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Ischemic acute renal failure induces the expression of a wide range of nephrogenic proteins

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Villanueva, Sandra, Carlos Céspedes, and Carlos P. Vio. Ischemic acute renal failure induces the expression of a wide range of nephrogenic proteins. Am J Physiol Regul Integr Comp Physiol 290: R861–R870, 2006. First published November 10, 2005; doi:10.1152/ajpregu.00384.2005.—Ischemia-induced acute renal failure (ARF) is a disorder with high morbidity and mortality. ARF is characterized by a regenerative phase, yet its molecular basis is still under study. Changes in gene expression have been reported in ARF, and some of these genes are specific for nephrogenic processes. We tested the hypothesis that the regenerative processes involved in ischemia-induced ARF can be characterized by the reexpression of important regulatory proteins of kidney development. The distribution pattern and levels of nephrogenic proteins in rat kidneys after ischemia were studied by immunohistochemistry and immunoblot analysis. Ischemic damage was assessed by conventional morphology, serum creatinine, and the apoptotic markers terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and caspase 3. The hypoxia levels induced by ischemia were assessed by specific markers: hypoxia induced factor (HIF)-1α and 2-pimonidazole. In kidneys with ARF, an important initial damage was observed through periodic acid Schiff staining, by the induction of damage markers α-smooth muscle actin (α-SMA) and macrophages (ED-1) and by apoptosis induction. In agreement with diminishing renal damage at the initial regeneration phase, the expression of the mesenchymal proteins vimentin, neural cell adhesion molecules (Ncam), and the epithelial markers, Pax-2, Noggin, and basic fibroblast growth factor was observed; after, in a second phase, the tubular markers bone morphogen protein 7, Engrailed, and Lim-1, as well as the transcription factors Smad and p-Smad, were observed. Additionally, the endothelial markers VEGF and Tie-2 were induced at the initial and middle stages of regeneration phase, respectively. The expression of these proteins was restricted in time and space, as well as spatially and temporally. Because all of these proteins are important in maintaining a functional kidney, these results suggest that during the regeneration process after induced hypoxia, these nephrogenic proteins can be reexpressed in a similar fashion to that observed during development, thus restoring mature kidney function.

kidney; morphogen; regeneration

ACUTE RENAL FAILURE (ARF) is a clinical syndrome characterized by a rapid decline in glomerular filtration rate and is associated with high morbidity and mortality rates; however, it is potentially reversible if patients survive the initial insult (36). Acute tubular necrosis (ATN) with prerenal disease is the most common cause of ARF accounting for two-thirds of intrinsic causes (12). The principal cause of ATN is hypoxia induced by ischemia/reperfusion (I/R), which can be induced by clinical conditions, such as hemorrhagic shock or sepsis (27). ATN is characterized by a regenerative phase (26), which is very important because it involves the recuperation of kidney function. Recovery of kidney function from ARF relies on a sequence of events, including epithelial cell dedifferentiation and proliferation followed by differentiation and restoration of the functional integrity of the nephron (1). Although the morphologic characteristics of the process have been described (42), the molecular bases of the events leading to regeneration after ATN are not understood (37).

Many genes have been shown to be modulated in response to kidney damage. The expression of transcription factors like c-myc (8), c-jun (3), and early growth response 1 (ERG-1) (19), growth factors, such as hepatocyte growth factor (HGF) and insulin-like growth factor (IGF) (35), Adam, HO1, UCP-2, Thimosin b4 (46) and proapoptotic factors FADD, DAXX, BAX, BAD, and p53 (38) is upregulated after induced kidney damage, whereas EGF, cytochrome p450, Id6, cyp 2d9, and ADH B2 expression is downregulated (49). However, the upregulated expression of these genes is a common event to several pathological processes and cannot explain the regeneration processes that occur during ATN.

Several reports have demonstrated the presence of nephrogenic proteins in the regeneration process after induced ATN. For example Pax-2 protein, which plays a crucial role during early metanephric development (34), remains detectable only in collecting duct cells in adulthood (8); however, it is reexpressed in regenerating tubular epithelium after renal damage (18). Another protein implicated in morphogenic process is Ncam, which is strongly detected in renal vesicles, s-shaped bodies, and early tubules in kidney development and has a minimal expression in normal tubules of the adult kidney; Ncam has been detected by immunohistochemistry in proximal tubule cells after ischemia (1). Vimentin, an intermediate filament protein that is expressed almost exclusively in mesenchymal cells, becomes detectable in mitotically active proximal tubular cells after I/R (48). In contrast the expression of Kid-1 protein, which is observed exclusively in proximal tubules of adult kidneys (47), is lost in animals with ATN induced by folic acid treatment, suggesting that its downregulation is due to a functional stage similar to early kidney

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development (47). However, there are many proteins involved in kidney development that have not been studied in the regeneration process induced by ATN.

Bone morphogen protein 7 (BMP-7) is the most abundant component of the BMP protein family in fetal and adult mammalian kidney, and it is expressed in the ureteric bud and mesenchyme derivatives (10). Recent data suggest that BMP-7 acts as a survival factor for undifferentiated mesenchyme, opposing apoptotic signals (11). BMP-7 activity is antagonized by proteins such as Noggin (6, 43), DAN (38), Drm (30), Chordin (13), Follistatin, and Gremlin (28). BMP-7 binds to a Ser-Thr kinase receptor constituted by two isoforms: type II, that binds to the agonist and type I that binds and phosphorylates the transcription factors Smad 1 and 5 (25). Similar to what has been described for BMP-7, Smad are expressed by mesenchymal cells in the nephrogenic zone and are downregulated once these cells begin to form epithelium (45).

Another protein secreted by the ureteric bud is the basic fibroblast growth factor (bFGF), which is necessary for the induction of mesenchymal cell aggregation; however, it is not capable of turning these aggregates into epithelial cells (22). The main effects of bFGF are the inhibition of apoptosis, the stimulation of mesenchymal cell condensation, and the maintenance of WT-1 synthesis, a transcription factor that induces the transformation of mesenchymal cells into metanephrogenic tissue (31). Only the presence of WT-1 has been reported in tubular cells after ARF (46); however, the role of other nephrogenic proteins has not been studied in adult kidney during the regeneration phase of ATN.

An important factor reported in kidney development is the vascular endothelial growth factor (VEGF), which is expressed in epithelial and endothelial cells of the renal corpuscle (5). The suppression of VEGF activity by neutralizing antibodies interrupts glomerulogenesis, although the structures of arteries and arterioles are not altered (23). These results suggest that VEGF stimulates glomerular development through the stimulation of endothelial cells (14). The angiopoietin receptor, Tie-2, is known to be expressed in later maturation stages of the mouse metanephiros, when interstitial and glomerular capillaries begin to form (24). Targeted disruption of Tie-2 gene causes embryonic death, as a result of defective modeling of primitive vascular plexus and lack of perivascular cells (20), suggesting that the Tie-2 receptor is essential for the maturation of blood vessels during embryonic development (24). There are no reports on the influence of VEGF and Tie-2 in the regeneration process after kidney damage.

We propose as a hypothesis a model of kidney regeneration after I/R induced ATN, where specific proteins appear as a recapitulation of kidney development. We have examined the expression pattern of nephrogenic proteins that have a pivotal role in kidney development. In addition, we evaluated the interaction of BMP-7 and Noggin by evaluating the expression and localization of the nonphosphorylated and phosphorylated Smad.

These results indicate that some nephrogenic proteins are transiently reexpressed in regenerating proximal tubular cells, similar to what has been described during the nephrogenic process of development. In addition, we have reported the expression of Noggin, a BMP-7 antagonist, during kidney regeneration.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (220–250 g, n = 7 for each I/R group; 24, 48, 72, and 96 h; n = 5 for each sham-operated group) were housed in a 12:12-h light-dark cycle. Control and treated animals were weighed at the time of initiation of bilateral ischemic injury and after completion of experiments. Animals were maintained at the animal care facilities at the Pontificia Universidad Católica de Chile; food and water were supplied ad libitum. All experimental procedures were in accordance with institutional and international standards for the humane care and use of laboratory animals [Animal Welfare Assurance Publication A5427–01, Office for Protection from Research Risks, Division of Animal Welfare, National Institutes of Health (NIH)]. In this study, the protocol of use of animals was reviewed and approved by the institutional and independent Ethical Committee of the Pontificia Universidad Católica de Chile.

Renal I/R injury. An established model of renal I/R injury was performed; this resembles structural and functional consequences of renal ischemia, including apoptotic tubular epithelial cells (4). Animals (n = 7 for each I/R group) were anesthetized with ketamine: xylazine (25:2.5 mg/kg ip), and body temperature was maintained at 37°C. Both kidneys were exposed by a flank incision, and both renal arteries were occluded with nontraumatic vascular clamps for 30 min. After completion, both clamps were removed, renal blood flow was reestablished, and the incisions were sutured. Rats were allowed to recover in a warm room with water and food ad libitum. Sham-operated rats were submitted to the same surgical procedure and conditions, without clamping the renal arteries. Rats were killed under anesthesia (ketamine:xylazine) at 24, 48, 72, and 96 h after reperfusion; both kidneys were removed and processed for immunohistochemistry and Western blot analysis.

Tissue processing and immunohistochemical analysis. Tissues processing for immunohistochemical studies in paraflin-embedded-sections were carried out according to methods previously described (44). For cryosections, the kidney sections (3 to 4 mm thick) were embedded in Tissue Tek, obtained from Sakura Finetek (Torrance, CA), frozen in liquid nitrogen, and stored at −80°C; the sections were cut (at 7-μm thickness) with a cryostat (Leica, Heidelberg, Germany), and stored until immunostaining.

Immunolocalization studies were performed using an indirect immunoperoxidase technique as previously described (44). Briefly, tissue sections were dewaxed, rehydrated, rinsed in 0.05 M tris-phosphate-saline (TPS) buffer (pH 7.6), and incubated with the primary antibody overnight at 22°C, followed by three washes (5 min each one) with TPS buffer, incubation with the corresponding secondary antibody, and with the peroxidase-antiperoxidase (PAP) complex was carried out for 30 min at 22°C. Immunoreactive sites were revealed using 3,3′-diaminobenzidine 0.1% (wt/vol) and 0.03% (vol/vol) hydrogen peroxide solution. For some specific antibodies, immunoreactivity was revealed using an alkaline-phosphatase-conjugated secondary antibody, in the presence of nitro blue tetrazolium chloride: 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (NBT/BCIP) in buffer Tris 100 mM pH = 9.5.

Antibodies and chemicals. The following primary antibodies were used: monoclonal antibodies against Lim 1–2 (clone 4F2), Engrailed (clone 4G11), vimentin (clone 40E-C), Ncam (clone 5B8), zona occludens-1 (ZO-1; clone R26.4C) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHID) and maintained by the University of Iowa, Department of Biological Sciences, (Iowa City, IA). The goat polyclonal antibodies against Pax 2–5–8, BMP-7, Smad 1–5–8, p-Smad 2–3, the rabbit polyclonal antibodies against Tie-2, bFGF, and monoclonal antibodies against VEGF (clone C-1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies against macrophages (clone ED-1) was obtained from Biosource (Camarillo, CA), α-smooth muscle actin (α-SMA) (clone 1A4) was obtained from...
Sigma Aldrich (St. Louis, MO), the hypoxia induced factor 1α-HIF-1α (clone H1α67) was obtained from Novus Biologicals (Littleton, CO), and Noggin was a gift of Dr. R. Harland. The 2-pimonidazole and a corresponding mouse monoclonal antibody were obtained from Chemicon (Temecula, CA).

Secondary antibodies and the corresponding peroxidase-antiperoxidase complexes were purchased from ICN Pharmaceuticals-Cappel (Aurora, OH). Triton X-100, 3,3'-diaminobenzidine, carrageenan, Tris·HCl, hydrogen peroxide, phosphate salts, and other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Fig. 1. Evidence of histological damage in hypoxic kidney induced by ischemia/reperfusion (I/R). The immunohistochemistry was made in kidney samples recovered at 24 h after ischemia/reperfusion (n = 7 for each I/R group). A clear induction of renal damage markers, such as macrophages (ED-1; A) or α-smooth muscle actin (α-SMA; B) can be observed in the interstitial space from the inner and outer medulla. Staining for ED-1 and α-SMA was done using peroxidase and was revealed with diaminobenzidine (brown color reaction). The histological damage was evaluated by periodic acid Schiff stain (C, D), and brush border, epithelial flattening, and mitosis are shown, indicating that I/R can induce renal damage. Kidneys from sham rats were also used to detect these markers (E-H) (n = 5 for each control group). Scale bar = 100 μm (A–C, E–G) and 50 μm (D, H). The arrows show the markers' location.

Sigma Aldrich (St. Louis, MO), the hypoxia induced factor 1α-HIF-1α (clone H1α67) was obtained from Novus Biologicals (Littleton, CO), and Noggin was a gift of Dr. R. Harland. The 2-pimonidazole and a corresponding mouse monoclonal antibody were obtained from Chemicon (Temecula, CA).

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Fig. 2. Distribution of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and caspase 3-positive cells in hypoxia induced by I/R. A and B: TUNEL techniques and immunohistochemistry for caspase 3 (C and D) was performed in kidney samples obtained 24 h after I/R (n = 7 for each I/R group) (B, D), or kidney samples from sham-operated rats (n = 5 for each control group) (A, C). A clear staining localized to proximal tubular cells from the inner and outer medulla area was observed at the first stage of damage induced by acute tubular necrosis (ATN). Staining for TUNEL and caspase 3 was done using fluorescence (Texas red and green colors reaction, respectively). Scale bar = 100 μm. The arrows show the markers' location.
2-Pimonidazole protein adduct immunohistochemistry. Renal hypoxia was assessed by using the hypoxia-sensitive marker 2-pimonidazole as was previously described (40). Ninety minutes before death, rats were given 2-pimonidazole (60 mg/kg ip). Anesthetized animals were opened with a midline incision, and the kidneys were quickly removed, cut transversally, and fixed by immersion in Bouin’s solution. The tissue was then prepared for routine histological examination.

The incorporation of 2-pimonidazole was assessed immunohistochemically from 5-μm paraffin sections by using standard staining procedures (39).

Immunoblotting. Whole medulla kidney sections (~1 mm thick) were homogenized with an Ultra-Turrax in buffer EDTA 0.05 M, PBS (pH 7.4) added with a protease inhibitor cocktail (Pierce, Rockford, IL). The protein concentration was determined through the Bradford method (Bio-Rad, Richmond, CA). Western blotting was performed as described by Harlow and Lane (17). Positive controls (embryonic murine kidney E15) were used in parallel. For SDS-PAGE, proteins were mixed with sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), boiled for 3 min, separated on 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Blocking was carried out by incubation in blocking solution [8% nonfat dry milk in Tris-buffered saline-0.1% Tween-20 (TBS-T)] for 2 h at room temperature. After blocking, the membranes were probed with the corresponding antibody for 18 h at 4°C, washed with TBS-T, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was detected using the enhanced chemiluminescence technique (PerkinElmer, Life Sciences, Boston, MA). The positive control was an embryo at 15 days of development, and the negative control was whole medulla proteins of a sham-operated animal.

Detection and quantification of renal cell apoptosis by in situ end labeling of fragmented DNA [terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method] and caspase 3 detection.

Apoptotic cells in kidney tissues slices were visualized using the Apop tag fluorescein in situ apoptosis detection kit by the indirect TUNEL method from Chemicon (Temecula, CA) following the manufacturer’s protocol. The paraffin-embedded tissue sections were hydrated and permeabilized with proteinase K (20 μg/ml) for 15 min, incubated with equilibration buffer for 5 min and with tdt enzyme for 60 min; the reactions were ended by a stop/wash buffer for 10 min. Sections were incubated with a antidigoxigenin conjugate for 30 min at room temperature, washed in PBS, and blocked with PBS/BSA for 60 min, after which they were incubated with an anticaspase 3 antibody (Promega, Madison, WI) at 4°C for 12 h. The sections were incubated with a green fluorofor (Alexa 488), polymer conjugated to goat anti-rabbit Ig (Molecular Probes, Eugene, OR). The immunoreactive fluorescein was visualized using a VECTASHIELDS + DAPI (Vector Laboratories, Burlingame, CA) as the substrate to stain nuclei.
added one drop of Anti-Fade solution (Molecular Probes, Eugene, OR). The fluorescence was viewed by microscopy using an appropriate excitation and emission filters.

**Determination of functional and tissue damage.** Functional damage was assessed through serum creatinine levels and creatinine clearance. Blood samples were obtained from the inferior vena cava. For 24-h urine collection, rats were placed in metabolic cages. Urine and plasma creatinine were assayed in a Beckman analyzer (Beckman Coulter, Fullerton, CA). Creatinine clearance over 24 h was calculated according to the standard formula $C = \frac{U \times V}{P}$, where $C$ is creatinine clearance, $U$ is creatinine urinary concentration, $V$ is the urine flow rate per minute, and $P$ is creatinine plasmatic concentration. Tissue damage was evaluated through periodic acid-Schiff (PAS) staining. Immunolocalization of ED-1 and $\alpha$-SMA were used as markers of tissue damage.

**Statistical analysis.** The differences were assessed with the non-parametric test of Mann-Whitney for pairwise comparisons when overall significance was detected. The significance level was $P < 0.05$.

**RESULTS**

**Determination of functional and histological damage.** The induction of renal damage by I/R was verified by the serum creatinine levels as an indicator of renal function. Serum creatinine levels in rats before I/R protocol was $0.6 \pm 0.1$ mg/dl and increased to $1.2 \pm 0.3$ mg/dl during the early phase of ATN (24 to 48 h after I/R). The creatinine clearance before I/R was $1.2 \pm 0.3$ ml/min and decreased to $0.8 \pm 0.2$ ml/min during the early phase of ATN. No changes were observed in control rats ($P < 0.05$).

Two indicators of renal damage were used after I/R: the presence of macrophages (ED-1) and $\alpha$-SMA. Immunostaining for these markers in postischemic rats showed an increase in these markers in interstitial space (Fig. 1, A and B). Periodic acid-Schiff staining of renal sections after the induced damage showed alterations in kidney morphology consistent with ATN, such as brush borders and epithelia flattening (Fig. 1, C and D). No changes were observed in sham-operated animals (Fig. 1, E–H). Additionally, in PAS-stained slides from 24–48 h I/R kidneys, a significant amount of mitosis could be observed in proximal tubule cells (Fig. 1, C and D) that was not observed in control rats (Fig. 1, G and H).

In situ cell death detection and caspase-3 immunohistochemistry. TUNEL was used to detect DNA double-strand breaks. Sham-operated nonischemic kidneys did not show a positive reaction in proximal tubular cells (Fig. 2A). In ischemic groups, TUNEL staining was predominantly localized to proximal tubular cells and some collecting duct cells from the inner and outer medulla area in ischemic groups (Fig. 2B). Signals above background level in TUNEL were detected in sham-operated animals [10.76 per high power field (hpf)]
and markedly increased at 24 h after I/R, when TUNEL stained the whole nucleus (about 23.56/hpf) (Fig. 2B) but decreased gradually thereafter. The majority of TUNEL-positive cells exhibited morphological features of apoptotic cell death (shrinkage of cytoplasm and condensation of nucleus). Additionally, we found a small portion that showed diffused cytosolic staining, which exhibited morphological changes, indicative of necrosis.

We examined caspase-3 to clarify the relationship between caspase-3 and renal apoptosis in ischemic rat kidney. Nonischemic cells from control group did not show caspase-3 staining (Fig. 2C). Caspase-3 immunostaining dramatically increased in the inner and outer medulla, at 24 h (24.85/hpf) after I/R (Fig. 2D), and decreased somewhat after 48 h after I/R. At 24 h, the majority of caspase-3-positive cells had apoptotic morphology, such as shrinkage of cell bodies and condensation of nuclei, and the staining was predominantly present in the proximal tubular cells and in a smaller degree in collecting duct cells. The correlation between caspase-3 and TUNEL was evident at 24 h postischemia. The positive correlation ($r = 0.721$) between caspase-3 protein and TUNEL was significant ($P < 0.05$).

Detection of hypoxic tissue by 2-pimonidazole and markers induced by hypoxia: HIF-1α, VEGF, and Tie-2. To establish the infarcted area affected by ischemia, we evaluated the 2-pimonidazole presence. Control or vehicle-treated rats showed no 2-pimonidazole staining (Fig. 3E). However, an intense 2-pimonidazole staining was observed at 24 and 48 h after I/R in the inner and outer medulla area of kidney (Fig. 3A). The staining decreased at 72 and 96 h after I/R (data not shown).

As reported previously, no staining for HIF-1α was observed in control kidneys (Fig. 3F). However, after renal artery clamping, strong nuclear accumulation of HIF-1α occurred within 30 min. HIF-1α staining increased and reached its maximum at 24 h after I/R (Figs. 3B and 7.1A); from 48 h onward, the number of HIF-1α-positive cell nuclei declined, disappearing at 96 h (Fig. 7.1A). The expression of both markers, 2-pimonidazole and HIF-1α, was observed in papillary collecting ducts, thick ascending limb, and proximal tubular cells, mainly localized in the inner and outer medulla of kidneys with I/R (Fig. 3, A and B).

Regarding induction of two markers induced by hypoxia VEGF and Tie-2, known by their angiogenic character, we observed a marked expression of VEGF at 24 h after I/R (Figs. 3C and 7.1B), which was significantly decreased by 72–96 h. Tie-2 had the highest expression at 48 h after I/R (Figs. 3D and 7.1C), decreased at 72 h and was completely inhibited at 96 h (Fig. 7.1C). The expression of both proteins was observed in proximal tubule cells mainly localized in the outer medulla (Fig. 3, C and D).
Expression of mesenchymal, epithelial, and tubular markers in kidney after I/R. Mesenchymal, epithelial, and tubular markers showed a differential expression pattern for sham-operated and I/R rats. ATN, induced by I/R, leads to an altered distribution pattern of mesenchymal markers: bFGF, Noggin, and Ncam; epithelial markers: BMP-7 and Engrailed; and tubular markers: Lim 1–2 and ZO-1 (Fig. 4, 5, 6). In the healthy adult kidney, these proteins could be detected in collecting duct cell nuclei, with the highest expression in the papilla. Cells from proximal tubules, glomeruli, or peritubular cells localized in the inner and outer medulla and in medullary rays were devoid of labeling. No change was observed after 30 min of I/R. In contrast, after 24–48 h of I/R, a marked expression of these proteins was observed in the outer medulla. bFGF and BMP-7 were localized in proximal tubule cells, and Ncam, Engrailed, Lim-1, ZO-1 and Noggin were localized in the peritubular area in the inner and outer medulla (Figs. 4, A and B, 5, A–D, and 6B). The highest expression for mesenchymal and early epithelial markers bFGF, Ncam, and Noggin was observed at 24 h after I/R (Fig. 7.2, D and E and 7.4L). This is the same pattern that has been previously reported for Pax-2 and vimentin in the regenerative phase, consistent with cell dedifferentiation and coincident with proliferation (18, 48) (Figs. 7.2, F and G, and 4, C and D). For later epithelial and tubular markers, BMP-7, Engrailed, Lim 1–2, and ZO-1, the highest expression was observed at 48 h after I/R (Figs. 5, D and H and 7.3, H–J). Positive cells could be identified as belonging to regenerating proximal tubules in inner and outer medulla, according to the high flattening epithelium and remnants of the damaged brush border detected in the lumen of the tubules. The expression of all proteins in proximal tubule cell and peritubular zone was found to be decreased at 72 h after I/R (Fig. 7.1 and 7.2). At this point, all proteins were barely detectable in the proximal tubules, and the expression was again restricted to collecting duct cells. No expression was observed in sham I/R rats (Figs. 4, E–H, 5, E–H, and 6F).

BMP-7, a survival factor for undifferentiated mesenchyme (11), is antagonized by Noggin protein (6, 43) and phosphorylates the transcription factors Smad 1 and 5 (25). The transcription factors Smad (total and phosphorylated) were studied in regenerating cells after I/R. Reexpression of both states, was observed in proximal tubular cells from inner and outer medulla; the maximum levels of Smad and p-Smad were observed at 24–48 h after I/R (Figs. 5, C and D and 7.4, M and N), decreasing at 72–96 h after I/R (Fig. 6, C and D).

Levels of differentiation markers on ATN kidneys. To study the effect of ATN induced by I/R on the levels of the different markers in rat kidneys, freshly prepared extracts from inner and outer medulla kidney sections were analyzed with the corresponding antibodies. Compared with control kidney ho-
Apoptosis is a programmed mode of cell death that plays an important role in the pathogenesis of the renal ischemia. This was upregulated in a time-dependent manner, as judged by a marked increase in its immunoreactivity. We observed a high degree of colocalization of TUNEL and caspase-3 in a time-dependent manner. The maximum expression of TUNEL and caspase-3 protein peaked at 24 h after I/R. Additionally, PAS staining indicated that ischemic tubular cells showed apoptotic features at 24–48 h after I/R, which was delayed when compared with the upregulation of TUNEL and caspase-3. These data are in agreement with previous reports in ischemic neurons in which was shown morphological changes several hours after caspase-3 upregulation (32). The regulation of caspase-3 was related to morphological changes of ischemic tubular cells and could play a regulatory role in renal injury after kidney ischemia, because when the expression of TUNEL and caspase 3 disappeared, the tissue began to express repairation proteins, in this case, morphogenic and epitheliogenic proteins.

Embryonic kidney development is characterized by the proliferation of dedifferentiated cells and later redifferentiation of daughter cells into specific cell phenotype. A similar sequence of events can be observed during the regeneration process, indicating the possibility that renal regeneration may repeat part of the kidney genetic program during organogenesis, including apoptosis (2).

Apoptosis is a programmed mode of cell death that plays an important role in the pathogenesis of the renal ischemia. Caspases play a key role in the mammalian apoptotic mechanism, caspase-3 being a prototypical component of this mechanism. This was upregulated in a time-dependent manner, as judged by a marked increase in its immunoreactivity. We observed a high degree of colocalization of TUNEL and caspase-3 in a time-dependent manner. The maximum expression of TUNEL and caspase-3 protein peaked at 24 h after I/R. Additionally, PAS staining indicated that ischemic tubular cells showed apoptotic features at 24–48 h after I/R, which was delayed when compared with the upregulation of TUNEL and caspase-3. These data are in agreement with previous reports in ischemic neurons in which was shown morphological changes several hours after caspase-3 upregulation (32). The regulation of caspase-3 was related to morphological changes of ischemic tubular cells and could play a regulatory role in renal injury after kidney ischemia, because when the expression of TUNEL and caspase 3 disappeared, the tissue began to express repairation proteins, in this case, morphogenic and epitheliogenic proteins.

Fig. 7. Immunoblot of nephrogenic proteins in acute renal failure ARF induced by I/R. 7.1 Expression of endothelial cell markers in kidney regeneration was studied by immunoblot for transcription factors: HIF-1α (A), VEGF (B), Tie-2 (C) at 24, 48, 72, and 96 h after a 30-min ischemia. An increase was observed in HIF-1α and VEGF levels, with a maximum at 24 h, followed by a decrease that was similar to control levels at 96 h. For Tie-2, the increment was maximum at 48 h and decreased at 96 h after ischemia to control levels. E15 is embryonic 15 day (positive control), and sham refers to kidney extracts from sham-operated rats. 7.2 Expression of metanephric mesenchymal and early epithelial markers in kidney regeneration was studied by immunoblot for morphogenic proteins bFGF (D), Ncam (E), Pax 2 (F), and vimentin (G) at 24, 48, 72, and 96 h after a 30-min ischemia. An increase of MM markers can be observed; vimentin and bFGF reach a maximum at 24 h after ischemia; however, the levels of both markers decreased at 72 h and returned to a basal level at 96 h. The early epithelial proteins Pax-2 and Ncam are expressed from 24 h to 72 h and disappeared at 96 h after ischemia. 7.3 Expression of epithelial and tubular markers in kidney regeneration was studied by immunoblot for morphogenic proteins BMP-7 (H), engrailed (I), and Lim 1–2 (J) at 24, 48, 72, and 96 h after a 30-min ischemia. An increase in epithelial and tubular proteins can be observed, with a maximum at 48 h and a decrease at 72 h after ischemia (n = 7). 7.4 Expression of transcription pathway for BMP-7 was studied by immunoblot for morphogenic proteins BMP-7 (K), Noggin (L), and the nonphosphorylated (M) and phosphorylated form (N) of the transcription factor Smad at 24, 48, 72, and 96 h after a 30-min ischemia. A maximum increase in BMP-7 and Noggin protein levels were observed at 48 and 24 h, respectively, followed by a decrease at 72–96 h after ischemia. The Smads proteins show an increase with a maximum at 48–72 h and decreased at 96 h after ischemia (n = 7 for each I/R group and n = 5 for each control group).
been implicated in several aspects of cell survival and induction of apoptosis (15). Because the area of HIF-1α expression overlapped with areas in which we found increased nephrogenic and epitheliogenic proteins (7), it is possible, that the upregulation of HIF-1α plays an important role in cell death and/or survival decision, inducing the cell to express morphogenetic proteins. VEGF and Tie-2 are known to be regulated by HIF-1α in an oxygen-dependent fashion. VEGF is an angiogenic factor that undergoes transcriptional and posttranscriptional induction by hypoxia. In several tissues, there is a direct relation between hypoxia and angiogenesis (48); for this reason, VEGF may have an important role in the vascular response to kidney ischemia (14). In our study, we examined the expression of VEGF after kidney injury; this factor was reexpressed in the regenerating proximal tubules and peaked at 24 h after I/R. This factor could contribute toward reestablishing the integrity of epithelial and endothelial cells after kidney damage. The angiopoietin receptor Tie-2, plays an important role in nephrogenesis, angiogenesis, and stabilization of vascular integrity. In our study, we observed a marked re-expression of Tie-2 at 48 h after injury induced by I/R. Because Tie-2 has a role in vascular growth in the early stages of mammalian nephrogenesis, we postulated that in kidney regeneration process Tie-2 could reestablish the integrity of interstitial and glomerular vessels. The differential expression of VEGF and Tie-2 is similar to the one observed in nephrogenesis and can be explained by the requirements for VEGF that has Tie-2 for its induction.

The results obtained indicate that renal adult cells have the capacity to reexpress specific proteins of kidney development during recovery from a transient episode of ischemia, suggesting that these cells participate in renal repair. bFGF, Ncam, BMP-7, Lim-1, Engrailed, and ZO-1 are known to play a crucial role during early metanephric kidney development (15). After kidney injury, these proteins were locally restricted and reexpressed in the regenerating proximal tubules. This expression was limited to a time interval of 24 to 72 h, peaking 24 h after I/R for bFGF and Ncam and 48 h after I/R for BMP-7, Lim-1, Engrailed, and ZO-1. These results are similar to other reported previously in kidney development (15), where bFGF and Ncam, which are nephrogenic proteins, are expressed first, and BMP-7 and ZO-1, which are epitheliogenic proteins, are induced secondly. In advanced processes of kidney development, Lim-1 protein acts as a molecule involved in the conversion of aggregated cells into nephron (21). This protein is found in the mesenchymal cells after they have condensed around the ureteric bud, and its expression persists in the developing nephron until embryonic day 19 (21). There are no reports on this protein in mature kidney; however, in this report, we have observed the induction of Lim-1 in the regeneration process, sequentially after the induction of nephrogenic proteins, indicating a temporal and spatial expression, similar to one reported in kidney development (15). In addition, its expression in proximal tubular cells declined after reconstitution of the tubule. This transient reexpression is similar to the one reported for Pax-2 (18).

Vimentin is a marker of mesenchymal cells and therefore is a marker of fully dedifferentiated renal epithelia. It is not present in healthy adult tubules but its reexpression occurs during tubular regeneration and proliferation (48). The early presence of these proteins in our model shows that these cells acquire a similar state to the one in early development, mimicking this process in the regeneration phase after I/R in ATN. The biological importance of nephrogenic proteins during development and adulthood includes the expression of bcl-2 and bax proteins, with antiapoptotic and proapoptotic roles during early metanephric development. In adulthood, these genes have been shown to be reexpressed in proximal tubular cells after ischemic damage (33). These examples are consistent with the hypothesis that during tissue regeneration a cascade of developmental gene pathways may be reactivated.

An important result was to demonstrate the presence of Noggin, a BMP-7 antagonist during reperfusion postischemia. In kidney, there have not been reports of any antagonist during development; however, we postulated that the presence of BMP-7 should implicate the presence of another protein with the capacity to regulate it. In another system, Noggin, Chordin, Follistatin, and Gremlin have been reported to regulate BMP-7 (6, 43, 13, 28). Noggin was clearly observed 24 h after damage. These data are relevant, because the high levels of Noggin are coincident with the low levels of BMP, indicating some kind of regulation. It is possible that Noggin could be generating specific signal to determine one particular cell type, or it could make cell groups sensitive to a specific morphogen; for example, BMP-7. Furthermore, this indicates that Noggin would be involved in kidney regeneration process after kidney damage.

Recent reports have shown that BMP-7 signaling, mediated by Smad proteins, is important during kidney development (45). The spatial and temporal expression patterns of the Smads have been described in mesenchymal cells of the nephrogenic zone during kidney development (45) during which Smads are downregulated once these cells begin to epithelialize. We observed Smad and p-Smad (the active form) reexpression after damage, with a maximum at 24–48 h after I/R, period in which we observed the presence of nephrogenic proteins and the highest levels of Noggin and BMP-7. Its lowest expression was at 72–96 h after I/R, coincident with the high levels of epitheliogenic proteins, similar to what was reported in embryo (21). On the basis of the observed patterns of expression, we speculate that individual or a combination of Smads could play specific roles in the early regeneration phase of ATN during kidney damage and be involved in nephrogenic process, in a similar way to the one described in kidney development.

In summary, our results suggest that during the regeneration processes, the nephrogenic proteins can be reexpressed to restore mature kidney function in a process similar to the one described in nephrogenesis during embryonic development, because proteins expressed at 24 h are mainly involved in morphogenic processes and early epitheliogenesis and proteins expressed at 48 h are involved in later epitheliogenesis and differentiation.

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