STRUCTURAL AND ARCHITECTURAL PATTERNS OF ASCENDING VASA RECTA BETWEEN THE INNER AND OUTER MEDULLARY BORDER OF THE MUNICH-WISTAR RAT KIDNEY

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Abstract

The goal of the following research project was to perform a three-dimensional reconstruction of selected CDs and associated AVR as they ascend through the IM/OM border, and determine whether AVR in this region coalesce to form giant vascular bundles, or whether they do so in a more dispersed pattern, interacting with components of the interbundle regions. The previously accepted model of vascular architecture in this region suggests that as AVR ascend through the IM/OM border, they coalesce to form dense vascular bundles (Figure 5, Pallone). Previous research performed in this lab, along with current three-dimensional reconstructions suggest that AVR are not confined to dense vascular bundles as they enter the OM. Instead, we hypothesize that as intracluster AVR ascend through the region of interest, they do so in a more dispersed pattern throughout the inner stripe of the OM. Analysis of the following experiment utilized methods including immunohistochemical preparation of the tissue samples, epifluorescent microscopy, and three-dimensional reconstructions of AVR through the region of interest. The three-dimensional reconstruction of three CDs, and five-associated AVR resulted in two AVR joining the vascular bundle, while the other three were found to ascend through the interbundle regions of the OM.
Introduction

The kidneys play a critical role in the body’s homeostatic regulation and function. This retroperitoneal pair of organs is responsible for removal of metabolic wastes and toxins, reabsorption of vital nutrient and electrolytes, maintenance of pH levels optimal for cellular and molecular function, and regulation of fluid volume and blood pressure (Sherwood). Inability to perform any of these functions can quickly result in a deadly disruptions in the body’s homeostatic state.

Longitudinal dissection of the kidney reveals its sectional composition. The human kidney consists of an outermost renal cortex, 7 pyramid-shaped units known as renal medulla, and the renal pelvis which is located at the medial aspect of the kidney and is responsible for collection of formed urine for excretion from the body. The renal medulla is further broken down into three sections; the outer medulla (OM), inner medulla (IM), and the renal papilla. The OM is adjacent to the renal cortex and lies on the periphery of the renal medulla. The IM abuts the OM and extends down to the medial aspect of the renal medulla known as the papilla (Figure 1). Contained within the inner and outer medulla are nephrons, which are the kidney’s functional unit. This implies that the nephron is the smallest unit capable of performing all of the kidneys functional processes. Each kidney contains approximately 1,000,000 nephrons attached to one another by connective tissue (Sherwood). Because the kidney acts as the body’s filter, all blood flowing through the body must pass through this organ. As blood passes through the kidney, it travels through the afferent arterioles which are blood vessels leading to a dense collection of capillaries known as the glomerulus. Fluid not filtered out by the
glomerulus is returned to the body via the efferent arterioles. The glomerular membrane is fenestrated, making it permeable to H2O and other small solutes. Red blood cells and albumin are unable to pass through the membranous pores due to either size, polarity, or both. Fluid filtered through the glomerular capsule is known as filtrate, which subsequently passes through Bowman’s capsule before entering the renal tubule. Filtrate first enters the proximal convoluted tubule which is the tubular region in which most water and solute reabsorption takes place. As filtrate continues through the renal tubule it passes through the water permeable descending thin limb (DTL) of the loop of Henle. This segment dips deep into the IM before turning towards the OM, and into the short Ascending Thin Limb (ATL). Remaining filtrate is then moved through the thick ascending limb of the loop of Henle before entering the distal convoluted tubule which dumps filtrate into the collecting duct to further concentrate the filtrate which will enter the renal pelvis as urine. Each tubular segment plays a different role in osmotic regulation by either reabsorbing solutes from the filtrate, or by secretion of solutes to the filtrate from adjacent capillaries. A labeled image of the previously described nephron structure is provided in figure 2.

Renal capillary vessels act as the main blood supply to renal tubules, creating a mode of nourishment to the tissues of the renal medulla. This vasculature, also known as vasa recta, additionally plays a major role in solute exchange and fluid transport from the IM to the OM. Fluid transport between renal tubules and vascular capillaries is thought to occur via conventional countercurrent exchange. The process of countercurrent exchange is responsible for the kidneys ability to maintain a medullary hypertonic gradient. Vasa recta accomplish this by looping back through the
osmotic gradient in reverse, preventing the dissolution of the concentration gradients as fluid moves throughout the medulla (Sherwood).

The medullary region of interest lies between the IM and OM. Specifically, the goal of this project is to determine the structural relationship between segments of the renal tubule, such as CDs, DTLs, and ATLs, and the accompanying vasculature through the IM/OM boundary. This boundary is identifiable by the presence of TALs that lie only in the OM and are positioned adjacent to CDs, both of which express CIC-K protein as viewed under epifluorescence microscopy (Figure 3).

The vasa recta associated with the renal tubules consist of descending vasa recta (DVR), and ascending vasa recta (AVR). CDs descend through the OM and clusters of CDs coalesce as they descend through the IM, eventually ending with about 10-13 CDs at the tip of the papilla. These terminal CDs form the ducts of Bellini through which final urine passes into the ureter. CD clusters form the organizing motif axially and transversely throughout the IM. DTLs are positioned transversely at the borders of these CD clusters (the intercluster region). ATLs lie uniformly within the CD clusters (intracluster region) as well as outside the clusters in the intercluster region.

AVR can be seen symmetrically abutting the CDs throughout almost the entire medullary region. These AVR lie in the intra-cluster region (Figure 4). Other AVR pass through the IM almost solely through the intercluster region. These latter AVR are largely unbranched AVR whereas the intercluster AVR appear to undergo a much greater degree of branching.

Little is known about the structural relationships between renal tubules and AVR as they travel through the IM/OM border, and into the OM. It is commonly believed that
upon ascension into the OM, branching AVR coalesce to form dense vascular bundles and lose their association with most CDs as they move further up through the OM towards the renal cortex (Pallone). This belief is based upon micrographs taken of the microvasculature of the desert rodent *psammomys obesus* (Figure 5).

Contrary to this accepted model, previous studies and current three-dimensional reconstructions from our laboratory reveal that this may not be true of the common laboratory rat which serves as a common model of the mammalian urinary concentrating mechanism. As AVR from the intra-cluster region move towards the OM, we hypothesize that branching AVR continue to interact with CDs, DTLs, and TALs by passing through inter-bundle regions of the OM.

**METHODS**

*Kidney Removal and Tissue Preparation*

The kidney utilized for functional three-dimensional reconstruction was obtained from a Munich Wistar rat. Euthanization of the rat was performed with CO2 gas. In preparation for immunocytochemistry, retrograde perfusion was performed through the aorta with 1X phosphate buffered saline (PBS) [pH 7.4] for 5 minutes. This was followed by periodate-lysine paraformaldehyde fixative [0.01M, 0.075M, 2%] in PBS for an additional 5 minutes. Upon removal of the rat kidney, the inner medulla was excised and immersed in fixative for 3 hours at 4°C before being washed in PBS and dehydrated using an ethanol series ranging from 50-100%. Upon excision, each medulla is trimmed so that the outer medullary region measures 2.0 x 1.4 mm. Obtained tissue
was set first in a solution of Spurr epoxy resin (Ted Pella, Inc.) and ethanol (1:1) for 16
hours at room temperature before being placed in 100% Spurr for 48 hours at 4°C.
Tissue was then embedded in 100% Spurr for 24 hours at 60°C. Sequential transverse
slices (1 μm thick) were cut from each medulla starting near the (OM)/(IM) border.
Transverse sections were sliced in such a way as to include portions of both OM and IM
regions. Every fifth tissue section was obtained and set onto glass microscope slides.
Each prepared slide contained 4 tissue slices.

*Immunohistochemistry*

The immunohistochemistry was performed as a method of visually and
chemically differentiating between (DTLs), (CDs), (ATLs), (TALs), (DVR), and (AVR).
This was accomplished using affinity-urified polyclonal antibodies against the COOH-
terminal region of human water channel aquaporin 1 (AQP1, chicken host, Dan Stamer,
University of Arizona), rat kidney-specific chloride channel (CIC-K, rabbit host,
Chemicon), and human water channel aquaporin 2 (AQP2, goat host, Santa Cruz), and
mouse monoclonal antibodies raised against amino acids 249-269 of rat AQP1 (Serotec)
and purified αB-crystallin from bovine eye lens (Stressgen). The ability to differentiate
between vascular structures was made possible by their differences in protein expression.
Because DTLs and CDs express AQP1 and AQP2 respectively, these protein structures
served as distinguishable markers. The antibody CIC-K binds to ATLs (CIC-K1) and
CDs (CIC-K2) in the IM, and to TALs and CDs in the OM. To differentiate AQP2
positive CDs from ATLs and TALs, CIC-K1 is utilized. Inner medullary prebends and
ATLs only bind the antibody CIC-K1 allowing easy identification. A common marker
for all tubules is αB-crystallin; this includes AQP1 positive and AQP1 negative DTLs. Labeling and identification of DVR is made possible by using affinity purified polyclonal antibodies raised in rabbits against the COOH-terminal region of the urea transporters UT-B (Jeff Sands and Janet Klein, Emory University). Labeling of AVR is made possible with a polyclonal antibody against the last 12 amino acid residues of the extracellular COOH-terminal region of the rat endothelial protein PV-1 (chicken host, Radu V. Stanford, Dartmouth Medical School). The protein PV-1 allows us to identify AVR and capillaries because it is found in diaphragms of the fenestrae of AVR. Before the antibodies were applied, Spurr resin was etched, treated with 1% SDS, then treated for 10 minutes with a blocking solution consisting of 5% BSA, 1% normal donkey serum (Jackson ImmunoResearch), and 0.2% Triton X-100 diluted into PBS. Primary antibodies are first diluted into blocking solution before their application overnight at 4°C. FITC-, TRITC-, CY5-, and DAPI- conjugated donkey immunoglobulins (Invitrogen/Molecular Probes or Jackson ImmunoResearch), were diluted in PBS/Triton and were applied simultaneously for 60 minutes at room temperature. Finally, sections were mounted with Dako fluorescent mounting medium (Carpinteria, CA)

*Epifluorescence Microscopy*

Two sets of images (3070 x 4093 pixels in grayscale) were created. The first set of images was gathered using four wavelengths from each tissue section, revealing expression of AQP1, AQP2, CLC-K, and αB-crystallin. The second set of images was obtained using three wavelengths from each tissue sample. Images were captured using an Olympus IX70 epifluorescence microscope, a 10X objective, and a motorized stage
(Applied Precision). Each individual piece of tissue was photographed as multiple images that were stitched together as a montage to obtain a single unified image.

Three-dimensional functional reconstruction

Images obtained through epifluorescence microscopy were adjusted so that tissue images were a uniform size. Image adjustments were performed using Photoshop version 9. Images were enumerated sequentially and aligned to aid in mapping the CDs and vasculature from one section to the next. Alignments and subsequent three-dimensional reconstructions were produced using Amira visualization and volume modeling software version 2.3 (Mercury, Chelmsford, MA). Three CDs were identified and mapped from their positions in the IM, through the OM. Position in the outer medulla was verified by the presence of TALs. AVR abutting labeled collecting ducts were mapped through the IM/OM border for an axial distance of approximately 1 to 1.5mm. Three-dimensional functional reconstructions of labeled CDs and AVR were produced and analyzed.
Results

1. Determining the presence of AVR within the interbundle region of the OM.

To determine that AVR do in fact exist within the interbundle regions of the OM, photomicrographs taken through epifluorescence microscopy were analyzed. During the immunohistochemical preparation of the kidney tissue, FITC-, TRITC-, CY5-, and DAPI- conjugated donkey immunoglobulins were added. Each of these immunoglobulins absorbs a different wavelength of light, allowing us to visualize only those specific structures to which it is bound. For example, when the epifluorescent microscope is set to the wavelength associated with the DAPI- conjugated immunoglobulin, only CDs within the tissue sample appear in the photograph. Because of this, each tissue slice was photographed four separate times, each of which imaged a different wavelength. Upon completion of the imaging process, CDs and AVR were assigned different colors to facilitate in their identification. The color of each renal tubule was altered in Photoshop, labeling CDs-blue, and AVR-red. After locating a tissue slice located in the IM/OM region, the two corresponding images of CDs, and AVR were combined into one composite image. This produced an image (Figures 3B and 3C) which revealed the presence of red AVR amongst the visible TAL of the OM region. This image not only proved the presence of AVR within the interbundle regions of the OM, but also provided evidence for our hypothesis that as AVR ascend into the OM from the IM, they do not all coalesce to form dense vascular bundles as previously thought.
2. **Proving the presence of AVR in the interbundle regions of the OM through three-dimensional reconstructions.**

In order to provide additional evidence of AVR presence in outer medullary interbundle regions, three-dimensional reconstructions of individual AVR were made. Previous studies in our laboratory have revealed that AVR in the IM abut CDs in symmetrical patterns as the tubular structures move from deep within the IM towards the OM (Figure 4). Utilizing this knowledge, we attempted to follow three CDs and a select number of abutting AVR through 100 sequential tissue images from the IM to the OM. To ensure that the CDs being followed eventually passed through the interbundle regions of the OM, tissue images of the OM were first analyzed. Three CDs located in the interbundle region of the OM were identified and labeled. These same three CDs were then mapped from the OM to the IM through all 100 images. Once these CDs had been satisfactorily mapped, five randomly abutting AVR were chosen and traced through each sequential image. While two of the intracluster AVR were found to pass through the IM/OM border and join a vascular bundle, the three remaining AVR ascend though an interbundle region (Figure 7). While this in no way gives a full picture of how the IM/OM renal architecture is structured, it does cast further doubt on the previously believed model (Pallone).
Discussion

Taken together, image analysis and the corresponding three-dimensional reconstructions prove that not all AVR coalesce into dense bundles, although there is not yet a sufficient amount of collected data to truly gain a clear picture of what the structural architecture of the vessels between the IM/OM border looks like. It does however provide evidence for the lesser accepted model which illustrates a more spread out pattern of AVR as they pass through this region (Figure 6).

By showing that AVR do in fact run through both the bundle and the interbundle regions of the OM, we have seemed to raise more questions than provided answers. Under the more accepted model the bundle AVR would participate directly in countercurrent solute exchange only with the DVR and short DTLs that are located within the bundles. In addition, the AVR positioned in the vascular bundle periphery would also be able to interact with the TALs located within the interbundle regions. Furthermore, under the accepted model the interaction of AVR with interbundle nephrons and vessels, including long DTLs, CDs, and most TALs is presently not believed to occur since all OM AVR are believed to exist only in the bundle region. The present research suggests that since AVR are also located within the interbundle regions, these AVR would be able to interact with all the vessels and nephrons contained within the interbundle regions. What the data reveals is that as some AVR ascend directly through the interbundle regions they must come in contact with the nephrons and vessels contained within. Depending on the permeability of the AVR and other nephrons and vessels contained within the interbundle region, knowledge of this architectural relationship may provide greater insight into the concentrating mechanisms and solute
exchange of vessels in this region. For example, solutes such as urea and/or NaCl carried towards the OM by the AVR might pass into descending segments such as the long DTLs and CDs and consequently be returned to the IM. This countercurrent exchange would tend to minimize washout of these solutes from the medulla.

During the course of this research experiment, there were a number of factors in each step of the process which proved challenging to overcome. Many of these issues became evident during epifluorescent imaging. Some of these issues included air bubbles under the slide’s cover slip or poor antibody expression, with the result that many of the images had to be reimaged numerous times before a useable image was produced.

When tracing individual AVR and CDs through each sequential image, it also became evident that the actual quality of slicing of the kidney tissue played an enormous factor in the ability to efficiently map these structures. During the slicing process, many of the tissue samples were damaged and as a result, were missing vital sections of the sample.

A specific problem encountered when tracing AVR from the IM through the OM was determining which intracluster AVR would result as interbundle AVR, or vascular bundle AVR. As AVR ascended further into the OM, it seemed as though the frequency of branching out and coalescing which took place increased with closer proximity to the OM region. It is speculated that the size of an individual AVR in the IM may directly correlate with whether that AVR will become part of the interbundle or the vascular bundle as is ascends through the IM/OM stripe. However, there is still insufficient evidence to confidently make this correlation.

Looking back on the methods and procedures employed in this research, it is
evident that there are several alterations that can be made to make future reconstructions both more effective and efficient. Once epifluorescent imaging is completed and all resulting pictures have been cropped to the appropriate size, a determination should be made as to which area will be the focus of the reconstructions. Images displaying any issues in the area of interest should be re-imaged immediately. Since images produced are often riddled with blurry patches, re-imaging should focus on achieving clarity in the desired regions. Also, if an area of interest, or even specific CDs are chosen for reconstruction, image alignments should focus only on this region to facilitate the mapping process. The act of mapping individual tubules is an arduous one that requires a great deal of concentration and patience. I believe through this process, a great deal of insight into the inner structure and function of the renal vessels can be achieved.
References


FIGURES

**Figure 1.** This illustration displays labeled regions of a longitudinally sectioned human kidney.
Figure 2. This diagram illustrates the labeled components of the functional unit of the kidney, the nephron.
Figure 3.  (A). Image illustrates immunofluorescent tubules located in the IM/OM stripe. TALs (CIC-K2) on the left are representative of the OM region, while ATLs (CIC-K1) on the right depict IM region. (B). Image illustrating the types of vasculature present at the OM (left) and IM (right) border. Present structures include CD (AQP2/blue), AVR (PV-1/red), and DVR bundles (UTB/yellow). (C) and (D). Enlarged images of medullary regions in figure 3B. This image reveals AVR that are dispersed throughout the IM/OM stripe and have not coalesced into large vascular bundles.
Figure 4. This three-dimensional functional reconstruction illustrates the architectural relationship between CDs (blue), and abutting AVR (red) in the intra-cluster region of the IM.
**Figure 5.** Image of the microvasculature of *Psammomys obesus*, a desert rodent. This image illustrates intra-cluster AVR coalescing into dense vascular bundles as they pass through the IM/OM stripe and into the OM.
Figure 6. In contrast to the microvasculature of the desert rodent *Psammomys obesus*, this micrograph reveals the vasculature of a more common mammalian rat. As vasculature ascends towards the IM/OM stripe, they do not coalesce into dense vascular bundles. It is believed that this is the model shared by other mammals such as humans.
Figure 7. (left) Three-dimensional reconstruction of CDs (blue) and associated AVR as they pass from the IM through the OM. Two AVR (yellow) are found to enter the OM through the vascular bundle. The three remaining AVR (red) enter the OM through the interbundle. (right) Image depicting CDs and AVR traced.