Interstitial interfaces show marked differences in regenerating tubules, matured tubules, and the renal stem/progenitor cell niche

Will W. Minuth, Lucia Denk

Molecular and Cellular Anatomy, University of Regensburg, D-93053 Regensburg, Germany

Abstract: Stem/progenitor cells are promising candidates for the regeneration of parenchyma in acute and chronic renal failure. After an implantation stem/progenitor cells must migrate through the interstitial space to concentrate at the site of damage. However, information is lacking to what extent the interstitial interface is influencing the development of stem/progenitor cells into nephron structures. In consequence, tubule regeneration within an artificial polyester interstitium was analyzed by electron microscopy in comparison with the interstitial interface of matured tubules and the interstitium within the renal stem/progenitor cell niche. The experiments demonstrate that fixation of specimens with glutaraldehyde (GA) is leading in all cases to inconspicuously looking interstitial interfaces. In contrast, fixation of regenerating tubules in GA containing ruthenium red and tannic acid shows a dense network of fibers lining along the basal lamina. In contrast, matured tubules reveal after ruthenium red label an extremely thickened basal lamina, while only a punctate pattern is obtained after tannic acid treatment. Finally, within the renal stem/progenitor cell niche ruthenium red and tannic acid label reveals large amounts of extracellular matrix spanning through the interstitium. Thus, fixation of tissue in GA containing ruthenium red and tannic acid exhibits an unexpectedly regional heterogeneity of the renal interstitial interface. This fact has to be considered for an optimal therapeutic repair of parenchyma, since contacts between stem/progenitor cells with the interstitial interface influence further development. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 100A: 1115–1125, 2012.

Key Words: regeneration, extracellular matrix, interstitium, artificial interstitium, kidney, ruthenium red, tannic acid, electron microscopy

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INTRODUCTION

An increasing number of investigations in biomedical research is focusing on the application of stem/progenitor cells for the repair of parenchyma in acute and chronic renal failure.[1,2] From the theoretical sight of view, the capacity of stem/progenitor cells for self-renewal and their ability to differentiate into functional parenchyma are ideal presuppositions to promote a process of regeneration.[3,4] However, in practical terms it shows that the initiation of a regeneration process is much more complex than believed and is influenced by multiple and up to date unknown molecular signals.

A frequently applied method for implantation is the infusion of stem/progenitor cells via the blood vessel system.[5] Other strategies are casual injection of stem/progenitor cells into diseased parenchyma[6] and subcapsular injection.[7] A new subcapsular implantation technique with stem/progenitor cells embedded in a fleece reservoir is under present research.[8] However, poor cell survival and missing cell concentration at the site of regeneration are the disadvantages.[9]

After an implantation has been performed, stem/progenitor cells must find a path to migrate from the site of application through the interstitial space until areas of the diseased renal parenchyma are reached. Here they are confronted with a harmful environment causing inflammation and cell degradation.[10] This process has to be terminated and turned into a milieu supporting regeneration.[3,4]

The main duty of stem/progenitor cells within diseased renal parenchyma is the repair and *de novo* development of functional nephron structures. It has been shown that the induction of nephrons depends on numerous reciprocal molecular interactions of morphogenetic factors.[11] However, little knowledge is available about rise of nephron segments and spatial orientation of the nephron. Cell contacts with extracellular matrix proteins at the interstitial interface seem to play a key role in this process.[12,13]

For example, coating of cultured renal cells with extracellular matrix proteins has demonstrated that the interface between arising tubules and the interstitium plays an essential role during spatial development.[14,15] Culture of stem/progenitor cells in a polyester fleece simulating an artificial interstitium has further elucidated that numerous tubules can be generated within I-7, Posi-4, or Posi-5 fleeces.[16,17] Although the material is the same, the spatial

Correspondence to: W. W. Minuth; e-mail: will.minuth@vkl.uni-regensburg.de

growth pattern of generated tubules is different due to fiber arrangement in applied polyester fleeces. The presented results suggest that the spatial development of tubules is strongly influenced by the interstitial interface comprising the basal lamina of tubules, the surface structure and orientation of neighboring fleece fibers, and the space between the fibers including the circulating fluid.

The focus of actual research is directed to specific features promoting the regeneration of renal tubules in the interstitium. Hence, in the present experiments this particular zone of regenerating tubules within an artificial interstitium was analyzed by histochemical methods, scanning, and transmission electron microscopy (TEM). To learn about the natural situation the results were compared with the interstitial interface of matured tubules and the interstitium of the renal stem/progenitor cell niche. A special fixation protocol with glutaraldehyde (GA) containing ruthenium red and tannic acid made it possible to illustrate for the first time unique extracellular matrix on regenerating tubules, matured tubules, and within the interstitium of the renal stem/progenitor cell niche.

EXPERIMENTS
Isolation of renal stem/progenitor cells
Both kidneys of 1-day old anesthetized and sacrificed New Zealand rabbits were removed and cut into two parts as described earlier.[18] Stripping off the capsula fibrosa with fine forceps a thin layer of stem/progenitor cells is adherent to the explant. Applying this simple isolation method an embryonic tissue layer of up to 1 cm^2 in square can be harvested. Up to date no other species is known for the isolation of renal stem/progenitor cells in such an amount necessarily needed for subsequent cell biological analysis.

Perfusion culture
For present culture experiments, the isolated layer containing stem/progenitor cells was placed between two punched out pieces of polyester fleece (I7, Walraf, Grevenbroich, Germany) [Fig. 1(a)] as described earlier.[19] This arrangement resulted in a sandwich set-up measuring 5 mm in diameter. To prevent damage during culture, a base ring of a Minusheet® tissue carrier (Minucells and Minutissue, Bad Abbach, Germany) with 13 mm inner diameter was used [Fig. 1(b)]. First, a polyester fleece measuring 13 mm in diameter was placed inside the tissue carrier. Then the sandwich set-up containing renal stem/progenitor cells was inserted. Finally, another fleece with 13 mm in diameter was placed on top. The tissue carrier was then transferred to a perfusion culture container with horizontal flow characteristics (Minucells and Minutissue) [Fig. 1(c)]. By closing the lid of the perfusion container the sandwich set-up was fixed in an exact position during culture.

For maintaining a constant temperature of 37°C the perfusion culture container was placed on a thermoplate (Medax-Nagel, Kiel, Germany) [Fig. 1(d)]. For a period of 13 days always fresh medium was continuously transported at a rate of 1.25 mL/h with an IPC N8 peristaltic pump
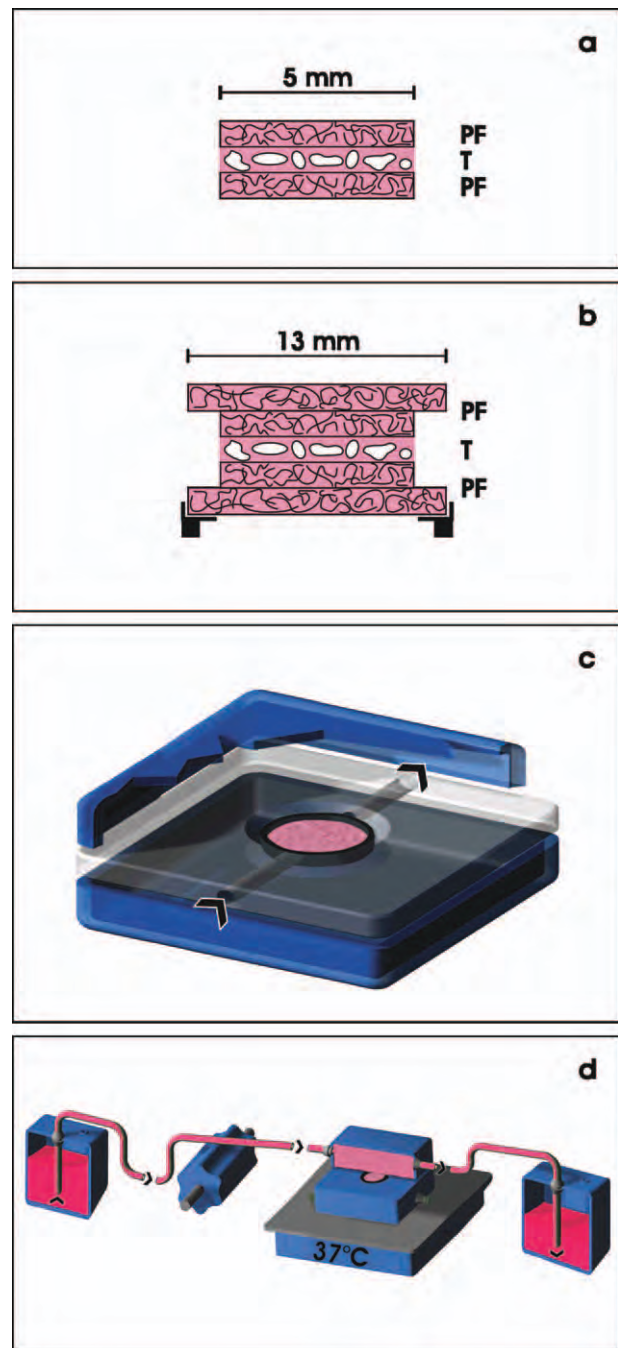

FIGURE 1. Generation of renal tubules (T) embedded in an artificial polyester interstitium for perfusion culture. (a) Stem/progenitor cells are isolated by stripping off the capsula fibrosa from neonatal rabbit kidney with adherent embryonic mesenchyme and collecting duct ampullae. To create an artificial interstitium, the tissue is placed between two layers of polyester fleece (PF). (b) For spatial orientation the tissue is covered by two further fleeces and placed in a tissue carrier. (c) Perfusion culture is performed in a container with horizontal flow characteristics (arrow heads). (d) While in perfusion culture, always fresh medium is transported (arrow head) from a storage to a waste bottle by a peristaltic pump. To maintain a constant temperature of 37°C, the culture container is placed on a thermoplate covered with a lid. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Ismatec, Wertheim, Germany). Applying this method medium is saturated up to 190 mmHg oxygen during transport. This content of oxygen is reached by a long thin-walled silicone tube, which was highly gas-permeable. It guaranteed optimal diffusion of respiratory gas between culture medium and surrounding atmosphere.

For the generation of tubules chemically defined IMDM (Iscove's Modified Dulbecco's Medium) including phenolred (GIBCO/Invitrogen, Karlsruhe, Germany) was used. Infections were prevented by adding an antibiotic–antimycotic cocktail (1%, GIBCO/Invitrogen). To induce tubulogenic development, aldosterone ($1 \times 10^{-7}M$, Fluka, Taufkirchen, Germany) was administered.

Histochemical labeling of cultures

After perfusion culture was terminated, the sandwich set-ups containing renal tissue within two layers of polyester fleece (5 mm diameter) were embedded in 1% agarose (Serva, Heidelberg, Germany), surrounded by TissueTek (O.C.T.™COMPOUND, Sakura Finetek, Zoeterwoude, the Netherlands) and frozen at $-80°$C. To analyze cell biological features, 20 μm thick cryosections were made and stained with toluidine blue.

Before lectin labeling, the specimens were fixed in ice-cold ethanol and washed several times with phosphate buffered saline (PBS). Then they were incubated for 30 min with blocking solution (PBS, pH 7.5, 10% horse serum, GIBCO, 1% bovine serum albumin, Serva). For soybean agglutinin labeling (SBA, Vector, Burlingame, USA) the samples were exposed to fluorescein-isothiocyanate (FITC)-conjugated lectin diluted 1:2000 in blocking solution for 45 min. Following several washes with PBS, the sections were embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, USA) and then analyzed using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany). Fluorescence images were taken with a digital camera at a standard exposure time of 1.3 s and thereafter processed with Corel DRAW Graphic Suite X5 (Corel Corporation, Otawa, Canada).

Scanning electron microscopy

For scanning electron microscopy (SEM), cultured specimens were fixed in 2% GA (Serva) buffered with PBS. After rinsing in PBS, dehydration was performed in a graded series of ethanols, then the specimens were transferred in acetone and critical point dried with CO_2 to sputter-coat them with gold (Polaron E 5100, Watford, GB). Examination was performed in a scanning electron microscope DSM 940 A (Zeiss, Oberkochen, Germany) as described earlier.[20] Images of the screen were taken by a Pentax SLR Digital camera and thereafter processed with Adobe Photoshop (Adobe) and Corel DRAW Graphic Suite X5 (Corel Corporation).

Transmission electron microscopy

Cultured specimens and the kidneys isolated from 1-day old New Zealand rabbits were transferred to immersion fixation for TEM. Previous investigations have demonstrated that classic histological procedures do not elucidate masked structures.[21] Thus, to illuminate hidden structures, in the present investigation, a protocol of fixation was applied, which was originally for the analysis of the adult mouse tectorial membrane matrix.[22] Without any modification the technique was applied. Following solutions were used:

Series 1: 5% GA (Serva) buffered with 0.15M sodium cacodylate, pH 7.4
Series 2: 5% GA with 0.5% ruthenium red (Fluka)
Series 3: 5% GA with 1% tannic acid (Sigma, Taufkirchen, Germany)

Fixation was performed for 1 day at room temperature. After several washes with 0.15M sodium cacodylate, the specimens were postfixed in the same buffer but containing 1% osmium tetroxide (Science Services, München, Germany). In the next step, the tissue was washed with sodium cacodylate buffer and dehydrated in graded series of ethanols. Finally, the specimens were embedded in Epon (Fluka), which was polymerized at $60°$C for 48 h.

Semithin and ultrathin sections were performed with a diamond knife on an ultramicrotome EM UC6 (Leica GmbH, Wetzlar, Germany). Sections were collected onto grids (200 mesh) and contrasted using 2% uranyl acetate and lead citrate as described earlier.[19] Sections were examined at 80 kV using an EM 902 transmission electron microscope (Zeiss). Electron micrographs were recorded digitally using a slow scan CCD camera and thereafter processed with Adobe Photoshop (Adobe, CA) and Corel DRAW Graphic Suite X5 (Corel Corporation).

Definition of cells within the renal stem/progenitor cell niche

In this article, the embryonic part of the growing kidney was described including the renal stem/progenitor cell niche. In consequence, the nomenclature of previously published articles was used.[23,24]

Amount of analyzed specimens

A total of 42 exactly orientated renal stem cell niches, 8 matured tubules, and 19 cultured specimens were analyzed for the present investigation.

Performed experiments are in accordance with the Animal Ethics Committee, University of Regensburg, Regensburg, Germany.

RESULTS

The interstitium is responsible to maintain essential physiological functions in the neighboring parenchymal cells. Since sound information about the regional heterogeneity of this import site is lacking, the aim of present experiments was to investigate the interstitial interface in the renal stem/progenitor cell niche, matured tubules, and regenerating tubules. It was considered that new information of the interstitial interface will help to improve the environment during therapeutic application of stem/progenitor cells.

Interstitial interface of regenerated tubules

In the first series of experiments, renal stem/progenitor cells were mounted in a polyester fleece so that spatial

generation of tubules can be investigated (Fig. 1). After a perfusion culture period of 13 days, the artificial interstitium was opened by tearing off the fleece layers to analyze the growth pattern of generated tubules (Fig. 2). The spatial area for tubule development between the fleeces was 5 mm in diameter and up to 250 μm in height. To recognize the extent of tubule development, cryosections were made and stained with toluidine blue [Fig. 2(a)]. It can be recognized that numerous tubules are growing between polyester fibers of the fleece.

For better analysis, whole mount label was performed by fluorescent SBA [Fig. 2(b–d)]. An individual example shows numerous tubules within a microscopic opening of 850 μm × 600 μm. Fluorescence microscopy of labeled specimens demonstrates that tubules were grown in a spatial arrangement but keeping distance to each other by an explicit interstitial space [Fig. 2(b)]. Part of tubules illustrated a straight forwarded growth [Fig. 2(c)], while others could be analyzed in a cross-section view [Fig. 2(d)]. Specimens further showed that generated tubules were exhibiting polarized cells, a visible lumen, and a basal lamina.

An artificial interstitium is replacing coating by extracellular matrix proteins. Since the surface of generated tubules was not sticked by proteins derived from a coating process, it was possible to analyze the basal aspect of generated tubules by SEM [Fig. 2(e)]. The overall view demonstrates that generated tubules have only a loose contact to the fibers of the polyester fleece. In part, tubules developed in a parallel fashion, in other cases curling or dichotomous branching is observed. All of the tubules are covered by an intact basal lamina. On the outer surface of the basal lamina, interstitial cells and bundles consisting of newly synthesized collagen and other extracellular matrix proteins are recognized. Also in this experiment it can be recognized that generated tubules are separated by a blunt interstitial space.

For detailed analysis of the interstitial interface TEM was performed (Fig. 3). A surface view after GA fixation depicts that generated tubules are standing in a more or less close neighborhood to each other and the surrounding polyester fibers of the fleece [Fig. 6(a)]. An explicit interstitial space is present. In the surrounding of tubules, synthesized extracellular matrix fibers and single interstitial cells can be found. The generated tubules contain a polarized epithelium. The apical plasma membrane of the isoprismatic cells borders a lumen. The cells contain in the centre a round nucleus. In the cytoplasm numerous lysosomes are found. Neighboring epithelial cells were connected by a tight junctional belt consisting of a typical zonula occludens, zonula adhaerens, and a desmosome. The basal lamina was consistently developed and exhibited a lamina rara, lamina densa, and lamina fibroreticularis as previously described.[19]

To obtain more detailed information exclusively, the interstitial interface of regenerating tubules was analyzed [Fig. 3(a)]. A cross-sectioned view in higher magnification demonstrates after GA fixation an inconspicuous basal aspect of the tubules [Fig. 3(b)]. The basal plasma membrane is in contact with a basal lamina consisting of a clear lamina rara, a lamina densa, and single strands of the lamina
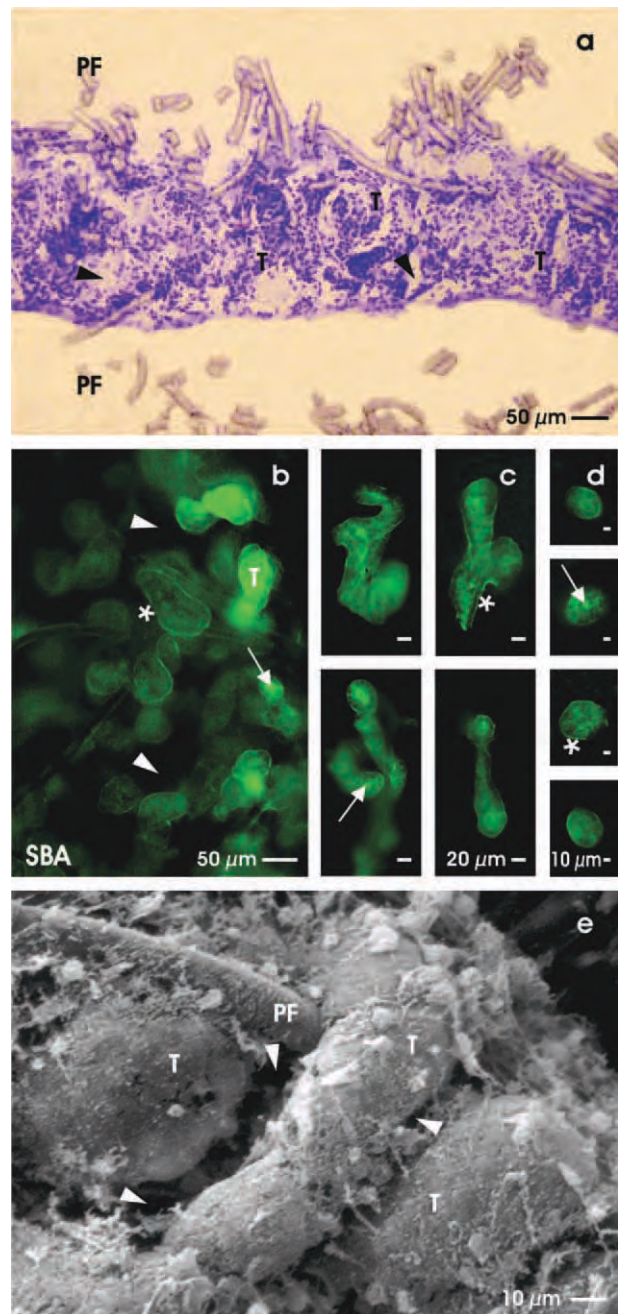

FIGURE 2. Analysis of renal tubules generated for 13 days in perfusion culture within an artificial interstitium. (a) A Toluidine blue stained cryosection shows numerous tubules (T) covered at the upper and lower side by I-7 fleece layers consisting of polyester fibers (PF). The tubules do not cluster but are separated by an interstitial space (arrow head). (b) SBA label of whole mount specimens depicts that tubules contain a lumen (arrow) and a basal lamina (asterisk). It is obvious that they are separated by a wide interstitial space (arrow head). (c) SBA label illuminates tubules in longitudinal course. (d) Cross view clearly exhibits that generated tubules contain a lumen and a basal lamina. (e) Scanning electon microscopy shows generated tubules (T) between polyester fibers (PF). Between tubules an interstitial space with interstitial cells and extracellular matrix fibers can be recognized (arrow head). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

fibroreticularis. In contrast, in specimens fixed with GA containing ruthenium red, a completely different view is obtained [Fig. 3(c)]. The lamina rara is barely visible exhibiting not a continuous but a punctual pattern. The lamina

FIGURE 3.

densa is seen as a thin line. Most impressive is the lamina fibroreticularis exhibiting a net of extremely long bundles of extracellular matrix spanning through the interstitial space. In contrast, fixation of generated tubules with GA containing tannic acid reveals that a lamina rara is barely visible, while the lamina densa is strongly labelled [Fig. 3(d)]. Striking features become visible in the lamina fibroreticularis. Numerous fibers protrude toward the interstitial space. They are orientated like numerous teeth in a fine-thoothed comb and exhibit a length of about 200 nm.

Interstitial interface of matured collecting duct tubules

The previous series of experiments has shown that fixation of regenerated tubules in GA shows a quite different pattern in the lamina fibroreticularis [Fig. 3(b)] than fixation in GA containing ruthenium red [Fig. 3(c)] or GA containing tannic acid [Fig. 3(d)]. It appears likely that such an arrangement is not accidently but seems to be caused by masked extracellular matrix as it was described for connective tissue and skin.[20] Furthermore, the peculiarities of the extracellular matrix might also be caused by the culture conditions. In consequence, for control and to obtain more information about structural features of the interstitium in adult renal parenchyma, exactly orientated vertical sections were made from matured tubules at the shaft of the collecting duct ampulla [Fig. 4(a)]. It can be recognized that all of the tubules are surrounded by a bright interstitium [Fig. 6(b)]. To obtain new information, the specimens were analyzed by TEM after fixation in GA [Fig. 4(b)] in comparison with GA containing ruthenium red [Fig. 4(c)] and GA containing tannic acid [Fig. 4(d)].

Fixation of tissue in GA revealed that the basal plasma membrane of tubules is consistently in contact with a basal lamina consisting of a lamina rara, a lamina densa, and only single but tiny strands of a lamina fibroreticularis protruding toward the interstitial space [Fig. 4(b)]. However, when specimens are fixed in GA containing ruthenium red a different view is obtained [Fig. 4(c)]. A continuously developed
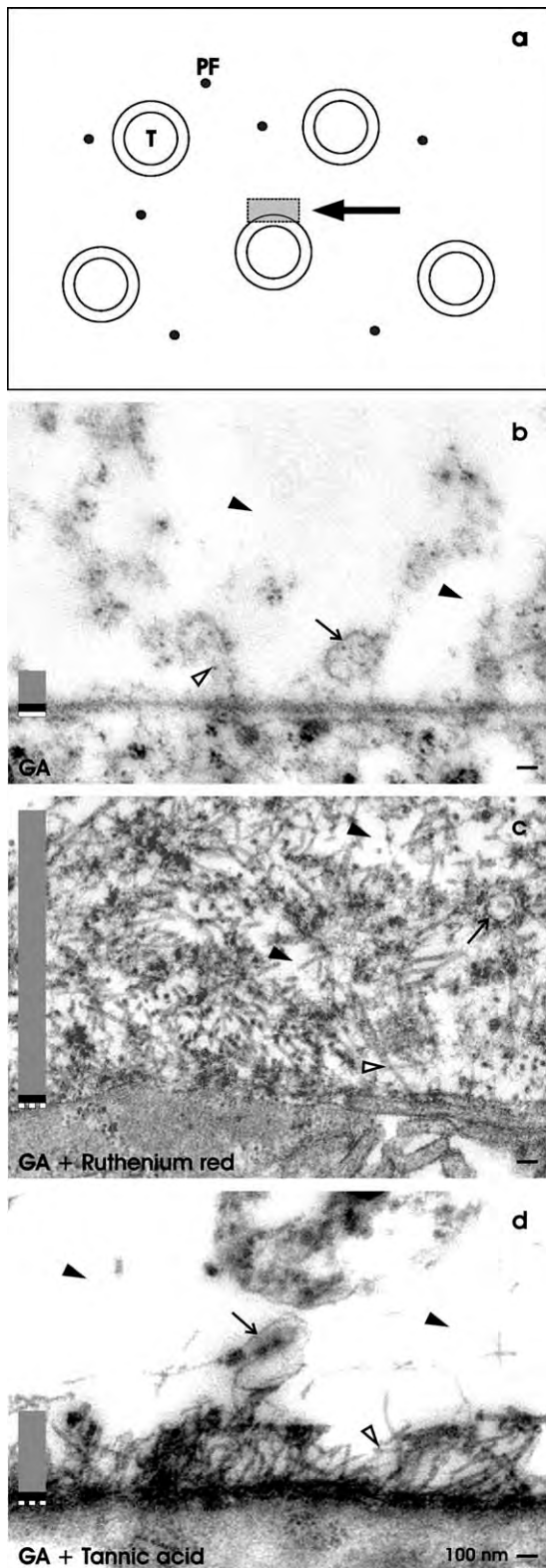
FIGURE 3. TEM of renal tubules generated for 13 days in perfusion culture after fixation in GA, GA containing ruthenium red, and GA containing tannic acid. (a) Schematic illustration demonstrates the area of section (thick arrow) at the basal aspect of generated tubules (T) embedded within polyester fibers (PF). (b) After GA fixation, a continuously developed basal lamina can be observed. As indicated by the bar (left) it consists of a continuous lamina rara, a clearly visible lamina densa, and a less extended lamina fibroreticularis. Single fibers (lighted arrow head) protrude to the bright and widely extended interstitial space (arrow head). Further single protrusions (thin arrow) from interstitial cells can be recognized. (c) In contrast, after fixation in GA containing ruthenium red a discontinuously developed lamina rara and a continuous lamina densa can be observed. Most conspicuous is the detection of numerous fibers (lighted arrow head) protruding from the lamina fibroreticularis toward the interstitial space (arrow head). (d) After fixation in GA containing tannic acid, a discontinuously developed lamina rara and a continuous lamina densa can be seen. Very impressive is the less extended lamina fibroreticularis. It consists of numerous comb-like fibers (lighted arrow head) protruding toward the bright and widely extended interstitial space (arrow head). Thin arrow indicates protrusions of interstitial/mesenchymal cells. Bar left side: white, lamina rara; black, lamina densa; gray, lamina fibroreticularis.

lamina rara is not anymore to recognize. Instead the lamina densa appears as a broad band with a thickness of 100 nm. In comparison to GA fixation [Fig. 4(b)], the lamina fibroreticularis appears after ruthenium red contrasting more
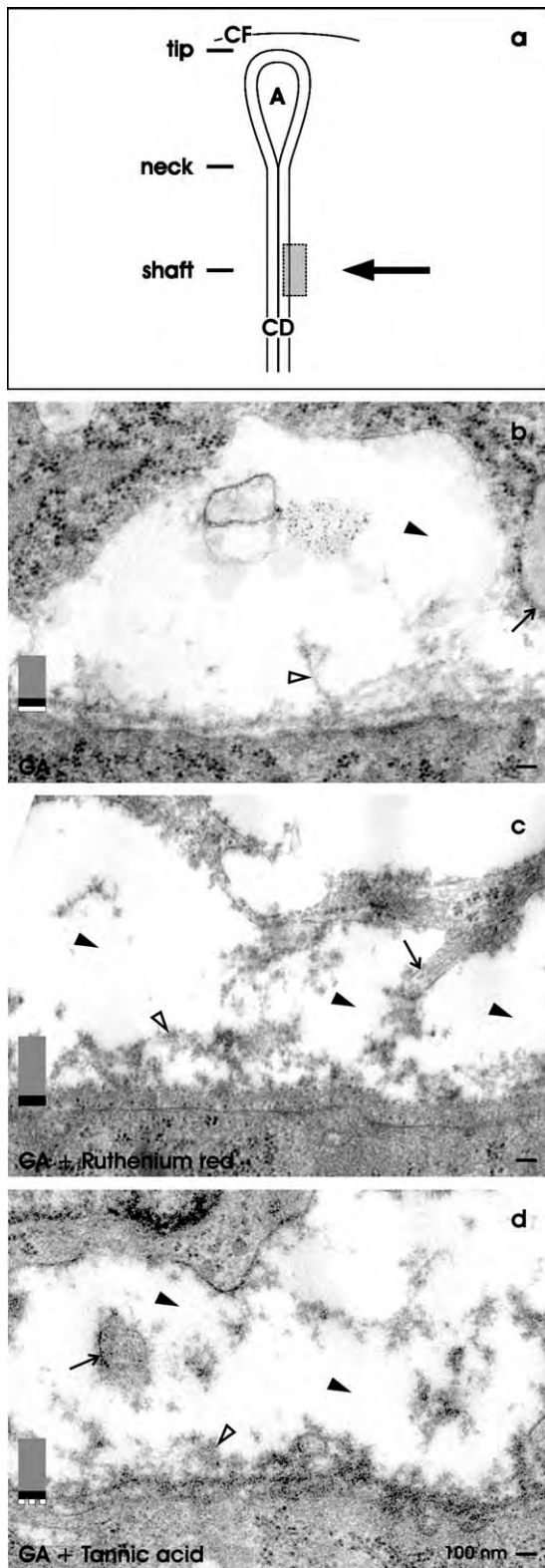
intensively labeled [Fig. 4(c)]. The fibers appear to be strongly thickened in diameter. Specimens fixed in GA containing tannic acid show a recognizable but punctuate lamina rara [Fig. 4(d)]. The lamina densa is clearly visible, but appears to exhibit a fine grained pattern. It is visible that the granular pattern is not restricted to the lamina densa, but protrudes casually to fibers of the lamina fibroreticularis.

In conclusion, fixation of matured tubules in GA [Fig. 4(b)], GA containing ruthenium red [Fig. 4(c)], and GA containing tannic acid [Fig. 4(d)] reveals differences in the labeling pattern of the basal lamina. In all of the cases a bright interstitial space is visible. Fibers of extracellular matrix are crossing occasionally through the interstitial space.

Interstitial interface within the renal stem/progenitor cell niche

In the following set of experiments, the interstitium within the renal stem/progenitor cell niche was analyzed (Fig. 5). To obtain comparable vertical views to the niche, a tissue block was always orientated along its exact cortico-medullary axis and in parallel to the lumen of lining collecting ducts (CD) [Fig. 5(a)]. Analysis was made exclusively at the top of the collecting duct ampulla including epithelial stem/progenitor cells derived from the ureter bud. At this specific site, the surrounding mesenchymal nephrogenic stem/progenitor cells are separated by a wide interstitial space [Fig. 6(c)].

Fixation of specimens in GA further illustrates that a consistently developed basal lamina covers epithelial stem/progenitor cells within the tip of the CD ampulla [Fig. 5(b)]. At this particular site, a complete basal lamina is present. The lamina rara and the lamina densa are consistently developed. Most interestingly, a broad and intensively labeled lamina fibroreticularis is developed. Their fibers exhibit more than 300 nm in length and protrude toward the interstitial space. It can be recognized that single protrusions from nephrogenic mesenchymal stem/progenitor cells are crossing the interstitial space to contact the lamina fibroreticularis at the basal lamina of the CD ampulla (Fig. 5).

FIGURE 4.

FIGURE 4. TEM of renal collecting duct (CD) tubules after fixation in GA, GA containing ruthenium red, and GA containing tannic acid. (a) Schematic illustration demonstrates the area of section (thick arrow) at the basal aspect of a matured CD tubule. (b) After GA fixation, a continuously developed basal lamina (asterisk) can be observed. As indicated by the bar (left) it consists of a continuous lamina rara, a clearly visible lamina densa and a less extended lamina fibroreticularis. Single fibers (lighted arrow head) protrude to the bright and widely extended interstitial space (arrow head). Further single protrusions (thin arrow) from interstitial cells can be recognized. (c) In contrast, after fixation in GA containing ruthenium red a missing lamina rara but an intense lamina densa can be observed. Some fibers (lighted arrow head) are protruding from the lamina fibroreticularis toward the interstitial space (arrow head). (d) After fixation in GA containing tannic acid a discontinuously developed lamina rara but a continuous lamina densa can be seen. Less impressive is the lamina fibroreticularis. It consists only of few and short fibers (lighted arrow head) protruding toward the bright and widely extended interstitial space (arrow head). Thin arrow indicates protrusions of interstitial/mesenchymal cells. Bar left side: white, lamina rara; black, lamina densa; gray, lamina fibroreticularis.

When the specimens are fixed in GA containing ruthenium red, a complete different view is obtained [Fig. 5(c)]. The lamina rara within the basal lamina at the tip of the collecting duct ampulla is not visible. Instead a broad band
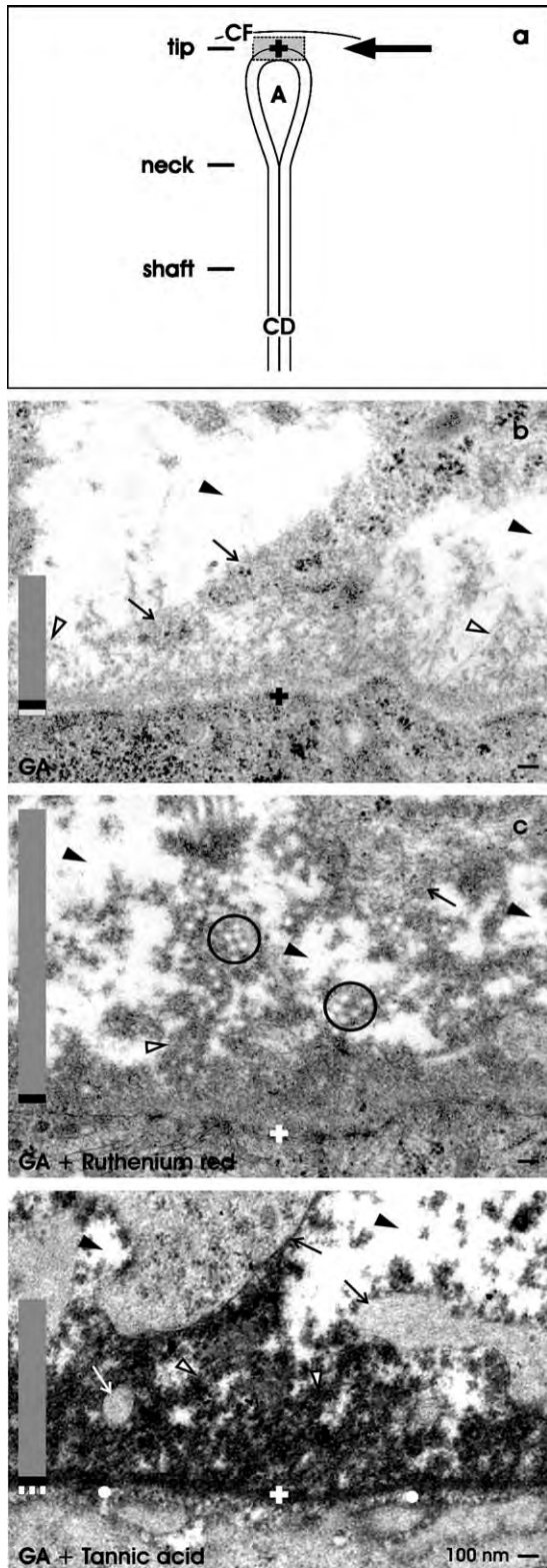

FIGURE 5.

at the site of the lamina densa is delimiting epithelial stem/progenitor cells within the CD ampulla. Long, strong, and intensely labeled fibers are lining from the lamina fibroreticularis toward the interstitial space contacting from time to time protrusions from mesenchymal stem/progenitor cells. In the interior of the ruthenium red material numerous microfibers are contained. These embrassed microfibers are lacking label with ruthenium red. Since these microfibers do not exhibit a repeating period in the electron microscope, they cannot be ascribed to a certain type of collagen. The complementary interstitial space occuring between material labeled by ruthenium red appears clear.

In the last series, fixation of the renal stem/progenitor cell niche was performed by GA containing tannic acid [Fig. 5(d)]. Most interestingly, the complete basal lamina of epithelial stem/progenitor cells is covered by an electron-dense coat as detected after fixation with GA containing ruthenium red [Fig. 5(c)]. It is apparent that the intensively stained pattern lines discontinuously from the basal lamina of the CD ampulla through the interstitial space toward cell protrusions and the surface of neighboring mesenchymal stem/progenitor cells.

The illustration further depicts that the tannic acid label at the basal lamina is intensive. Only a punctualy developed lamina rara becomes visible [Fig. 5(d)]. The lamina densa comprising the lamina fibroreticularis appear as a broad and intensively labeled band. Most impressive is that tannic acid labels to a high degree roughly structured bundles of extracellular matrix within the interstitial space. In addition, numerous protrusions and the cell surface of neighboring mesenchymal stem/progenitor cells are labeled. Thus, fixation with GA containing tannic acid exhibits that to an unexpected degree large amount of extracellular matrix is contained within the interstitium of the renal stem/progenitor cell niche. It is obvious that not the complete interstitial space but only part of it is labeled by tannic acid. This

FIGURE 5. TEM of the renal stem/progenitor cell niche after fixation in GA, GA containing ruthenium red, and GA containing tannic acid. (a) Accurate orientation of tissue for sectioning (thick arrow) of the renal stem/progenitor cell niche beyond the organ capsule (CF) in parallel to the lumen of a lining collecting duct (CD). For clear identification, the basal lamina at the tip of a CD ampulla (A) is marked by a cross (+). (b) After GA fixation, a continuously developed basal lamina can be observed. As indicated by the bar (left) it consists of a continuous lamina rara, a clearly visible and thickened lamina densa, and an extended lamina fibroreticularis. Single fibers (lighted arrow head) protrude to the bright and widely extended interstitial space (arrow head). (c) In contrast, after fixation in GA containing ruthenium red a lamina rara cannot been detected but an intense lamina densa is observed. Numerous and extended fibers (lighted arrow head) are protruding from the lamina fibroreticularis toward the interstitial space (arrow head). The ruthenium red is covering bundles of unstained but bright fibers (circle). (d) After fixation in GA containing tannic acid a discontinously developed lamina rara but an intense lamina densa can be seen. Impressive is the lamina fibroreticularis. It consists of densely packed and irregular extracellular matrix (lighted arrow head) protruding toward the interstitial space (arrow head). Thin arrow indicates protrusions of interstitial/mesenchymal cells. Bar left side: white, lamina rara; black, lamina densa; gray, lamina fibroreticularis.
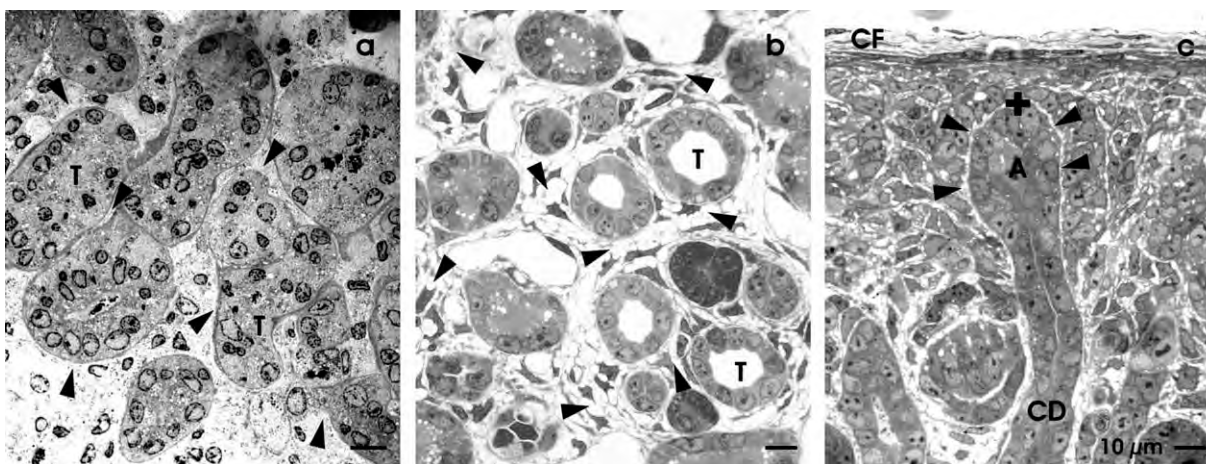
FIGURE 6. Microscopic surface view of the interstitial interface between generated tubules, matured tubules, and within the renal stem/progenitor cell niche after fixation in GA. (a) Tubules (T) generated for 13 days within a polyester fleece do not cluster, but develop a clearly visible interstitial interface (arrow head). (b) In the matured kidney tubules (T) are separated by a wide interstitial space (arrow head). (c) A section through of the embryonic cortex of neonatal kidney underneath the organ capsule (CF) depicts the tip (+) of a CD ampulla (A) containing epithelial stem/progenitor cells. A thin layer of mesenchymal stem/progenitor cells is detected between the CD ampulla and the organ capsule. Mesenchymal stem/progenitor cells are separated from epithelial stem/progenitor cells within the CD ampulla by an astonishingly wide interstitial space (arrow head). In all of the cases the interstitial space appears bright and looks inconspicuously after fixation in GA.

important result speaks in favour for a tannic acid-specific label and not for a unspecific background signal.

DISCUSSION

Although the interstitium looks inconspicuously from the morphological point of view, it is of great physiological and pathophysiological relevance for the kidney (Fig. 6).[25,26]

Multifaceted functions of the renal interstitium

The interstitium can be divided into two different parts. Its structural elements consist of mainly collagen type III fibers maintaining the outer form and the spatial orientation of nephron structures in the interior of the organ.[27] Within the complementary space interstitial fluid is crossing between collagen fibers, tubules, and blood vessels to provide the parenchyma with nutrition, respiratory gas, hormones, and morphogenetic factors.

By light microscopy, the interstitium can be recognized as the small distance between the basal lamina of tubules and the outer surface of blood vessels (Fig. 6).[28,29] Two types of cells are contained. Fibroblasts form the skeletal elements, while dendritic cells belong to the mononuclear system and fulfill sentinel functions. Information about the origin of renal fibroblasts is barely available. It is assumed that they develop from mesenchymal cells within the cap condensate that fails to be converted to nephron epithelia.[30–32] During organ growth, abundant interstitial cells are connected via cellular processes to form a widely communicating network.

During development, the interstitium within the stem/progenitor cell niche is of special importance. It is the site, where epithelial stem/progenitor cells within the tip of the CD ampulla are surrounded by the cap condensate containing mesenchymal nephrogenic stem/progenitor cells.[33] As long as the nephron anlagen are developing, a specific microenvironment within the interstitium promotes stem/progenitor cells to remain in a competent state. In turn, numerous reciprocal interactions such as cellular communication, exchange of morphogenetic information, and spatial orientation between epithelial and surrounding mesenchymal stem/progenitor cells are leading to induction so that exactly at this site the next generation of nephrons is formed.[34]

In the diseased kidney, numerous inflammatory cells infiltrate the interstitium. The resulting increase of interstitial cells and an enhanced synthesis of extracellular matrix cause obstructive nephropathy and fibrosis.[35] During this process, tubule cells loose epithelial features and convert to fibroblast-like cells.[36] Although under actual debate,[37] the epithelial–mesenchymal transition (EMT) is paralleled by the expression of fibroblast-specific protein-1(FSB1),[38] heat shock proteins, and α-smooth muscle actin.[39–41]

When stem/progenitor cells are implanted into a diseased kidney, they have to concentrate at sites with damaged parenchyma.[9] Independently from the kind of surgical application, stem/progenitor cells will migrate through the interstitial space until they find diseased parenchyma so that they can start with regeneration. In contrast, during the development of the kidney stem/progenitor cells do not migrate but stay for the period of development within the stem/progenitor cell niche located constantly underneath the organ capsule. It indicates that the interstitial environment is keeping stem/progenitor cells during the developmental phase in position. This continuous homing makes it possible that only at this specific site new nephrons are successively formed.

Thus, numerous articles demonstrate that the renal interstitium fulfills multiple functional roles. However, performing light and electron microscopy, the interstitium of a healthy kidney appears bright and does not reflect these
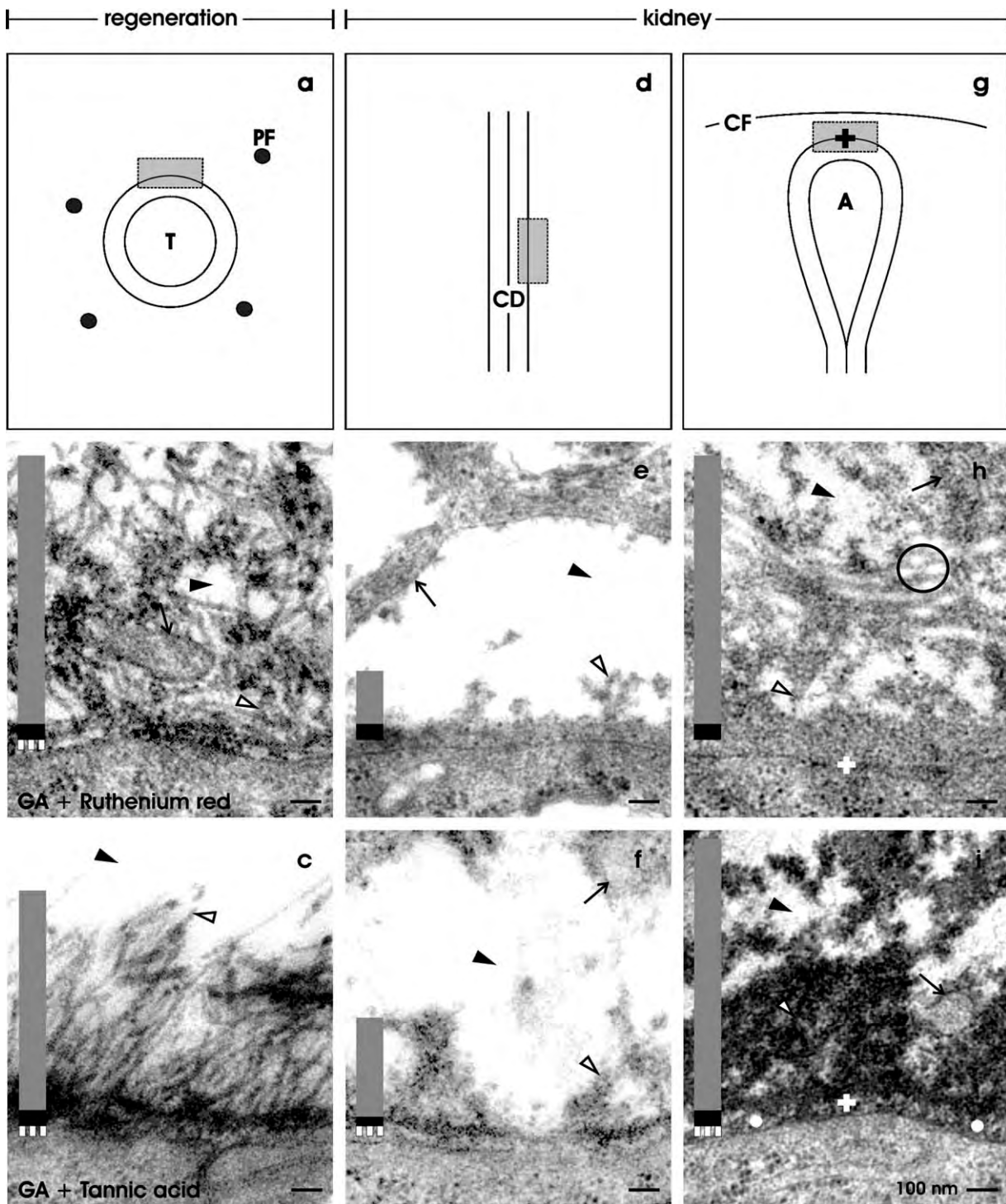
FIGURE 7. TEM of renal parenchyma illuminates microheterogeneity of the interstitial interfaces after fixation in GA containing ruthenium red and tannic acid. (a) Area of section at the basal aspect of generated tubules (T) growing within polyester fibers (PF). (b) Fixation with GA containing ruthenium red depicts an extended network of fibers (lighted arrow head) between the lamina fibroreticularis and the interstitial space (arrow head). (c) In contrast, fixation of specimens in GA containing tannic acid shows a comb-like lamina fibroreticularis (lighted arrow head), while the interstitial space is free of fibers (arrow head). (d) Area of section at the basal aspect of a matured collecting duct (CD) tubule. (e) Fixation of parenchyma with GA containing ruthenium red illustrates an extended basal lamina with few fibers (lighted arrow head), while the interstitial space is bright (arrow head). (f) Fixation in GA containing tannic acid demonstrates few fibers (lighted arrow head) in a wide and bright interstitial space (arrow head). (g) Area of section within the renal stem/progenitor cell niche. (h) Fixation in GA containing ruthenium red demonstrates an extended basal lamina at the tip ($+$) of the CD ampulla (A). The interstitial space (arrow head) is filled with a large amount of ruthenium red labeled fibers (lighted arrow head). Ruthenium red is covering bundles of unlabeled fibers (circle). (i) In contrast, fixation of GA containing tannic acid depicts bundles of extracellular matrix (lighted arrow head) spanning through the interstitial space. (arrow head). Arrow is indicating protrusions of interstitial/mesenchymal cells. Bar left side: white, lamina rara; black, lamina densa, gray, lamina fibroreticularis.

different tasks [Figs. 4(b) and 6(b)]. This obvious discrepancy was the reason to reinvestigate by electron microscopic techniques the interstitial interface of generated tubules, matured tubules, and the renal stem/progenitor cell niche. The presented results show for the first time that the interface between the renal parenchyma and the interstitium is regionally different and heterogeneously composed. This important finding will be of outmost relevance for the promotion of nephron regeneration in a diseased kidney, since adapted biomaterials can be co-implanted with stem/progenitor cells to support regeneration.

Masked interstitial interfaces

When regenerating tubules were fixed in a classical solution containing GA, it can be recognized under low microscopic magnification that no clustering occurs but individual distances between tubules in the form of an interstitium are developing [Fig. 6(a)]. Regarding a section of the matured kidney after fixation in GA, numerous tubules with interspersed vessels and interstitial cells can be seen [Fig. 6(b)]. The interstitial space is recognized as a bright and widely dispersed network separating the tubules and blood vessels from each other. A similar situation is given within the renal stem/progenitor cell niche [Fig. 6(c)]. Although an intense reciprocal molecular interaction of morphogenetic information for nephron induction occurs at this site, both stem/progenitor cell populations are separated by an astonishingly wide but inconspicuously looking interstitial space.

Morphological microheterogeneity of the interstitium

Thus, classical fixation in GA does not deliver new information. In consequence, alternative fixation with GA containing ruthenium red [Fig. 7(b,e,h)] and GA containing tannic acid [Fig. 7(c,f,j)] was applied. For the first time it is demonstrated that fixation with GA containing ruthenium red [Figs. 3(c), 4(c), 5(c), and 7(b,e,h)] and GA containing tannic acid [Figs. 3(d), 4(d), 5(d), and 7(c,f,j)] illuminates structured extracellular matrix at the interface between tubules and the interstitium, which was not visible in specimens fixed by GA [Figs. 3(b), 4(b), and 5(b)]. Most impressive is that the label of ruthenium red and tannic acid treated specimens is not identical.

In regenerating tubules, GA containing ruthenium red shows striking features in form of numerous fibers spanning in a three-dimensional manner along an extended lamina fibroreticularis [Figs. 3(c) and 7(b)]. In contrast, fixation in GA containing tannic acid exhibits more or less parallel lining fibers extending from the lamina fibroreticularis toward the interstitial space [Figs. 3(d) and 7(c)].

Matured renal collecting duct tubules depict after fixation in GA containing ruthenium red a thickened lamina densa, but only a barely visible lamina fibroreticularis [Figs. 4(c) and 7(e)]. In contrast, a thin but punctuate labeled lamina densa is found in specimens treated with GA containing tannic acid [Figs. 4(d) and 7(f)]. It is obvious that in both cases the interstitial space is free of extracellular matrix fibers.

As described earlier, epithelial stem/progenitor cells are covered by a special basal lamina.[23] It consists of a thickened lamina densa and an intense lamina fibroreticularis contacting protrusions of mesenchymal stem/progenitor cells [Fig. 5(b)]. On fixation in GA within the interstitial space, broader extracellular matrix is not visible. However, fixation of specimens with GA containing ruthenium red illuminates that numerous and amorphous extracellular matrix is contained within the interstitial space [Figs. 5(c) and 7(h)]. In addition, numerous bundles of unstained fibers mantled by ruthenium red become visible. Very exclusive is the label in samples fixed with GA containing tannic acid [Figs. 5(d) and 7(j)]. The thickness of the lamina densa at the tip of the collecting duct ampulla is increased [Fig. 7(j)] as compared to matured tubules [Fig. 7(f)]. Unexpected amounts of intensively labeled extracellular matrix are found within the interstitial space at this site. By the first view labeled material appears amorphous but in higher magnification punctuate and structured patterns can be observed [Fig. 7(j)].

It is obvious that the morphological view to the detected interstitial extracellular matrix de-masked by GA containing ruthenium red and tannic acid does not inform about its molecular composition. For that reason immunohistochemical experiments are in progress to identify on the electron microscopic level the contained molecules.

CONCLUSIONS

The classical fixation of renal parenchyma in GA results in an inconspicuously morphological view to the interstitium free of fibers and amorphous extracellular matrix. In contrast, application of GA containing ruthenium red and tannic acid reveals that numerous fibers and big amounts of amorphous extracellular matrix become visible after unmasking. For example, applying fixation with GA containing ruthenium red or tannic acid for the first time illustrates that the interstitial interface of regenerated tubules, matured tubules, and the renal stem/progenitor cell niche is heterogeneously composed. This important result opens a new possibility to optimize the molecular composition of the interface between regenerating cells and new biomaterials.

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