Research Paper

Development of the Human Fetal Kidney from Mid to Late Gestation in Male and Female Infants

Danica Ryan [a], Megan R. Sutherland [a], Tracey J. Flores [a], Alison L. Kent [b], Jane E. Dahlstrom [c],
Victor G. Puelles [a,d,i,j], John F. Bertram [a], Andrew P. McMahon [e], Melissa H. Little [f,g],
Lynette Moore [h], Mary Jane Black [a,*]

[a] Biomedicine Discovery Institute, Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria, Australia
[b] Departments of Neonatology, Canberra Hospital, Australian National University Medical School, Australian Capital Territory, Australia
[c] Anatomical Pathology, Canberra Hospital, Australian National University Medical School, Australian Capital Territory, Australia
[d] Department of Nephrology and Immunology, RWTH Aachen University, Aachen, Germany
[e] Department of Biological Sciences, University of Southern California, Los Angeles, California, USA
[f] Murdoch Children's Research Institute, Parkville, Melbourne, Australia
[g] Department of Pediatrics, University of Melbourne, Parkville, Melbourne, Australia
[h] Department of Surgical Pathology, South Australia Pathology, Women's and Children's Hospital, North Adelaide and the University of Adelaide, South Australia, Australia
[i] Department of Nephrology, Monash Health, Clayton, Victoria, Australia
[j] Centre for Inflammatory Diseases, Monash University, Clayton, Victoria, Australia

ARTICLE INFO

ABSTRACT

Background: During normal human kidney development, nephrogenesis (the formation of nephrons) is complete by term birth, with the majority of nephrons formed late in gestation. The aim of this study was to morphologically examine nephrogenesis in fetal human kidneys from 20 to 41 weeks of gestation.
Methods: Kidney samples were obtained at autopsy from 71 infants that died acutely in utero or within 24 h after birth. Using image analysis, nephrogenic zone width, the number of glomerular generations, renal corpuscle cross-sectional area and the cellular composition of glomeruli were examined. Kidneys from female and male infants were analysed separately.
Findings: The number of glomerular generations formed within the fetal kidneys was directly proportional to gestational age, body weight and kidney weight, with variability between individuals in the ultimate number of generations (8 to 12) and in the timing of the cessation of nephrogenesis (still ongoing at 37 weeks gestation in one infant). There was a slight but significant ($r^2 = 0.30$, P = 0.001) increase in renal corpuscle cross-sectional area from mid gestation to term in females, but this was not evident in males. The proportions of podocytes, endothelial and non-epithelial cells within mature glomeruli were stable throughout gestation.
Interpretation: These findings highlight spatial and temporal variability in nephrogenesis in the developing human kidney, whereas the relative cellular composition of glomeruli does not appear to be influenced by gestational age.

1. Introduction

Nephrogenesis (the formation of nephrons) commences in early development and is complete by birth in the term born human infant (Cullen-Mcewen et al., 2016), with the majority of nephrons (approximately 60%) formed in the second half of gestation (Hinchliffe et al., 1991). Studies of autopsied kidneys have shown that there is a wide range in the number of nephrons in the normal human kidney, from approximately 250,000 to over 2 million (Puelles et al., 2011). The mechanisms leading to such a wide variation in nephron number are currently unknown, but genetic variability, differences in the in utero environment, the rate of nephron loss with aging, as well as exposure to postnatal renal insults throughout life are likely key factors (Cosgrove and Goodyer, 2016; Hoy et al., 2005; Puelles et al., 2011). Since loss of glomeruli ultimately leads to renal disease (Hoy et al., 2005), it is likely that individuals born with a high nephron endowment will be relatively protected from renal disease later in life, whereas individuals born with a low nephron endowment are likely to be more vulnerable. Hence, in order to preserve long-term renal health it is imperative to maximize nephron endowment at birth. In order to develop strategies to do this, it is essential to first develop an

* Corresponding author at: Department of Anatomy and Developmental Biology, Monash Biomedicine Discovery Institute, Monash University, Clayton 3800, Victoria, Australia.
E-mail address: Jane.black@monash.edu (M.J. Black).

understanding of normal kidney development, and how factors during pregnancy influence nephron formation.

Over recent years valuable knowledge relating to the regulation of nephrogenesis has been derived from animal models (predominantly rodents) (Takasato and Little, 2015); however, whether these findings can be fully extrapolated to the developing human infant is equivocal given that the temporal and spatial development of nephrogenesis differs markedly between species (Cullen-McEwen et al., 2016). Apart from anatomical microdissection studies conducted many decades ago (Osathanondh and Potter, 1963a; Osathanondh and Potter, 1963b; Oliver, 1968; Saxen, 1987) there have been few studies of nephrogenesis in the fetal human kidney, and these have mainly been conducted in small numbers of infants (Hinchliffe et al., 1991; Faa et al., 2010; Sutherland et al., 2011; dos Santos et al., 2006; Fonseca Ferraz et al., 2008; Souster and Emery, 1980; Crobe et al., 2014; Chikkannaiah et al., 2012; Hinchliffe et al., 1992). One exception is an early study by Potter et al. 1943 (Potter and Thierstein, 1943), where the kidneys from 1000 deceased fetuses and infants were analysed. However, there were a number of confounding factors in that study relating to in utero growth, exposure to intrauterine inflammation, preterm birth (some infants lived for two to 69 days after birth), and cause of death. Furthermore, only one parameter of kidney growth (nephrogenic zone width) was assessed (Potter and Thierstein, 1943). Importantly, no studies to date have compared nephron development throughout gestation between male and female fetuses.

Therefore, to further enhance our knowledge of the normal development of the human kidney we conducted a comprehensive histological examination of kidney growth from 20 weeks in gestation until term, the developmental period when the majority of nephrons are formed. The aims were to examine: nephrogenic zone width, the number of glomerular generations, glomerular size, and the proportions of different cell types within glomeruli, as well as to explore variability in the timing of the cessation of nephrogenesis. The kidneys from male and female infants were analysed separately, and only the kidneys from infants that were normally grown in utero and died acutely were analysed.

2. Materials and Methods

2.1. Study Groups

In this retrospective study, archived fetal and newborn kidney tissue was obtained from the Women's and Children's Hospital in North Adelaide, South Australia, and the Canberra Hospital in the Australian Capital Territory. The kidneys were collected at autopsy from 71 appropriately grown infants who died suddenly in utero or within 24 h after birth. Sixty eight (95.8%) of the infants were born of caucasian mothers, 1 (1.4%) of a Sri Lankan mother, 1 (1.4%) of a Vietnamese mother, and 1 infant (1.4%) was born of an Indigenous Australian mother. The causes of death included asphyxia/cord entanglement (14/71 [20%]), placental abruption (12/71 [17%]), placental infarction/placental thrombosis (8/71 [11%]), extreme prematurity/ respiratory failure (6/71 [8%]), and termination of pregnancy (2/71 [3%]). In 29 of the cases (41%) the cause of sudden death was not determined at autopsy. Five of the infants (7.0%) were exposed to preeclampsia, and 1 infant (1.4%) was exposed to antenatal corticosteroids; in 2 cases (2.8%) the mothers were induced to deliver with prostaglandin treatment. The infants ranged in age from 20 to 41 weeks of gestation (n = 33 female; n = 38 male), whereby gestational age was primarily defined according to early ultrasounds and the date of the mother's last menstrual period. Autopsies were performed between the years 1996 and 2013, and written informed consent from the parents was obtained for autopsy and archival of tissue. Infants were excluded from the study if there was evidence of congenital abnormalities, intrauterine growth restriction, cardiovascular or renal complications, or exposure to chorioamnionitis or diabetes in utero. Kidneys were also excluded if they were severely macerated. Ethical approval for this study was

obtained from the Children, Youth and Women's Health Service Research Ethics Committee of South Australia and the Australian Capital Territory Human Research Ethics Committee.

2.2. Tissue Preparation and Processing

Kidneys collected at autopsy were weighed and cut into two in the longitudinal plane; large kidneys were further cut transversely. The kidneys were embedded in paraffin and sectioned at 5 μm. Sections were stained with haematoxylin and eosin for the assessment of nephrogenic zone width, number of glomerular generations and renal corpuscle area. Analyses of glomerular cell types were undertaken in a subset of the archived kidneys by immunofluorescence; this was restricted to kidneys where immunofluorescent staining was successful.

2.3. Assessment of Nephrogenesis

In all kidneys it was noted whether nephrogenesis was ongoing or complete. Nephrogenesis was considered ongoing if there was evidence of metanephric mesenchyme and immature nephrons in the form of comma and S-shaped bodies in the outer renal cortex.

2.4. Assessment of Nephrogenic Zone Width and Glomerular Generation Number

In kidneys where nephrogenesis was ongoing, the width of the nephrogenic zone was measured in four randomly sampled regions of the cortex using image analysis software (Image Pro Plus v. 6·0 for Windows, Media Cybernetics; Silver Spring, MD, USA), and the average width per kidney was determined (Gubhaju et al., 2009; Sutherland et al., 2011).

The number of glomerular generations formed within all kidneys was assessed using a medullary ray glomerular counting method (Hinchliffe et al., 1991; Faa et al., 2010; Sutherland et al., 2011). Mature glomeruli were counted along five clearly defined medullary rays per kidney, and the average number of generations per kidney was determined.

2.5. Assessment of Glomerular Size

Glomerular size was assessed by measuring the cross-sectional area of renal corpuscles. To do this, kidney sections were systematically sampled throughout the cortex (inner, middle and outer cortex) at 400× magnification with a step length of 1 mm. At each field of view, one glomerulus was measured using image analysis software (Image Pro Plus v. 6·0 for Windows, Media Cybernetics; Silver Spring, MD, USA). If more than one was observed in the field of view, the renal corpuscle for analysis was selected according to the method of Nyengaard and Marcussen (Nyengaard and Marcussen, 1993). At least 100 renal corpuscles were measured per kidney, and the average was then calculated.

2.6. Immunofluorescent Identification of Glomerular Cell Types

A standard immunofluorescence protocol (Puelles et al., 2014; Puelles et al., 2015) was used to identify podocytes, endothelial and non-epithelial cells within mature glomeruli; glomeruli in stages I, II and III of development were analysed (Naruse et al., 2000; Sutherland et al., 2011). Briefly, sections were first subjected to heat-induced antigen retrieval in sodium citrate buffer. Immunofluorescent staining was then performed using a DAKO Autostainer Plus Staining System. Wilms' Tumour-1 (WT-1) antibody (monoclonal mouse M356101, Dako; 1:50, (RRID:AB_564063)) with Alexa Fluor 488 conjugated secondary antibody (A-11001, Invitrogen; 1:2000) was used for the identification of podocytes (previously shown to stain podocyte cytoplasm and foot processes) (Puelles et al., 2014, Puelles et al., 2015), and von Willebrand Factor (vWF) antibody (polyclonal rabbit A008202, Dako;