The Opisthonephric Blood Vascular System of the Chicken Embryo as Studied by Scanning Electron Microscopy of Microvascular Corrosion Casts and Critical Point Dried Preparations

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SCANNING ELECTRON MICROSCOPY OF MICROVASCULAR CORROSION CASTS AND
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Abstract

Microvascular corrosion casts of chicken embryos between four and 19 days after fertilization have been prepared. The developing kidney was investigated with scanning electron microscopy (SEM). The injection technique and resin composition were modified in order to facilitate the complete replication of native blood vascular systems of specimens as small as 15 mm body length. The development of the opisthonephros was followed from near the beginning of its function until a vascular development comparable to the adult situation was reached. Critical point dried glomeruli show the differentiation of the glomerular visceral epithelium (podocytes) from initially epithelioid to highly branched forms. The embryonic kidney (cranial part of the opisthonephros - mesonephros) shows a construction - principle resembling amphibians that is entirely different from the definitive excretory organ (caudal part of the opisthonephros - metanephros).

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Introduction

Numerous studies on renal vascular systems use the microvascular corrosion casting technique in connection with SEM (for Reviews see e.g., Gannon, 1978; Hodde and Nowell, 1980; Lametschwandtner et al., 1984; Ditrich and Splechtna, 1987). The kidney of several adult avian species, e.g., chicken (Sperber, 1948; Siller and Hindle, 1969; Siller, 1971), dove (Ditrich and Splechtna, 1985), duck (Ditrich and Splechtna, 1986) and finches (Johnson, et al., 1972) were studied in light- and scanning electron microscopy using injection methods. However, studies using the microcorrosion casting technique on the developing kidney are scarce. Only the studies of Evan et al. (1979) on newborn dogs, Kazimierczak (1980) on the juvenile rat and of Naito (1984) on the development of the glomerulus of the Bullfrog have been made so far. Studies on other embryonic organs (e.g., pig liver - Hasselager and Leiser, 1988;) are rare as well and usually limited to relatively large embryos and/or later stages of development. The formation of the glomerular visceral epithelium (podocytes) and of the vascular endothelium of chicken embryonic kidneys was studied by Jacob et al. (1977) and Narbaitz and Kapal (1986) using transmission electron microscopy and/or scanning electron microscopy of critical point dried specimens. In this study an attempt was made to modify the well established corrosion casting technique for preparations of very small specimens. Furthermore, an attempt was made to follow the onto-genetic development of the opisthonephric vasculature which shows drastic morphological and functional changes.

Materials and Methods

The domestic chicken (Gallus domesticus) was chosen as a model for this study as the material can be obtained easily and the ontogenesis is documented extensively in the literature (e.g., Lillie, 1952; Russo-Caia, 1966). Eggs for hatching were incubated from four to 19 days. The embryos were removed from the egg-shells, cleared of yolk without destruction of the omphalo-mesenteric vessels and covered in a petri dish with 0.9% NaCl saline warmed at 38°C. The injection method proposed earlier for larger animals (Ditrich and Splechtna, 1987) had to be modified to apply to such delicate objects. No vasodilatators or fixative agents were applied. 0.5% heparin (5000 I.E./ml) was added to the saline as an anticoagulant. The embryos showed heart-beat and an apparently functioning circulation. For injecting the whole vascular system, three different ways were chosen.

In very small animals (approx., day 4 - 6) a larger omphalomesenteric vein was punctured with a glass micropipette using a micromanipulator under microscopic control. The micropipette was previously filled with a small amount (approx. 0.3 ml) of flushing saline immediately followed by the casting medium. The tip of the capillary was secured by a ligature, flushing of the vascular system had to be limited due to the small volumes involved. Also the mixture of the casting resin (Mercox - Jap. Vilene Co. diluted with 20% v./v. methyl-methacrylate - Ohtani and Murakami; 1978) was modified by decreasing the amount of catalyst - hardener to less than 1% to obtain a longer injectable time of the medium (about 15 min.) and by increasing the amount of methyl-methacrylate to 25%, thus obtaining a very low viscosity injecting medium. Injection was carried out by hand under microscopic control with a 1 ml disposable syringe connected to the micropipette. The injection was stopped when the transparent skin of the embryos showed filling of the superficial vessels.

For larger embryos (day 7 - 11), the injecting procedure was carried out as above, but the ventricle of the heart was chosen as the site of injection. The heart was exposed by dissection with fine forceps and no ligature was applied as the contracting muscles of the heart seal the tip of the capillary sufficiently.

Embryos after that stage (from 12 - 18 days) were already large enough to be injected by hand with two ml disposable syringes and steel needles (Nr.20; 0.4x19), directly into the surgically exposed ventricle. Flushing in this case was carried out by injecting approx. 0.5 ml of saline immediately preceding the casting resin.

The preparations were subsequently placed in a water bath at 70°C with addition of a neutral domestic cleaning agent and cured for 12 - 14 h. Remaining tissue was macerated in 5% KOH (usually less than 24 h). In addition the casts were rinsed in tap- and distilled water, frozen, sectioned with a cutting disc, mounted and sputtered with gold (10 min.; 12-17 mA;) as described earlier (Ditrich and Splechtna, 1987).

Other embryos were immersed after quick dissection in 4% formaldehyde (0.1M phosphate - buffered), dehydrated in ethanol and paraffin sectioned for light-microscopy or critical point dried (Acetone/CO_2) by conventional methods.

The specimens were investigated in a ZEISS DSM-950 scanning electron micros-cope or under a REICHERT POLYVAR light microscope.

Results

The embryos can be split in three groups for investigation. Group 1 (day 4 to 11) shows an apparently functional cranial part of the opisthonephros (meso-nephros - see "Discussion" for remarks on the terminology) and a rapid craniad growth of the caudal part of the opisthonephros (metanephros). Group 2 (day 11 to approx. 16) shows degeneration of the cranial part. In this time the cranial part loses its former filtering capacity and later becomes the epididymis of the male or the epoophoron in the female. The caudal part differentiates to the lobate structure of avian kidneys and begins its function as the definitive excretory organ. Group 3 (after approx. day 17) shows a situation that is similar to the adult, although the topographical and size relations are still different.

In group 1, the cranial part reaches its full excretory function as a very large organ when compared to the adult situation (Fig. 1). No external signs of lobulation can be found in this temporary kidney. The organ is drained by the Wolffian duct, running dorsolaterally along the surface. The venous portal system which is mainly supplied by the cardinal veins is very prominent. Starting at about day six, the caudal part of the opisthonephros becomes apparent in the corrosion casts as a biconical outgrowth running dorsomedial to the Wolffian duct (Fig. 2). The cranial part of the opisthonephros shows a simple inner structure. The venous portal supply forms numerous large sinusoidal branchings that run dorsoventrally through the kidney. Glomeruli are scarce and usually situated medioventrally (Fig. 3). These are supplied directly by small branchings

from the aorta and drained via short efferent vessels into the venous system (Vv. subcardinales - V. cava posterior) that leave the kidney ventrocranially. A conspicuous feature of the glomeruli of the cranial part is their size (Fig. 4). Although only few of these glomeruli can be found, they are about two to four times larger than the later formed glomeruli of the caudal part. In CPD preparations, the podocytes of these "temporary" glomeruli already show a well developed process structure, comparable to the situation found in adults (Fig.5). Glomeruli of the caudal part that are formed from about day 6 on are not only smaller than the glomeruli of the cranial part, they also show an epithelioid appearance of the podocytes (Fig. 6) with only very few areas where simple processes and filtration slits are already present. It can therefore be concluded that the contribution of these glomeruli to the whole filtering capacity of the embryonic kidney is low at that stage of development.

In group 2, the cranial part loses its glomeruli and is reduced to an inconspicuous loose anastomosing vascular network. The caudal part grew dorso-craniad so that the portal vessels now run ventrally along the surface of the kidney. The three lobes of the avian kidney are already visible externally and the internal lobular structure is formed. The glomerular visceral epithelium has developed its foot processes in most glomeruli, though several of them still show an epithelioid appearance indicating that glomerular maturation is still taking place.
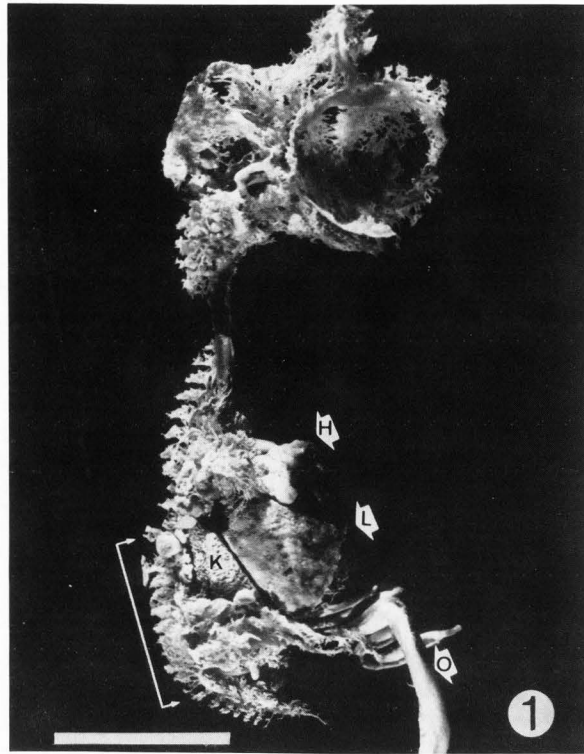

Fig. 1. Macrophotograph of a cast preparation of an 8 day embryo. Note the comparatively large extent of the embryonic kidney (↑_K_↓). L = Liver; H = Heart, O = Omphalomesenteric vessel; Bar=2 mm.
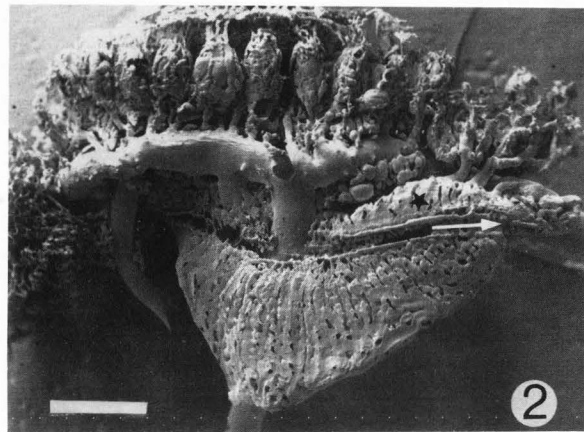
Fig. 2. Low power lateral SEM view of the cast kidney of a 7 day embryo. Note the smooth surface of the embryonic kidney and the impression of the Wolffian duct (arrow). The caudal part of the opisthonephros (asterisk) starts to grow anteriorly. Bar=1 mm.
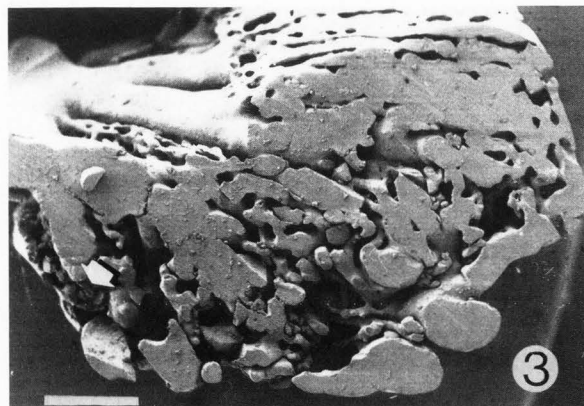
Fig. 3. Frontal section through the cranial part of the right kidney of an 8 day embryo. The afferent (portal) venous network is strongly developed giving a sinusoidal appearance. No lobular structure of the vasculature can be found. A glomerulus (arrow) is situated ventrally, the aorta and posterior caval vein were removed in this preparation. Bar=500 μm.

The structure of the kidney in group 3 is very similar to the adult situation. The organ is still very large relative to the rest of the body. The inner structure shows several small functional glomeruli as in adult chicken although numerous newly formed renal corpuscles can still be found.

Discussion

About 50% of the cast embryos showed complete filling of the vascular system. This may seem a low yield, however, as the whole vascular system is still developing, several unpredictable effects seem to take place during injection. The most common artifact in small embryos (before day 11) was a complete filling of the venous system while the arteries failed to be cast. A possible explanation for this effect might be the absence of functional venous valves, thus leading to a partial retrograde filling of the vessels. The "trapped" flushing liquid in distal parts of the system might then lead to incomplete filling. Another possible explanation might be that the more elastic arteries empty themselves after injection as the casting resin remains liquid and the site of injection was not sealed. Vasospasms may also be considered as a possible explanation for these artifacts. Furthermore, the omphalomesenteric (yolk-) vessels provide an additional very large drainage in close vicinity to the kidney. More experiments are needed to understand the flow relationships in an embryonic vascular system during injection.

The term opisthonephros was chosen to describe the organ developing from the nephric blastema according to Kerr (1919). This includes the cranial part that becomes the non-excretory, sperm draining system in the male (epididymis) and the rudimentary epoophoron in the female. According to Felix and Bühler (1906) and others, this part is often referred to as mesonephros, a term that seems not appropriate for comparative anatomical studies. The caudal part of the opisthonephros, induced by the ureter buds, that later becomes the definitive excretory organ is frequently called metanephros. Although this terminology is well established in the biological and medical literature (e.g., Jollie, 1973), the terms pars cranialis and pars caudalis of the opisthonephros seem more appropriate to describe their ontogenetic connection (see also Starck, 1982).

The structure of the cranial embryonic kidney in its excretory state resembles the kidney of amphibians in many aspects. The vascular system is composed of a dorsolateral venous (portal) input a medial arterial supply to the glomeruli and a medioventrad

efflux. Comparatively few, very large glomeruli are situated in the ventral portion of the kidney. No internal lobular structure is developed and the renal tubules are drained by the Wolffian duct. This situation is very similar to descriptions of the adult kidney of Amphibia (e.g., Wake, 1970; Lametschwandtner et al., 1978; Ohtani and Naito, 1980; Naito, 1984; Ditrich and Splechtna, 1987;). The caudal part of the kidney shows an entirely different structure. Its development involves interaction of nephrogenic tissue, vascular endothelial cells and especially the anteriorly growing ureter bud (Ekblom and Sariola, 1985). The latter induces the three-lobate external form and the complex internal lobulation of the caudal part of the avian kidney (Siller, 1971 ; Nickel, et al., 1973; Kurihara and Yasuda, 1975; Ditrich and Splechtna, 1986), i.e., the definitive excretory organ. Connected with this change in the excretory structures is a shift in the chemical composition of the excretory products. While the embryonic kidney excretes predominantly urea, the main adult excretory product is uric acid (Sykes, 1971). Naito (1984) studied in detail the formation of glomeruli in the Bullfrog using microvascular corrosion casting. His description of glomerular formation by endothelial sprouting and infolding of vessels seems also applicable for the chicken, as blind ending buds were frequently found at the cast glomeruli in this study, especially in the caudal part. However, these buds may also be a sign of incomplete replication and an in situ development of the glomeruli should also be taken into account. In this case a replication with the corrosion casting technique would be possible when the glomerulus becomes functional, i.e., connected to the extra-glomerular capillary system by the Vas efferens which is reported in the literature (e.g., Evan, et al., 1979) to be the last in development of the vessels supporting the vascular tuft. Before that, a developing glomerulus would only be seen in CPD and light microscopical preparations. This possibility seems very likely from our experiments as the cast glomeruli usually show a good differentiation and beginning stages as described by Naito (straight vessels with two or three loops) have not been observed. With more evolved and reliable corrosion casting techniques, supplemented by light and transmission electron microscope the problem of glomerulogenesis in birds will further be investigated.

Fig. 4. Section through a cast glomerulus of a 12 day embryo. Note the numerous, partly cut large glomerular vessels. Bar=50 μm.

Fig. 5. Glomerular visceral epithelium of a 9.5 day embryo in a CPD preparation. The processes of the podocyte cells are already well developed indicating that this glomerulus is in a functional state. Bar=10 μm.

Fig. 6. The epithelium of a glomerulus of the caudal part of the kidney is still of epithelioid appearance at 19 days. Bar=5 μm.
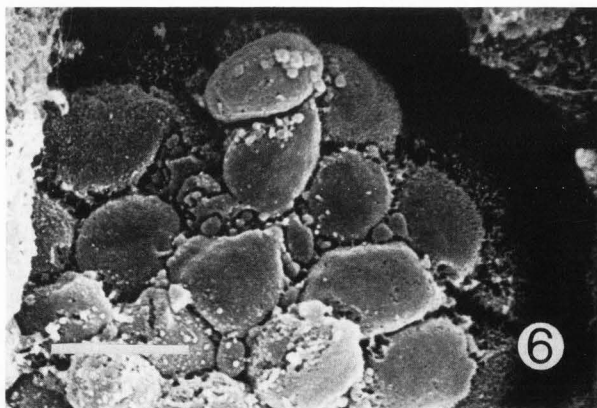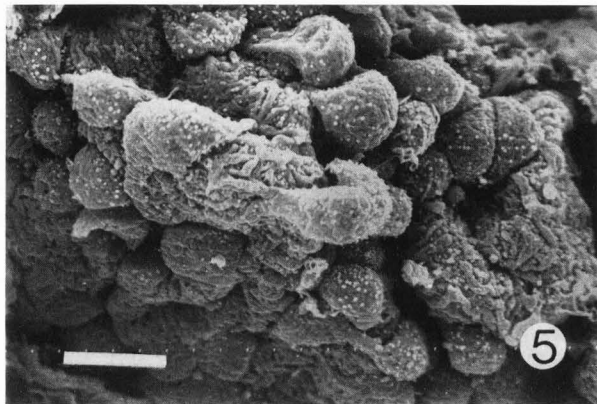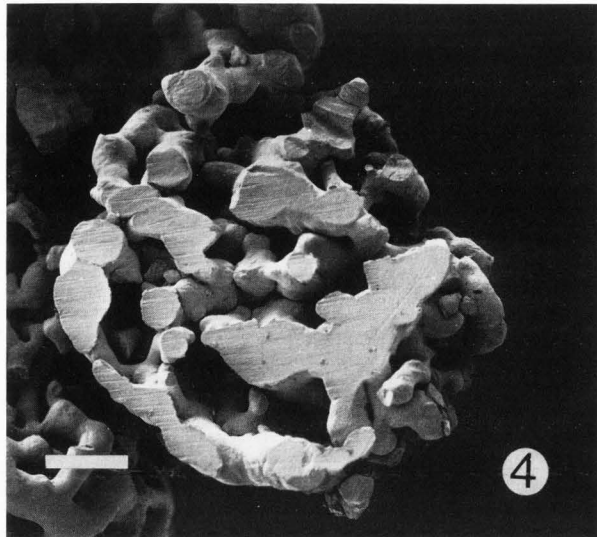
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Discussion with Reviewers

Reviewer I: Please provide more information on the strain of chicks used. The total number of embryos injected vs. number of embryos actually studied with SEM after corrosion casting should be given.

V.H. Gattone: Was the number of chicks sufficiently large to have actually quantitatively determined the glomerular sizes to support the statement that the cranial embryonic glomeruli are larger?

Authors: The eggs are derived from LSL (Lohmann Selected Leghorn) chicken. 35 satisfactory cast preparations were investigated in the SEM, these were made daily between embryonic day 4 and 19. More than half of the incompletely filled vascular systems occurred in the group between day 4 - 6, due to the difficult preparation. The latter were briefly inspected, and not used for further investigations. Two or three additional embryos of each day were fixed for LM and CPD. The size differences between the glomeruli of the cranial part (approx. 160 μm) and the adult glomeruli (63 μm $\pm$ 8.6 std; n=26) is so obvious that no statistical tests were applied.

A.Kikuta: The blood vessels of embryos are fragile and injected resin highly inclines to leak out of the vessels and to produce artificial anastomosis with adjacent vessels. Furthermore, as the authors mentioned, in cases, incomplete filling of injected resin occurs. What criteria do the authors use for distinguishing between true anastomosis and an artificial one, and between true budding and incomplete filling?

Authors: Incomplete filling is usually found more to cause absence of larger areas than of single capillary branches.

However, for a single vascular bud, the presence of endothelial impressions in its vicinity and the form of the tip (conical instead of round) give some confidence in the completeness of filling. Vascular leakage can also be identified by absence of endothelial marks and a usually round or drop-like appearance. Elongate cast structures with irregular course and diameters below those of capillaries may also represent plasma filled capillary sprouts (see also Rhodin and Fujita, 1989), however, exact interpretation of single tips remains difficult.

V.H. Gattone: In group 1, do the cranial glomeruli efferent vessels all end directly in the subcardinal vein or do they connect with the venous portal sinusoids as well?
Authors: As far as we traced the Vas efferens of the glomeruli, these always join the portal peritubular vessels, however, this fusion occurs near to the ventral surface of the kidney, i.e., close to the drainage into the subcardinal vein.

Reviewer I: All of the injections might have been done from the heart. Why was the peripheral portion of the omphalomesenteric vessels not tied off? To solve the problem of incomplete filling (50% of the specimens) the authors should inject from arterial side.
Authors: In the group of very young embryos, the tissue is still very soft and flexible. Accordingly it turned out to be very difficult to insert the micropipette into the heart without major ruptures and to seal the tip capillary with a ligature (which is in this case necessary, as the muscles are not yet strong enough to seal the site of injection by their own contraction). The omphalomesenteric vein is comparatively easily accessed and the ligature securing the tip of the micropipette prevents reflux of the casting medium to distal portions of the yolk vessels. Injection from the omphalomesenteric artery would lead to an unphysiologic retrograde flow, a situation we try to avoid whenever possible (see Ditrich and Splechtna, 1987).

V.H. Gattone: Could you comment on the appearance of endothelial fenestrae in either the glomerular or renal venous network in the cranial embryonic kidney or the caudal part which becomes the definitive kidney.
Authors: We have no own data on this question, the work of Jacob, et al. (1977), contains TEM micrographs of glomerular endothelial fenestrae of an apparently normal morphology.

Reviewer I: What is the justification for not using perfusion fixation for the specimens prepared by CPD for conventional SEM?
Authors: Quick immersion fixation was used because: a) the animals are small and soft enough to be penetrated by aldehydes in a short time, the difficulties and risks of perfusion fixation can therefore be avoided, b) as we did not study the luminal aspect of the vessels, the physiologic blood pressure probably renders more "natural" appearances of glomerular and tubular epithelia.

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