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Normal Tubular Regeneration and Differentiation of the Post-Ischemic Kidney in Mice Lacking Vimentin

Fabiola Terzi,* Roger Maunoury,* Emma Colucci-Guyon,† Charles Babinet,† Pierre Federici,* Pascale Briand,* and Gérard Friedlander‡

From INSERM U 380, Institut Cochin de Génétique Moléculaire, URA 1960 CNRS,† Biologie du Developpement, Institut Pasteur, and INSERM U 426 and Department de Physiologie,‡ Faculté X. Bichat, Paris, France*

Proliferation and dedifferentiation of tubular cells are the hallmark of early regeneration after renal ischemic injury. Vimentin, a class III intermediate filament expressed only in mesenchymal cells of mature mammals, was shown to be transiently expressed in post-ischemic renal tubular epithelial cells. Vimentin re-expression was interpreted as a marker of cellular dedifferentiation, but its role in tubular regeneration after renal ischemia has also been hypothesized. This role was evaluated in mice bearing a null mutation of the vimentin gene. Expression of vimentin, proliferating cell nuclear antigen (a marker of cellular proliferation), and villin (a marker of differentiated brush-border membranes) was studied in wild-type ($Vim^{+/+}$), heterozygous ($Vim^{+/-}$), and homozygous ($Vim^{-/-}$) mice subjected to transient ischemia of the left kidney. As expected, vimentin was detected by immunohistochemistry at the basal pole of proximal tubular cells from post-ischemic kidney in $Vim^{+/+}$ and $Vim^{+/-}$ mice from day 2 to day 28. The expression of the reporter gene β-galactosidase in $Vim^{+/-}$ and $Vim^{-/-}$ mice confirmed the tubular origin of vimentin. No compensatory expression of keratin could be demonstrated in $Vim^{-/-}$ mice. The intensity of proliferating cell nuclear antigen labeling and the pattern of villin expression were comparable in $Vim^{-/-}$, $Vim^{+/-}$ and $Vim^{+/+}$ mice at any time of the study. After 60 days, the structure of post-ischemic kidneys in $Vim^{-/-}$ mice was indistinguishable from that of normal non-operated kidneys in $Vim^{+/+}$ mice. In conclusion, 1) the pattern of post-ischemic proximal tubular cell proliferation, differentiation, and tubular organization was not impaired in mice lacking vimentin and 2) these results suggest that the transient tubular expression of vimentin is not instrumental in tubular regeneration after renal ischemic injury. (Am J Pathol 1997, 150:1361–1371)

Vimentin is an intermediate filament of class III that is expressed, in the adult and under normal conditions, mainly in cells of mesenchymal origin. In the mature kidney, vimentin is detected in glomeruli, vessels, and interstitial cells but not in tubular epithelial cells.[1,2] However, vimentin expression has been observed in tubular epithelial cells under three different conditions: 1) in the early stage of renal development,[2] 2) in renal cell carcinomas that are derived from the cortical tubules,[3–6] and 3) in the regenerating phase after ischemic or toxic renal injury characterized by tubular necrosis.[7–12] In the post-ischemic kidney, vimentin expression was recently reported to be restricted to the late proximal tubule and to disappear by 2 weeks after ischemia.[10] In all three conditions mentioned above, in which cellular proliferation is higher than in the normal mature kidney, the transient vimentin expression in tubular cells was interpreted as a marker of cell dedifferentiation.

Like other intermediate filament proteins of the cytoskeleton, vimentin has been suspected to be involved in transcription,[13] in mRNA tracking,[14] and in the cell cycle.[14,15] Indeed, vimentin is considered

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Address reprint requests to Dr. Fabiola Terzi, INSERM U 380, Institut Cochin de Génétique Moléculaire, 22, Rue Mechain, 75014 Paris, France.

as an intermediate-early gene, as its expression is increased in G0/G1 transition.[16]

Despite the numerous studies reporting coincidence between tubular vimentin expression and tubular regeneration, the question still remains of whether vimentin is instrumental in cell proliferation or is only a marker of cellular dedifferentiation. To address this question, we studied the impact of vimentin inactivation on the early and late events after renal ischemic injury. For this purpose, we used mice bearing a null mutation in the vimentin gene. This strain was recently established using homologous recombination by Colucci-Guyon et al,[17] and mice appear to be normal and are fertile.

We show that cellular proliferation, cellular differentiation, and structural organization of the renal parenchyma after renal ischemia were not impaired in homozygous mutant animals as compared with wild-type mice. These results rule out a predominant role for vimentin in tubular regeneration in the post-ischemic kidney.

Materials and Methods

Animals

Mice Lacking Vimentin

The details of targeted inactivation of the vimentin gene in mice have been reported previously.[17] We used the *Vim*^{1} mice, in which the endogenous vimentin gene has been disrupted by an in-frame insertion of *Escherichia coli* β-galactosidase coding sequences into exon 1 of vimentin gene. Thus, X-gal staining, which reveals β-galactosidase activity (see below), could permit analysis of the pattern of renal vimentin expression during the post-ischemic regeneration phase.

To identify wild-type (*Vim*$^{+/+}$), heterozygous (*Vim*$^{+/-}$), and homozygous (*Vim*$^{-/-}$) mice of different crosses, DNA from mouse tail was extracted, and the presence of targeted vimentin alleles was detected using the polymerase chain reaction (PCR) method. A mixture of three oligonucleotide PCR primers was used; primer 1 (5′-TGTCCTCGTCCTCCTACCGC-3′) and primer 2 (5′-AGCTGCTCGAGCTCAGCCAGC-3′) are located upstream and downstream, respectively, of the *Nar*I site in exon 1 of the vimentin gene, and primer 3 (5′-CTGTTCGCCAGGCTCAAGGC-3′) is located in the *neo* coding sequence (for details on the targeting construct, see Colucci-Guyon et al[17]). The primer 1 and primer 2 pair allows the amplification of a 398-bp fragment when a wild-type allele is present and of a 4.8-kb fragment, usually not detected, when the *Vim*^{1} disrupted allele is present. The primer 2 and primer 3 pair allows the amplification of a 530-bp fragment when a disrupted allele is present. PCR amplification was performed with *Taq* polymerase (Eurobio, Les Ulis, France) under the conditions recommended by the supplier on a Hybaid thermal reactor (Cesa Labo, Aubervilliers, France) and involved 30 cycles consisting of 1 minute at 94°C, 1 minute at 65°C, and 1 minute at 72°C.

Protocol

Adult (3 to 4 months) *Vim*$^{+/+}$, *Vim*$^{+/-}$, and *Vim*$^{-/-}$ mice, originating from the same litter, were studied. Animals were anesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (Janssen, Beerse, Belgium; 37 μg/g of body weight). A left flank incision was made and the renal pedicle was clamped with a microaneurysm clamp (Moria, Paris, France). Blood vessel clamping was maintained for 50 minutes. Reperfusion was assessed by visual examination of the kidney, which recovered the usual color, within 20 to 30 seconds.

Mice were sacrificed at 2, 7, 14, 28, 42, and 60 days after surgery. At each time, five *Vim*$^{+/+}$, five *Vim*$^{+/-}$, and five *Vim*$^{-/-}$ mice were examined.

To determine the rate of cellular proliferation in normal kidney, three normal non-operated littermates were studied at 2, 7, 14, and 28 days after surgery.

At the time of sacrifice, the left post-ischemic kidney and the right control kidney were removed, cut into two halves, and either immediately frozen (one half) in 2-methylbutane (Sigma-Aldrich, St. Quentin Fallavier, France) for further X-gal staining or fixed in Kryofix (Merck, Nogent-sur-Marne, France) for morphological and immunohistochemical studies.

In animals sacrificed at day 7, urine samples were collected using Tecniplast metabolic cages during the 24 hours before sacrifice and blood samples were obtained by intracardiac puncture at the time of sacrifice for determination of creatinine, urea, and electrolyte concentrations. Values were compared with those obtained from five normal non-operated littermates.

Methods

Localization of β-Galactosidase Activity

The renal tissue, frozen in 2-methylbutane and kept at −80°C, was cut on a cryostat (Shandon, Dublin, Ireland) to obtain 7-μm-thick sections. Sec-

tions were put on poly-L-lysine-coated glass slides and immediately fixed in fresh 2% formaldehyde/0.2% glutaraldehyde for 15 minutes at 4°C. After three rinses in phosphate-buffered saline (PBS), the slides were incubated overnight at 35°C in a staining solution containing 2 mmol/L $MgCl_2$, 4 mmol/L $K_3Fe(CN)_6$, 4 mmol/L $K_4Fe(CN)_6$, and 0.4 mg/ml 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-gal, Gibco BRL, Cergy Pontoise, France) buffered with PBS. The slides were then washed in PBS, counterstained with eosin, and mounted in Eukitt (Labonord, Villeneuve d'Ascq, France).

Immunohistochemistry

Immunostaining: Immunostaining was performed using the ABC procedure[18] as modified by Maunoury et al.[19] Briefly, kidneys were fixed in Kryofix (Merck), ethanol dehydrated, and paraffin embedded with an automated Tissue-Tek Vip (Bayer Diagnostic, Puteaux, France). The 5-μm-thick sections were cut and mounted on silane-coated glass slides. Then, sections were incubated for 30 minutes with 10% donkey nonimmune serum to reduce nonspecific background staining, followed by an overnight incubation at room temperature with the specific antibody diluted in 10% donkey serum. After a 10-minute rinse in PBS, sections were incubated for 30 minutes at room temperature with a biotinylated donkey anti-rabbit antibody (Jackson Research Laboratories, West Grove, PA) diluted 1/200, followed by the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). After extensive washing, sections were incubated for 4 minutes in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Fluka, Mulhouse, France) and 0.01% freshly prepared hydrogen peroxide (Perhydrol; Merck). As proliferating cell nuclear antigen (PCNA) antibody was conjugated with peroxidase, sections treated with this antibody were directly incubated with diaminobenzidine. The sections were counterstained with alcian blue and mounted in Eukitt (Labonord).

Negative controls were obtained by replacing specific antisera with normal nonimmune sera; no labeling was observed, indicating that all of the procedures and reagents used resulted in specific labeling.

Antibodies: The primary antibodies used in the present study were 1) a rabbit polyclonal anti-mouse villin antibody (reported by Maunoury et al[20]), diluted 1/1000 (the specificity of this antibody was assessed in a previous study),[19,20] 2) a mouse monoclonal anti-rat PCNA antibody (PC10 Epos; Dako, Trappes, France), diluted 1/20, 3) a rabbit polyclonal anti-human keratin antiserum (Europa, Cambridge, UK), diluted 1/500, and 3) a rabbit polyclonal anti-rat vimentin antiserum (provided by Dr. A. M. Hill), diluted 1/2000. To eliminate undesired cross-reacting antibodies of this anti-vimentin antiserum, we purified the anti-vimentin antibodies. Briefly, organs from mice lacking vimentin were dissected, fixed in Kryofix, and homogenized (Polytron; Bioblock Scientific, Illkirch, France). After centrifugation and washing in PBS, the pellet was incubated with anti-vimentin antibodies for 24 hours at 4°C and the supernatant was collected. After this treatment, an absolute negative staining was observed by immunohistochemical analysis on sections from $Vim^{-/-}$ mice, whereas a specific labeling was preserved on sections from normal mice.

Quantification of Immunostaining: The number of tubular PCNA-labeled nuclei was determined in 25 randomly selected microscopic fields moving from cortex to medulla by direct examination in a Microphot-Fxa microscope (Nikon, Charenton-le-Pont, France) with a 20× objective. All sections were evaluated by a pathologist who was unaware of the group examined.

Renal Morphology

Serial sections, processed as for immunohistochemistry, were stained with hematoxylin and eosin and periodic acid Schiff.

Renal Function

All plasma and urine determinations were performed with a multiparametric autoanalyzer Hitachi 717 (Boehringer-Mannheim, Melun, France).

Expression of Data and Statistical Analysis

Data are expressed as means ± SEM. Differences between the experimental groups were evaluated using one-way analysis of variance, which was followed, when significant, by the Dunnett test.

Results

All the experiments described hereafter were performed on animals from intercrosses between $Vim^{+/-}$ mice, which bear a mutated allele of the vimentin gene from which bacterial β-galactosidase was expressed in place of vimentin.

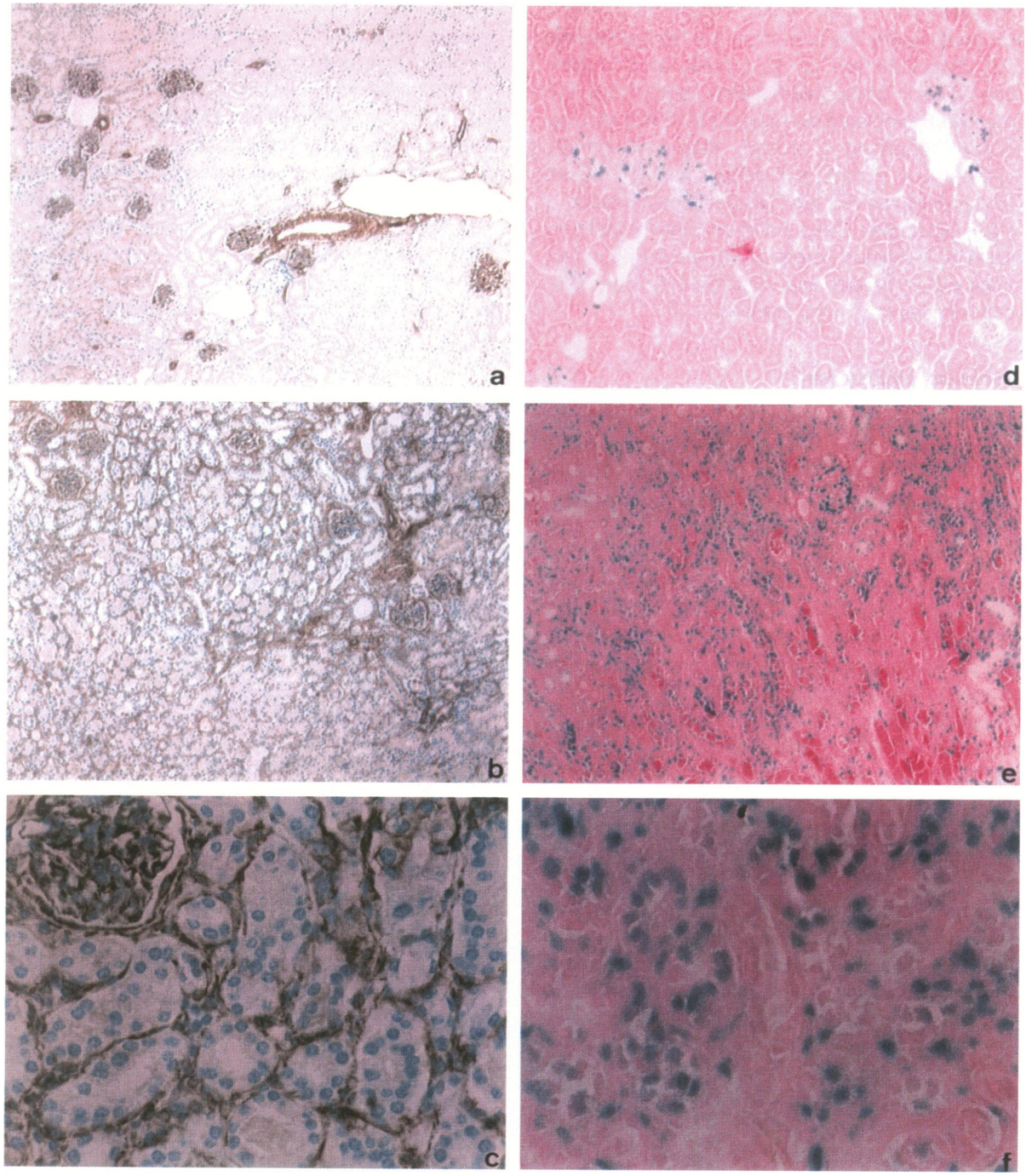

Figure 1. *Vimentin expression in contralateral control kidney* (a *and* d) *and post-ischemic kidney* (b *to* c *and* e *to* f) *from* $Vim^{+/-}$ *mice, 7 days after ischemia.* a *to* c: *Vimentin immunostaining.* d *to* f: *X-gal staining* (β-*galactosidase activity was put under the control of the vimentin promoter*). *Note the marked increase in staining of proximal tubules in post-ischemic kidney* (*compare* b *with* a *and* e *with* d *as regards immunostaining and X-gal staining, respectively*). *At higher magnification* (c *and* f), *X-gal staining of tubular nuclei* (f) *confirms the tubular origin of the basolateral vimentin expression* (c). *In* a *to* c, *the blue nuclear labeling results from counterstaining with alcian blue, whereas in* d *to* f, *it results from X-gal staining* (*these sections were counterstained with eosin, exclusively*). *Magnification,* ×75 (a, b, d, *and* e) *and* ×300 (c *and* f).

Vimentin Expression

Vimentin expression was evaluated in $Vim^{+/+}$, $Vim^{+/-}$, and $Vim^{-/-}$ animals by immunohistochemistry. As expected from the original study,[17] reporting the establishment of the vimentin-null mice strain, vimentin protein could not be detected by anti-vimentin antibodies in any structure of both contralateral control and post-ischemic kidneys from $Vim^{-/-}$ mice. In contrast, vimentin was revealed in glomeruli,

vessels, and interstitial cells of both post-ischemic and contralateral control kidney in $Vim^{+/+}$ (data not shown) and $Vim^{+/-}$ mice (Figure 1, a and b). In both groups, vimentin expression was also detected in most proximal tubules and in a few distal tubules and was found to increase in glomerular parietal cells of Bowman's capsule of post-ischemic kidney (Figure 1b). The staining predominated in the deep cortex and the outer stripe of outer medulla and was located at the vicinity of the basal cell membrane (Figure 1c). The distribution and the intensity of staining were maximal at day 7 after ischemia. Vimentin tubular staining was decreased at 28 days after ischemia and could not be detected in any renal section at 42 days after ischemia. This time course was similar in $Vim^{+/+}$ and $Vim^{+/-}$ animals. In contrast, vimentin was undetectable in tubules of contralateral control kidney of both $Vim^{+/+}$ and $Vim^{+/-}$ mice at any time of the study (Figure 1a).

To further assess the cellular origin of vimentin, X-gal staining was performed in $Vim^{+/-}$ (Figure 1, d–f) and $Vim^{-/-}$ mice (Figure 2, a and b). As expected, glomerular, vascular, and interstitial cells showed β-galactosidase activity in both kidneys (Figures 1, d and e, and 2, a and b). In contrast, intense nuclear labeling of tubular cells was observed exclusively in post-ischemic kidneys (Figures 1, e and f, and 2b).

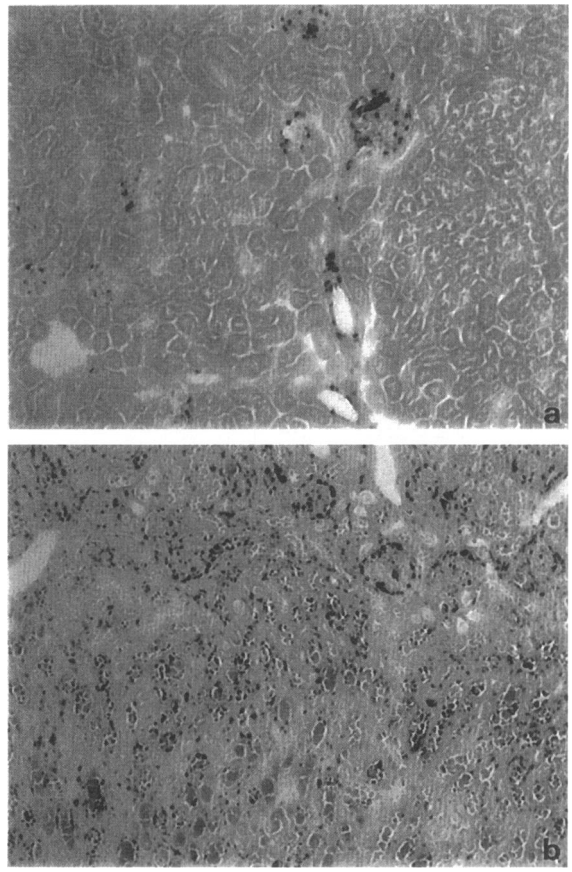

Figure 2. *X-gal staining (β-galactosidase activity was put under the control of vimentin promoter) in contralateral control kidney* (**a**) *and post-ischemic kidney* (**b**) *from* $Vim^{-/-}$ *mice, 7 days after ischemia. Note the strong staining of proximal tubular nuclei in post-ischemic kidney. Magnification,* ×75.

PCNA Expression

PCNA staining was used as a marker of cellular proliferation. In $Vim^{+/+}$ mice, the number of PCNA-labeled tubular nuclei increased significantly after ischemia (Figure 3, a and b). Proliferative activity concerned mainly the proximal tubules. As shown in Figure 4, the increment was maximal at day 2 in the injured kidney. The labeling was still higher than in the contralateral control kidney at day 14 after surgery and returned to normal values by 28 days after ischemia. Labeling remained unchanged thereafter. A similar pattern in terms of time course and intensity of staining was observed in $Vim^{+/-}$ and $Vim^{-/-}$ animals, in which the number of labeled nuclei was not different from that of $Vim^{+/+}$ mice at any time of the study (Figures 3, b–d, and 4).

Because the number of labeled tubular nuclei in contralateral control kidney seemed higher than those reported in normal kidney,[21] we compared these values to those of intact non-operated kidneys from animals of the same litter. Tubular PCNA staining was comparable in $Vim^{-/-}$, $Vim^{+/-}$ and $Vim^{+/+}$ non-operated animals at any time of the study. As shown in Figure 4, PCNA labelling of the right control kidney was significantly higher than that of non-operated animals at day 2, but not at any other time of the study.

Villin Expression

Villin staining was used as a marker of differentiation of polarized proximal tubular cells. Villin expression was similar in contralateral control and in normal non-operated kidneys, and no difference could be detected when comparing $Vim^{+/+}$ and $Vim^{-/-}$ animals (Figure 5, a and b). In both $Vim^{+/+}$ and $Vim^{-/-}$ mice, the number of villin-labeled tubules was dramatically reduced in the first days after ischemia. Villin staining was heterogeneous among tubules, from the almost complete absence to marked expression. The reduction was maximal at day 7 (Figure 5c), at the time when vimentin expression was highest, and was accompanied by a staining of tubular casts resulting from shedding of brush borders into the lumen. Villin expression was progressively

Figure 3. *Immunohistochemical analysis of PCNA expression of cortex and outer stripe of outer medulla in contralateral control kidney from* $Vim^{+/+}$ *mice* (a) *and in post-ischemic kidney from* $Vim^{+/+}$ (b), $Vim^{+/-}$ (c), *and* $Vim^{-/-}$ (d) *mice, 2 days after ischemia. The number of labeled nuclei (dark dots) in post-ischemic kidney, increased when compared with contralateral control kidney, was comparable in* $Vim^{+/+}$, $Vim^{+/-}$, *and* $Vim^{-/-}$ *mice. Magnification,* ×300.

restored (Figure 5d) and had returned to normal in post-ischemic kidney by 28 days after ischemia (Figure 5e). The aspect remained unchanged until 60 days after ischemia (Figure 5f) and was comparable in $Vim^{+/+}$ and $Vim^{-/-}$ mice throughout the study (Figure 5, c–e). In $Vim^{+/-}$ mice, the pattern of villin expression was comparable to those observed in the other two groups.

Keratin Expression

To investigate whether the absence of vimentin was compensated by other intermediate filaments, we analyzed the expression of keratin in $Vim^{+/+}$, $Vim^{+/-}$, and $Vim^{-/-}$ contralateral control and post-ischemic kidneys. As previously reported,[3] the anti-keratin antibodies used reacted with collecting ducts but not with other epithelial structures of normal non-operated kidneys. The staining was not affected in $Vim^{+/+}$ contralateral control kidney (Figure 6a). By contrast, the intensity and the number of labeled collecting ducts increased progressively from day 2 to day 14 (Figure 6b) in post-ischemic kidneys from $Vim^{+/+}$ mice. Moreover, keratin reactivity was detected in several damaged proximal tubules, particularly at day 2 after ischemia. Like vimentin, keratin staining returned to normal by 42 days after surgery. The changes in keratin expression were similar for intensity and distribution in $Vim^{+/+}$, $Vim^{+/-}$, and $Vim^{-/-}$ animals (Figure 6, b–d) at any time of the study.

Renal Structure

We then evaluated whether the absence of vimentin influenced the severity of early lesions and the long-term completion of tubular regeneration. Tubular lesions were comparable in terms of distribution, severity, and timing in the three experimental groups at any time of the study. In the first week after ischemia, numerous tubular sections exhibited evidence of necrotic lesions, with dying cells desquamating and

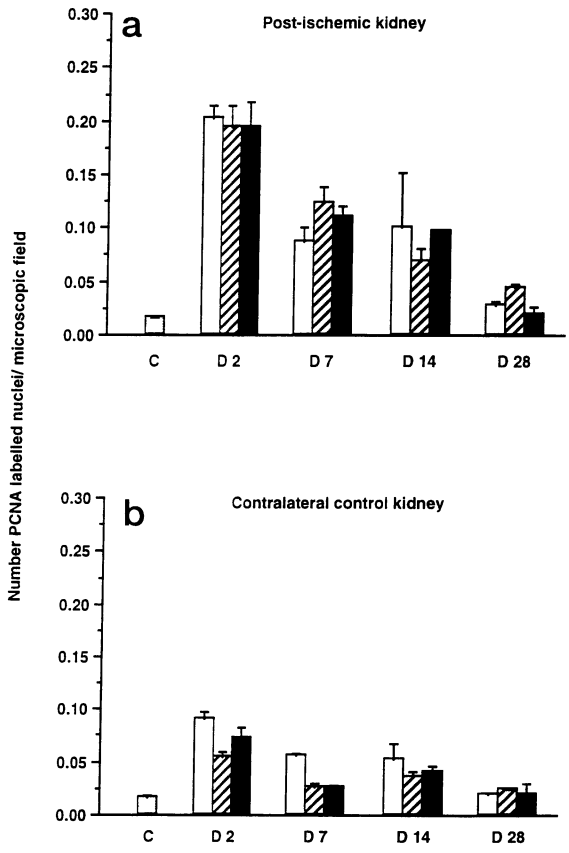

Figure 4. *Tubular cellular proliferation in post-ischemic* (a) *and contralateral control* (b) *kidneys from* $Vim^{+/+}$ (open bars), $Vim^{+/-}$ (hatched bars), *and* $Vim^{-/-}$ (solid bars) *mice and of normal non-operated littermates* (dotted bars). *The number of PCNA-labeled cells was determined in 25 microscopic fields randomly selected moving from cortex to medulla. Data are means ± SEM. Analysis of variance revealed that, in the post-ischemic kidney, cell proliferation was significantly different from control (C) value ($P < 0.05$) at days 2, 7, and 14 but not at day 28, whereas in the contralateral control kidney, only the day 2 value was significantly different from control ($P < 0.05$). No intergroup statistical difference appeared at any time of the study in either kidney.*

leaving denuded sites on the basement membrane. Cell debris and intratubular proteins formed casts and obstructed tubules. A marked interstitial inflammatory cell infiltrate was evident, particularly at day 2. Tubular necrosis culminated at day 7 and was followed by a progressive restoration to tubular epithelial integrity. By that time, most tubular profiles were lined by a flattened epithelium devoid of brush border. By day 28, the histological appearance of post-ischemic kidney was comparable to that of contralateral control kidney in most microscopic fields, although patches of interstitial hyperplasia and degenerated tubules still appeared focally in the injured kidney. By 2 months after ischemia, the kidney structure of $Vim^{-/-}$ mice was restored *ad integrum* (Figure 7b) and could not be distinguished from that of a contralateral control kidney or of a normal non-operated $Vim^{+/+}$ kidney (Figure 7a).

Renal Function

Finally, parameters of renal function were evaluated in $Vim^{+/+}$ and $Vim^{-/-}$ mice, at day 7 after surgery, when tubular vimentin expression was maximal, and were compared with those obtained from normal non-operated littermates. Plasma creatinine, urea, and electrolytes were not significantly affected by unilateral ischemia in $Vim^{+/+}$ animals. Moreover, values obtained from operated $Vim^{-/-}$ mice were not different from those of the two other groups. Similarly, glomerular filtration rate, as assessed by creatinine clearance, was similar in non-operated $Vim^{+/+}$ (0.12 ± 0.01 ml/minute), in operated $Vim^{+/+}$ (0.14 ± 0.01 ml/minute), and in operated $Vim^{-/-}$ (0.11 ± 0.01 ml/minute) mice.

Discussion

In the present study, we have demonstrated that the absence of vimentin did not affect cellular proliferation, differentiation, and tubular organization in the post-ischemic kidney.

As expected in $Vim^{-/-}$ mice, which were obtained by targeted mutagenesis in embryonic stem cells,[17] vimentin could not be detected by immunohistochemistry in any structure of the kidney either under basal conditions or after ischemia. However, changes in β-galactosidase activity, which mimicked vimentin expression in $Vim^{-/-}$ mice, assessed vimentin promoter stimulation by ischemia in these animals. Therefore, $Vim^{-/-}$ mice are the adequate tool to address the question of the role of vimentin in proliferation and differentiation after ischemia.

As noted previously by other groups,[10,12] organization of vimentin filaments in post-ischemic tubules was strikingly different from that reported in mesenchymal cells. Thus, the accumulation of vimentin at the basal pole of the cells could raise the question of the cellular origin of vimentin filaments. Because β-galactosidase was put under the control of the vimentin promoter, data from $Vim^{+/-}$ mice provided the unique opportunity to demonstrate unambiguously that proximal tubular cells were indeed expressing vimentin. First, the vimentin synthesis in tubular cells was attested by the fact that the majority of proximal cells exhibited X-gal nuclear staining, and second, nuclear X-gal labeling was apparent in those cells that were stained with anti-vimentin antibody at their basal

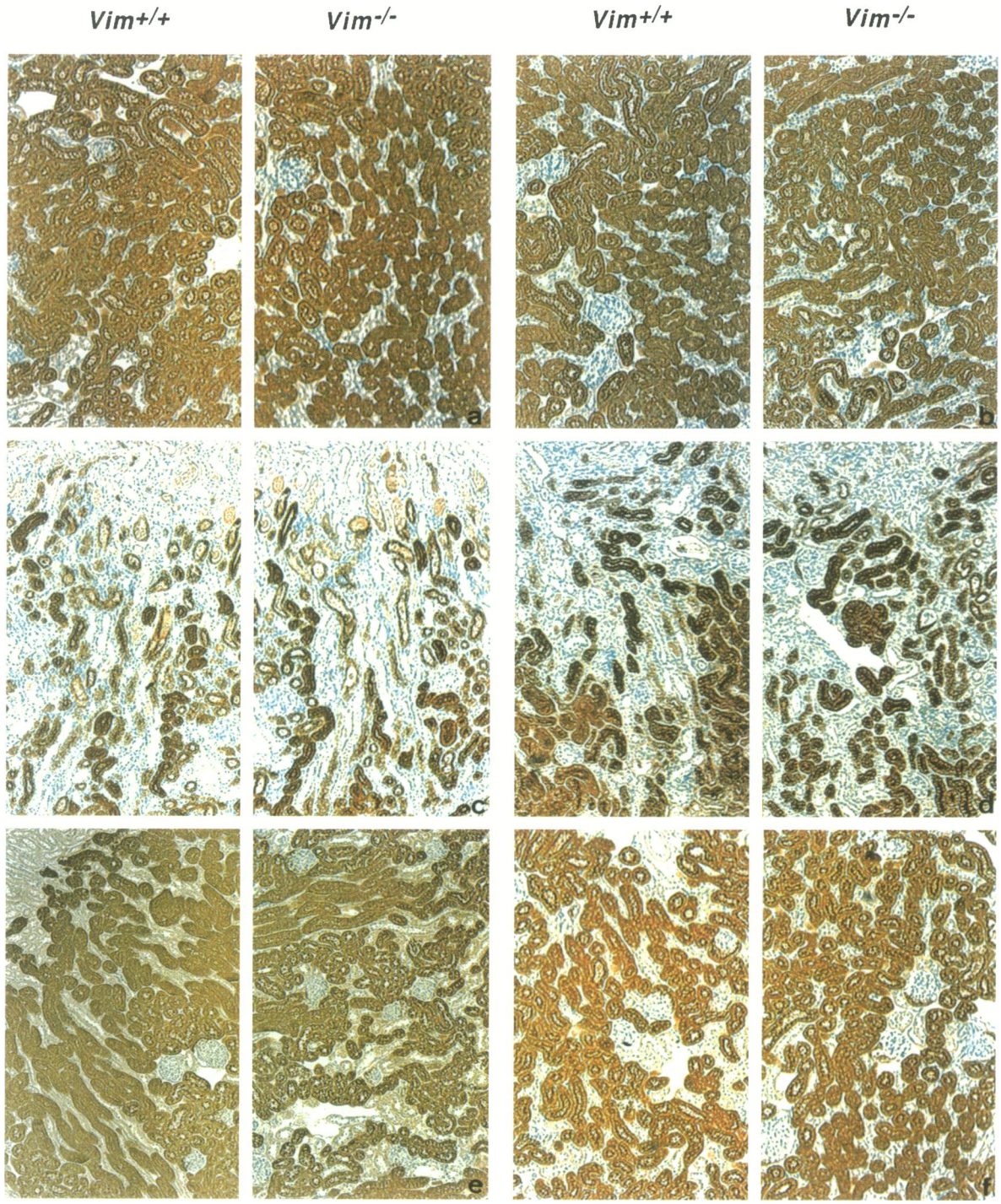

Figure 5. *Immunohistochemical analysis of villin expression in normal non-operated* (a) *and in contralateral control* (b) *kidney by 7 days after ischemia, and in post-ischemic kidney* (c *to* f) *by 7* (c), *14* (d), *28* (e), *and 60* (f) *days after ischemia from* $Vim^{+/+}$ *and* $Vim^{-/-}$ *mice. Villin staining was unchanged in contralateral control kidney* (b) *as compared with normal non-operated kidney* (a). *By contrast, the staining of post-ischemic kidney changed progressively after reperfusion; markedly reduced at day 7* (c), *it increased thereafter* (d) *to restore the normal pattern at day 28* (e). *This pattern remained unchanged until 60 days* (f). *At any time of the study, the staining of both contralateral control and post-ischemic kidneys was comparable in* $Vim^{+/+}$ *and* $Vim^{-/-}$ *mice* (b *to* f). *Magnification,* ×75.

pole. That interstitial cells may participate in vimentin accumulation at the basal tubular pole cannot be definitely ruled out as X-gal-stained interstitial cells attached to tubules. It is noteworthy that vimentin expression was not restricted to proximal tubular epithelial cells in the post-ischemic kidney

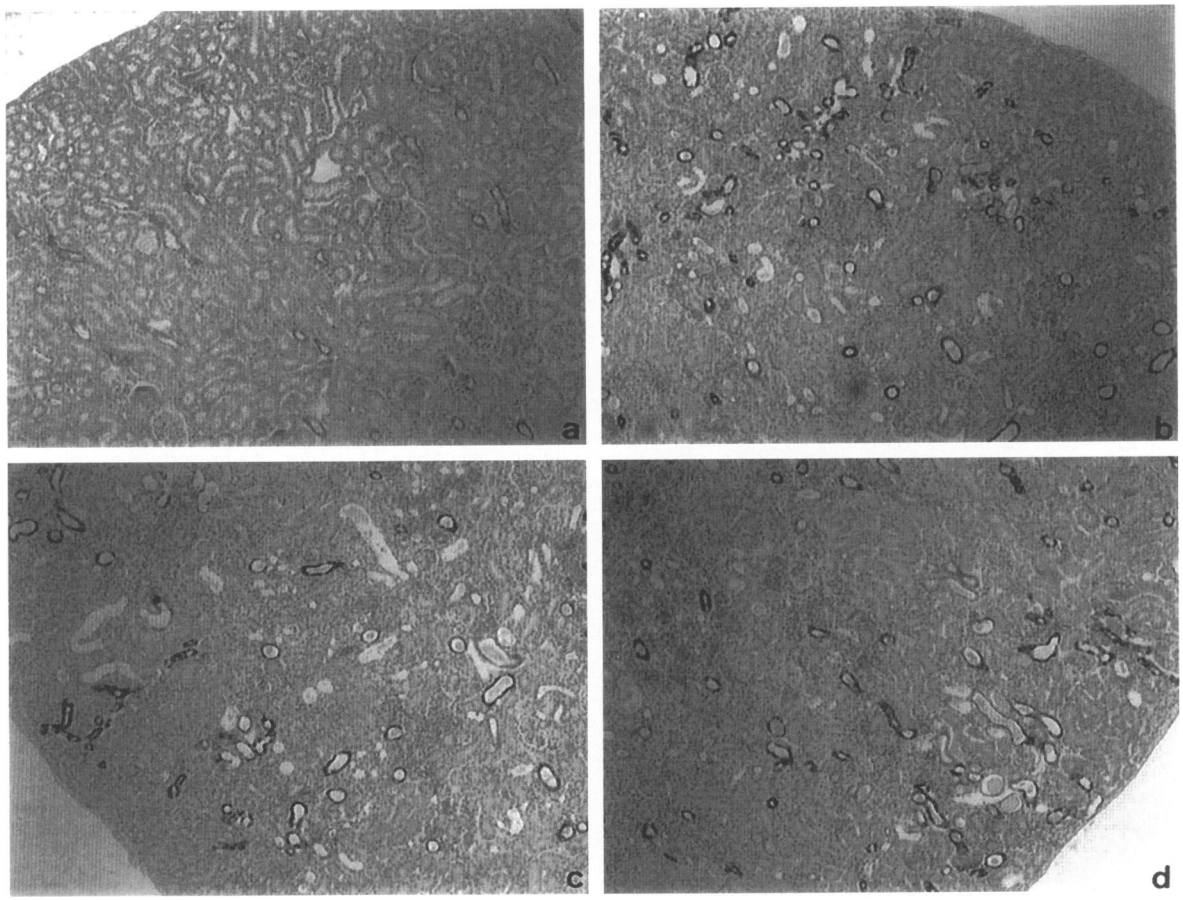

Figure 6. *Immunohistochemical analysis of keratin expression in contralateral control kidney from* $Vim^{+/+}$ *mice* (a) *and in post-ischemic kidney from* $Vim^{+/+}$ (b), $Vim^{+/-}$ (c), *and* $Vim^{-/-}$ (d) *mice, 7 days after ischemia. The intensity of staining and the number of labeled tubules increased in post-ischemic kidney when compared with contralateral control kidney and were comparable in* $Vim^{+/+}$, $Vim^{+/-}$, *and* $Vim^{-/-}$ *mice. Magnification,* $\times 75$.

but was also present in, to a smaller extent, distal tubular cells and glomerular epithelial parietal cells of Bowman's capsule. In the latter, the labeling was weak in normal kidney, but it increased in terms of distribution and intensity after ischemia. This pattern is slightly different from that reported by Witzgall et al,[10] who detected vimentin expression in S_3 segments of proximal tubules exclusively. These apparent discrepancies may be accounted for by differences in animal species, in duration of ischemia, and/or in sensitivity of methods used.

As regards proliferation, previous studies[7,10,11] showed that vimentin and PCNA, a widely used marked of cellular proliferation, were co-localized in the same cells of regenerating tubules, raising the possibility that expression of vimentin could be instrumental in cell proliferation. This possibility was also suggested by indications that vimentin could play a role in the cell cycle.[14,15] That both the magnitude and time course of PCNA expression were similar in $Vim^{+/+}$, $Vim^{+/-}$, and $Vim^{-/-}$ mice rules out this possibility and demonstrates that vimentin is not a major determinant of tubular cell proliferation after ischemia.

Extensive tubular necrosis, such as that achieved in our model, is associated with partial denudation of basement membrane. Regeneration is therefore accomplished by combination of cellular proliferation, spreading, and/or migration. Zhu et al[22] suggested in a recent study on toxic tubular injury that vimentin expression may be important in cell mobility. Although we cannot exclude that vimentin expression is linked to partial denudation of the basement membrane rather than to cell proliferation in proximal tubules, our results argue against a major role of vimentin in cell migration, at least in the model used. Indeed, the re-epithelialization of denuded proximal tubules was complete and the rate of restitution *ad integrum* of tubular structure was normal in $Vim^{-/-}$ mice, in which the appearance of the experimental

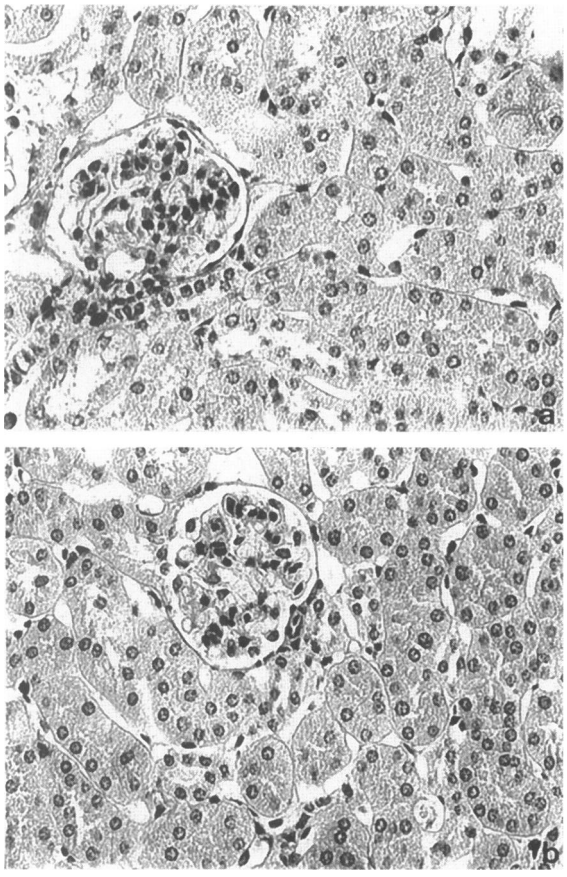

Figure 7. *Renal structure at 60 days after ischemia of normal non-operated kidney from* $Vim^{+/+}$ *mice* (**a**) *and of post-ischemic kidney from* $Vim^{-/-}$ *mice* (**b**). *The structure of the two kidneys was similar. Periodic acid Schiff; magnification,* ×*300.*

kidney was indistinguishable from that of control kidneys by 2 months after ischemia.

A hallmark of tubular ischemic injury is the loss of surface membrane polarity.[23] During recovery, proximal tubular cells undergo remodeling of membrane surface and reconstitution of apical and basolateral domains of the plasma membrane.[24] Villin, a specific constituent of brush-border membranes, was used as a marker of differentiated apical membranes. As expected, a drastic and progressive loss of apical staining with anti-villin antibodies was observed in most tubules after ischemia, whereas cellular debris were detected in the tubular lumen, indicating the presence of microvilli fragments. However, villin staining was completely recovered 28 days after ischemia. That recovery of villin expression was delayed to that extent may result from the combination of proliferation and migration of regenerating tubular cells. Vimentin inactivation affected neither dedifferentiation nor remodeling. This suggests that, in dedifferentiated cells, expression of vimentin cannot be proposed as a causative factor for loss of cell polarity.

Surprisingly, villin staining was detectable not only in brush border membranes but also in cytoplasm of proximal tubular cells. This pattern was already reported in normal embryonic renal cells[20] and in renal cell carcinomas[25] and may probably result from the detection of newly synthesized villin before it is targeted to the apical membrane. It is noteworthy that, in our study, tissue was fixed with Kryofix, at variance with other studies in which acetic acid or Methacarn were used.

As regards renal function, it should be pointed out that minimal changes had to be expected after unilateral ischemia. However, the important observation that glomerular filtration rate, as assessed by creatinine clearance, was not altered in $Vim^{-/-}$ animals suggests that the contralateral kidney compensates the function of the ischemic one as efficiently as in $Vim^{+/+}$ mice.

The absence of a pathological phenotype after ischemia in mice lacking vimentin raised the question of whether another cytoplasmic intermediate filament could compensate the absence of vimentin. This issue was already addressed by Colucci-Guyon et al[17] in tissues that normally express vimentin. These authors could not detect the presence of other intermediate filaments by immunohistochemistry and Western blot or of the corresponding network by electronic microscopy in both fibroblasts and lens cells of $Vim^{-/-}$ mice, whereas these cells frankly expressed vimentin in $Vim^{+/+}$ animals. It should be pointed out that, in the present study, the model used (paraffin-embedded sections of mouse kidney) limited the use of available anti-keratin antibodies, precluding any definitive conclusions as regards the expression of keratin subtypes present in the proximal tubules. However, we show that ischemia increased keratin expression in distal segments of the nephron and that this increment was similar whether vimentin was present or absent. This suggests that the normal phenotype observed during the reperfusion phase is not due to compensatory expression of another intermediate filament.

In summary, although vimentin is usually expressed in tubular cells during the recovery of ischemic tubular necrosis, cell proliferation, differentiation, and tubular organization are not impaired in mice lacking vimentin. These data suggest that, after acute tubular necrosis, the transient tubular expression of vimentin is not instrumental in the tubular regeneration process. The role of tubular vimentin expression during regeneration, if any, remains to be elucidated.

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References

1. Bachmann S, Kriz W, Kuhn C, Franke WW: Differentiation of cell types in the mammalian kidney in immunofluorescence microscopy using antibodies to intermediate filament proteins and desmoplakins. Histochemistry 1983, 77:365–394
2. Holthöfer H, Miettinen A, Lehto VP, Lehtonen E, Virtanen I: Expression of vimentin and cytokeratin types of intermediate filament proteins in developing and adult human kidneys. Lab Invest 1984, 50:552–559
3. Holthöfer H, Miettinen A, Paasivuo R, Lehto VP, Linder E, Alfthan O, Virtanen I: Cellular origin and differentiation of renal carcinomas. Lab Invest 1983, 49:317–326
4. Waldherr R, Schwechheimer K: Co-expression of cytokeratin and vimentin intermediate-sized filaments in renal cell carcinomas. Virchows Arch (Pathol Anat) 1985, 408:15–27
5. Dierick AM, Praet M, Verbeeck P, Robyns C, Oosterlinck W: Vimentin expression of renal cell carcinoma in relation to DNA content and histological grading: a combined light microscopy, immunocytochemical, and cytophotometrical analysis. Histopathology 1991, 18: 315–322
6. Ward JM, Stevens JL, Konishi N, Kurata Y, Uno H, Diwan BA, Ohmori T: Vimentin metaplasia in renal cortical tubules of preneoplastic, neoplastic, aging, and regenerative lesions of rats and humans. Am J Pathol 1992, 141:955–964
7. Gröne HJ, Weber K, Gröne E, Helmchen U, Osborn M: Coexpression of keratin and vimentin in damaged and regenerating tubular epithelia of the kidney. Am J Pathol 1987, 129:1–8
8. Gossrau R, Günther T, Graf R: Enhancement of gentamicin-induced nephrotoxicity by Mg deficiency in non-pregnant rats. Histochemistry 1989, 90:489–496
9. Wallin A, Zhang G, Jones TW, Jaken S, Stevens JL: Mechanism of nephrogenic repair response: studies on proliferation and vimentin expression after ^{35}S-1,2-dichlorovinyl-L-cysteine nephrotoxicity *in vivo* and in cultured proximal tubule epithelial cells. Lab Invest 1992, 66:474–484
10. Witzgall R, Brown D, Schwarz C, Bonventre J: Localization of proliferating nuclear cell antigen, vimentin, c-fos, and clusterin in the postischemic kidney. J Clin Invest 1994, 93:2175–2188
11. Nouwen EJ, Verstrepen WA, Buyssens N, Zhu MQ, De Broe ME: Hyperplasia, hypertrophy, and phenotypic alterations in the distal nephron after acute proximal tubular injury in the rat. Lab Invest 1994, 70:479–493
12. Toubeau G, Nonclercq D, Zanen J, Laurent G, Schaudies PR, Heuson-Stiennon JA: Renal tissue expression of EGF and EGF receptor after ischaemic tubular injury: an immunohistochemical study. Exp Nephrol 1994, 2:229–239
13. Traub P, Shoeman RL: Intermediate filament and related proteins: potential activators of nucleosomes during transcription initiation and elongation. Bioessays 1994, 16:349–355
14. Skalli O, Goldman RD: Recent insights into assembly, dynamics, and function of intermediate filament networks. Cell Motil Cytoskeleton 1991, 19:67–79
15. Klymkowsky MW: Intermediate filaments: some answers, more questions. Curr Opin Cell Biol 1995, 7:46–54
16. Rittling SR, Baserga R: Functional analysis and growth factor regulation of the human vimentin promoter. Mol Cell Biol 1987, 7:3908–3915
17. Colucci-Guyon E, Portier MM, Dunia I, Paulin D, Pournin S, Babinet C: Mice lacking vimentin develop and reproduce without an obvious phenotype. Cell 1994, 79:679–694
18. Hsu S, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J Histochem Cytochem 1981, 29:577–580
19. Maunoury R, Robine S, Pringault E, Huet C, Guénet JL, Gaillard JA, Louvard D: Villin expression in the visceral endoderm and in the gut anlage during early mouse embryogenesis. EMBO J 1988, 7:3321–3329
20. Maunoury R, Robine S, Pringault E, Léonard N, Gaillard JA, Louvard D: Developmental regulation of villin gene expression in the epithelial cell lineages of mouse digestive and urogenital tracts. Development 1992, 115: 717–728
21. Goss RJ: Kinetics of compensatory growth. Q Rev Biol 1965, 40:123–146
22. Zhu MQ, De Broe ME, Nouwen EJ: Vimentin expression and distal tubular damage in the rat kidney. Exp Nephrol 1996 (in press)
23. Molitoris BA, Falk AS, Dahl RH: Ischemia-induced loss of epithelial polarity. J Clin Invest 1989, 84:1334–1339
24. Spiegel DM, Wilson PH, Molitoris BA: Epithelial polarity following ischemia: a requirement for normal cell function. Am J Physiol 1989, 256:F430–F436
25. Bacchi CE, Gown AM: Distribution and pattern expression of villin, a gastrointestinal-associated cytoskeletal protein, in human carcinomas: a study employing paraffin-embedded tissue. Lab Invest 1991, 64:418–424