesting that apoptosis occurs uniformly in the kidney. The MI, however, was significantly greater in the collecting ducts than in the stem cells and stroma ($p<0.05$) and also greater than in the tubules and glomeruli. The MI seem to increase in each compartment with age, except in the stem cell compartment, where it decreased ($p<0.05$).

Table 2. Total data of each compartment for mitosis and apoptosis.

	Stem	CD	Stroma	Tubules	Glomeruli
Total cells	7823	6373	47239	11338	1585
Mitotic cells	52	93	372	126	18
Apoptotic cells	10	13	133	34	1
MI	0.66%	1.46%	0.79%	1.11%	1.14%
PI	0.13%	0.20%	0.28%	0.30%	0.06%

Discussion

Previous studies reported clusters of apoptotic cells in the developing kidney, close to, but not in, tubular structures. Based on these findings, and the fact that epidermal growth factor (EGF) dramatically reduced the extent of apoptosis in the kidney, it was suggested that this pattern reflected competition for limited amounts of survival factors (Koseki et al., 1992; Coles et al., 1993). This could act as a means of eliminating uninduced cells (histiogenic cell death), sculpting the appropriate morphological features (morphogenetic cell death), or matching the numbers of cells in the bud to those of the metanephrogenic mesenchyme (Gluksmann, 1951).

In this study, however, we found no evidence of such clustering, and in fact, the PI was uniform in all compartments. In Coles' study the PI was 3% in the developing rat metanephros and the proportion and clearance rate of apoptotic cells was similar to those in neural tissues, where 50% of cells die by apoptosis. Coles concluded that due to the similar rapid clearance of dead cells between the two biological systems, apoptosis must be underestimated in metanephrogenesis (Coles et al., 1993).

Our PI was one tenth of Coles' value, so it therefore seems unlikely that apoptosis plays as significant a role as previous studies suggest. Nevertheless, it is possible that apoptosis during normal metanephric development reflects not a histiogenic or morphogenetic function, but rather, a defect whereby cells are unable to respond to apoptosis-inhibition signals, such as that facilitated by bcl-2.

Our ability to identify pyknotic cells correctly was critical for the validation of our results. As described earlier, cells with a typically apoptotic morphology were easy to identify using confocal microscopy, and bias was eliminated by the verification of all mitotic and apoptotic cells by a second viewer, thus standardising all cell counts. The use of other staining methods could be used to identify apoptotic cells such as TUNEL and BrdU staining for mitotic cells. However, we found that we were unable to produce consistent validated results with these methods despite using differing protocols, so these techniques did not form part of our methods.

The MI was found to be 3.4 times greater than the PI in the renal cortex as a whole between E12.5 and E16.5. The PI in the renal cortex was negligible at E12.5, and reached a maximum at E13.5, after which it plateaud. A possible explanation for this feature is that the

metanephros would grow at an exponential rate and that apoptosis (as shown by our study) would have a diminished role compared to mitosis in the formation of the internal architecture of the kidney.

The greatest MI was found in the collecting duct. This was perhaps to be expected, as the ureteric bud grows into, and invades the MM, which secretes growth factors to induce the characteristic pattern of branching bifurcation (Herzlinger, 1994).

Stem cells behaved differently from the other compartments as there was a significant decrease in the MI as development proceeded. Stem cells are induced to differentiate into both nephrogenic structures and the supporting stroma by the invading bud as it extends and bifurcates (Herzlinger, 1994). As the kidney approaches the completion of its mature structure, the rate of new nephron formation slows and eventually ceases, using up the last of the stem cells as it does so (Davies and Bard, 1998). The stem cells therefore disappear as they are induced to differentiate.

Our findings highlight several distinct patterns of growth within the normal metanephros, and suggest that apoptosis plays a less significant role in nephrogenesis than is currently believed. Mitosis is greatest in the collecting duct and least in stem cells. Apoptosis is uniform throughout all compartments. Also, in general, mitosis is 3.4 times greater than apoptosis, which is to be expected in an organ growing exponentially.

Owing to this complex pattern of growth, any interruption of reciprocal induction between the ureteric bud and MM may lead to maldevelopment of the renal system by altering the fine balance between apoptosis and mitosis. Failure of apoptosis of unneeded MM or undifferentiated mesenchyme may lead to an abnormal structure of the renal system such as ectopic ureters (leading to vesico uretric reflux) or renal hypoplasia or dysplasia (Pope et al., 1999). Conversely, a rapid rate of apoptosis may lead the ureteric bud fail to develop sufficiently to induce the MM, causing a congenital absence of one kidney (Koseki et al., 1992). It has been shown that following mutations of the transcription factors involved in metanephrogenesis, the rate of apoptosis in the kidneys of mice is increased, accounting for renal hypoplasia (Porteus et al., 2000).

In a review by Woolf, the author postulated that deregulation of the fine control of metanephrogenesis can lead to congenital renal disease, stating; (i) that there is a fine regulation of apoptosis in normal metanephric development, (ii) simple physical insults can cause changes in metanephric development by deregulation of mitosis and apoptosis (Woolf and Welham, 2002).

One sequela of congenital renal disease is the risk of progression of renal dysfunction to chronic renal failure. Human fetal bladder outflow obstruction is a major cause of chronic renal failure in children. It is associated with complex congenital renal abnormalities. Studies of urinary outflow obstruction have revealed that apoptosis outweighs mitosis by 2-8 fold in the affected kidneys. It is therefore suggested that apoptosis has become a driving mechanism of renal damage and renal remodelling, with an eventual progression to chronic renal impairment (Thomas et al., 1998; Thiruchelvam et al., 2003).

We suggest from our study that this initial pathological mechanism suggested by Woolf, can be added upon; (i) apoptosis plays a less significant role in normal nephrogenesis, (ii) if intrinsic abnormalities of the developmental mechanisms regulating metanephrogenesis are present, the fine balance between mitosis and apoptosis will be altered, (iii) a subsequent increase or decrease in the role of apoptosis may lead to renal maldevelopment, with the formation of congenital abnormalities, (iv) congenital defects predispose the patient to renal impairment, and (v) as renal impairment begins, the role of apoptosis may be exaggerated in the kidney, leading to early chronic renal failure.

Further characterisation of the molecular pathways responsible for kidney growth, and perhaps a re-evaluation of the role of apoptosis, may provide treatment modalities to decrease the chronic renal impairment found in congenital renal disease.

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