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NEPHROLOGY FORUM

Actin cytoskeleton in ischemic acute renal failure

Principal discussant: BRUCE A. MOLITORIS

Indiana University School of Medicine, Indianapolis, Indiana

CASE PRESENTATION

A 36-year-old African American man was evaluated in the emergency room at Wishard Memorial Hospital following a motor vehicle accident. The patient's car was hit from behind, and as he fled the scene of the accident, he fell and rolled down a hill. He was subsequently detained by police and brought to the hospital for evaluation of diffuse trauma and myalgias. In the emergency room, he was awake and alert but tachycardic, with a heart rate of 136 beats/min, a respiratory rate of 20 breaths/min, and a blood pressure of 104/57 mm Hg. The trauma team's evaluation disclosed no significant injuries. However, he was admitted for evaluation and hydration, and because of an elevated serum creatinine level.

His medical history was remarkable for a solitary right kidney, excessive alcohol intake, and use of crack cocaine, but he denied illicit intravenous drug use. He was taking no prescribed medications and had no history of diabetes, hypertension, or past surgeries. He had no known allergies. The baseline serum creatinine had been 1.2 mg/dL 8 years prior to admission. A review of systems was negative, with the exception of diarrhea over the previous 48 hours.

Physical examination revealed a somewhat anxious, mildly obese black man in no apparent distress. The examination was otherwise within normal limits except for diffuse mild muscle pain.

Laboratory values on admission revealed: serum sodium, 139 mEq/L; potassium, 4.9 mEq/L; chloride, 103 mEq/L; bicarbonate, 9 mEg/L; creatinine, 2.0 mg/dL; total protein, 6.5 g/dL; albumin, 3.5 g/dL; calcium, 9.2 mg/dL; bilirubin, 1.7 mg/dL; hemoglobin, 14.1 g/dL; white blood cell count, 12,600/mm^2 with a normal differential; platelets, 245,000; a normal peripheral smear; and lactate, 4.7 mEq/L. Urinalysis disclosed cloudy urine containing >300 mg/dL protein; pH, 5.5; 4+ hemoglobin on dipstick; 5 red blood cells/high-power field; and no casts, but the urine was qualitatively positive for myoglobin. The initial serum creatine phosphokinase (CPK) was 14,565 units/mL; aspartate aminotransferase (AST), 2470; and alanine aminotransferase (ALT), 1785 U/mL.

The patient was aggressively hydrated with normal saline at 200 mL/hour. After receiving 1 L, the infusion was altered to 5% glucose with 3 ampules of bicarbonate added per liter and administered at 300 mL/hour. Twenty-four hours following admission, the patient's laboratory values were: sodium, 133 mEq/L; potassium, 4.9 mEq/L; chloride, 96 mEq/L; bicarbonate, 22 mEq/L; blood urea nitrogen (BUN), 36 mg/dL; and creatinine, 2.3 mg/dL. The CPK was 134,470 U/mL; calcium, 6.1 mg/dL; magnesium, 3.7 mg/dL; and phosphate, 6.3 mg/mL. The albumin and total protein had decreased to 2.5 g/dL and 5.0 g/dL, respectively, and the hemoglobin had decreased to 11.7 g/dL without evidence of bleeding; the level remained stable thereafter. Over the first 24 hours, the patient had received several liters of fluid and had produced 2.2 L of urine. Repeat urinalysis was unchanged, except that the pH had increased to 7.0 and numerous amorphous casts were present. Indirect immunofluorescent staining revealed fragmented and vesicular material both within casts and free floating that stained for F-actin, actin depolymerizing factor, and cofilin (Fig. 1).

Over the course of his hospital stay, he remained nonoliguric. The CPK, AST, and ALT levels steadily declined,

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and the serum creatinine peaked at 6.4 mg/dL on hospital day 6. He did not undergo renal replacement therapy and was discharged on day 8 from the hospital with a serum creatinine of 4.3 mg/dL that was decreasing daily.

DISCUSSION

DR. BRUCE A. MOLITORIS (*Director, Nephrology Division, Professor of Medicine, and Director, Indiana Center for Biological Microscopy, Indiana University School of Medicine, Indianapolis, Indiana, USA*): This case represents a typical presentation of multifactorial acute renal failure (ARF). Prerenal azotemia, cocaine-mediated vasoconstriction and trauma resulted in acute tubular necrosis (ATN) secondary to rhabodmyolysis and renal hypoperfusion. The pathophysiology and clinical aspects of rhabdomyolysis-induced ATN have recently been reviewed [1] and is little changed from the excellent 1996 article by Zager [2]. Therefore, this case will be used as a model of renal ischemia resulting in cellular actin alterations in epithelial, endothelial, and smooth muscle cells as a mechanism of cellular injury and death mediating kidney dysfunction.

The patient was treated aggressively with volume replacement and bicarbonate therapy but he remained nonoliguric for the duration of his hospital stay. Since the level of serum creatinine increased by greater than 1.5 mg/dL, but the patient did not require renal replacement therapy, I would describe this as moderate ARF. Although the initial urine analysis was nondiagnostic, the subsequent presence of hemoglobin, myoglobin, and muddy brown casts (Fig. 1A) left little doubt as to the diagnosis and etiology of ATN in this patient. In a previous case of rhabdomyolysis, secondary to rigorous exercise, we had the highly unusual advantage of having obtained an early renal biopsy, taken within 24 hours of the initiating event, to help delineate the pathophysiology of clinical ATN. As Figure 2 illustrates, there was severe proximal tubule injury with loss of apical microvilli, accumulation of proteinaceous material within the lumen, detachment of proximal tubule cells (PTCs), and increased white blood cells within the peritubular microvascular space. Unfortunately, the biopsy material was limited to the cortical region, so the outer medullary area could not be evaluated.

The pathophysiology of ischemic ARF involves a complex interplay among cell injury, inflammation, and altered renal hemodynamics. While no single overarching theme can tie the myriad of injurious consequences together, alterations in the actin cytoskeleton have been shown to affect multiple cell types and physiologic processes within the kidney during and following ischemic injury. Furthermore, these actin alterations play a well-documented role in the initiation and extension phases of ischemic ARF in animal models. Therefore, the purpose of this Forum is to describe and interrelate the known abnormalities that occur in epithelial, endothelial, and smooth muscle cell actin cytoskeletons during ischemia and reperfusion to what is known about the pathophysiology of ischemic ARF. Finally, exciting actin cytoskeletal pathways have been documented recently and might provide yet a further integrative step in the role of actin alterations, cellular injury, and organ dysfunction in ischemic ARF.

Physiology

The actin cytoskeleton plays an ever increasingly understood role in mediating interactive processes necessary for cellular and organ structure and function. New and exciting information regarding the dynamic aspects of the actin cytoskeleton and its intracellular regulation is unfolding at a rapid rate. Actin cytoskeletal-surface membrane interactions mediating such diverse cellular events as cell polarity, endocytosis, exocytosis, division, migration, cell-cell and cell-extracellular fluid (ECF) adhesion, signal transduction, and ion channel activation are part of an ever-growing list of cellular processes dependent upon precise polarization and regulation of actin assembly and disassembly [3–5]. Ischemia rapidly induces duration-dependent structural alterations in the cellular actin cytoskeleton in both in vivo and in vitro studies [5–9]. Furthermore, recent data now implicate these alterations as fundamental to the cellular and organ dysfunction that ensue.

Actin filaments, in concert with actin-binding proteins, mediate actin-dependent cellular processes. Actin filaments are arranged into "higher order" forms including a cortical actin network (actin gel), stress fibers (loose nonparallel bundles), and tight parallel bundles seen in microvilli [5]. These F-actin structures have different functions, lengths, and cellular locations. They are influenced differently by cellular regulatory processes, such as Rho guanosine triphosphatases (GTPases), but their generation and maintenance comprise a dynamic, energy-dependent process requiring F-actin assembly and disassembly. Assembly and disassembly of actin filaments are the central steps in actin-dependent cellular processes. The rate of this turnover determines sensitivity to cellular adenosine triphosphate (ATP) depletion, with more rapid turnover increasing sensitivity [10]. Actin filaments are polar, with a rapid-growing barbed end and a slow-growing pointed end [11]. Disassembly occurs primarily by dissociation at the pointed end, releasing G-actin bound to adenosine diphosphate (ADP). Once released, ADP-G-actin undergoes nucleotide exchange with abundant cytosolic ATP, and the resulting ATP-G-actin is rapidly bound by thymosin-sequestering proteins and stored as a high-energy intermediate until needed for actin polymerization [4, 5, 11].

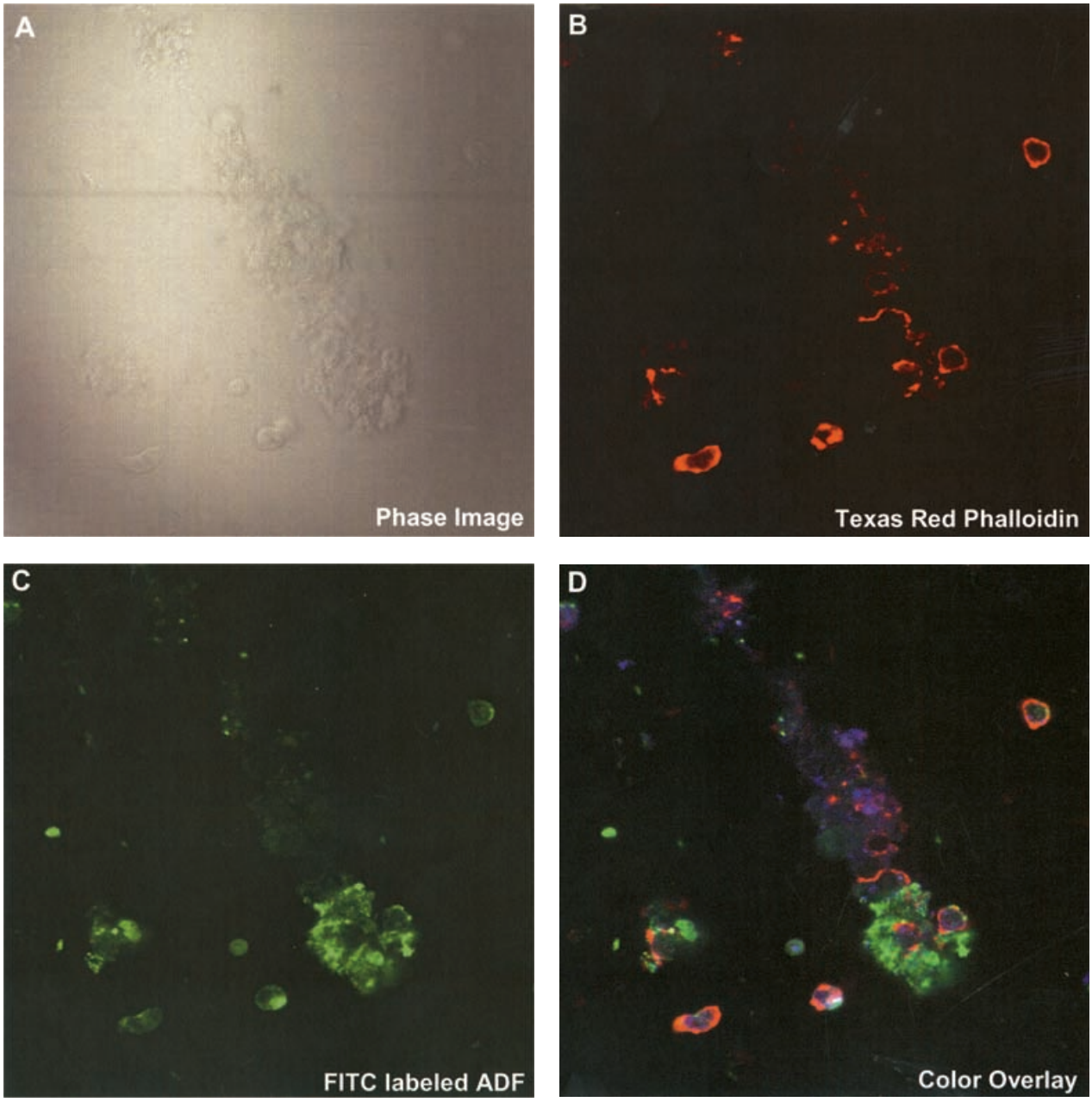

Fig. 1. Urinary casts and cellular debris contain actin and actin depolymerizing factor proteins. On day 2 of the hospitalization a fresh urine sample was processed for urinalysis and the sediment fixed with 4% paraformaldehyde. The sample was stained for F-actin utilizing Texas red phalloidin. Indirect immnofluorescent staining was used to identify actin depolymerizing factor and also cofilin using specific primary antibodies and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for ADF and a CY-5 conjugated secondary antibody for cofilin. Images were obtained using confocal microscopy with sequential excitation. (*A*) A phase contrast image of a cellular cast and cellular debris. (*B*) Texas red phalloidin labeling indicates the cast has fragments and intact vesicles containing F-actin. (*C*) Staining for ADF, but selective cofilin staining is not shown separately. (*D*) An overlay shows close association of F-actin, ADF, and cofilin. This considerable overlap between F-actin, ADF, and cofilin in casts and debris is consistent with staining as previously reported in tubular lumens and the urine following ischemic injury in a rat model [20, 36].

In all cells, the assembly and function of actin are controlled by actin-binding proteins as well as by the inherent properties of actin [5]. The state of actin assembly depends upon four groups of actin-binding proteins. Sequestering proteins, like thymosins, bind G-actin and inhibit polymerization by reducing the "effective" or "free" G-actin concentrations. Other monomer-binding proteins enhance addition of G-actin at the barbed end (profilin) or stimulate dissociation at the pointed end [actin depolymerizing factor (ADF) or cofilin]. Capping proteins bind to filament ends and stabilize either the barbed or pointed ends. Severing proteins mediate filament cleavage, and dynamizer proteins (ADF/cofilin) function by enhancing filament growth and turnover.

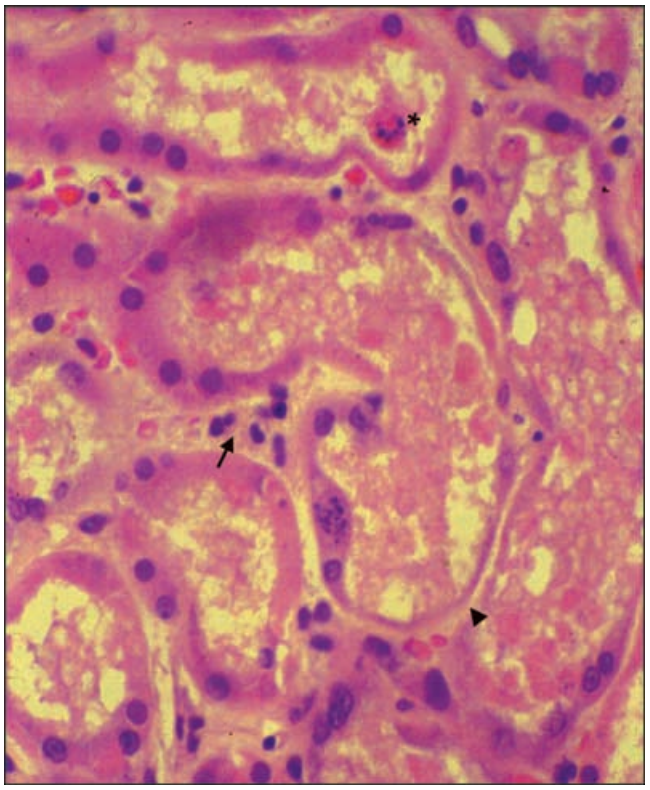

Fig. 2. Human renal biopsy showing proximal tubule injury. This image is a representative sample of a kidney biopsy for ARF, kindly provided by Dr. James Hasbargen, following exercise-induced rhabdomyolysis. The biopsy, obtained within 24 hours of the event, revealed significant proximal tubule cell damage with intraluminal accumulation of apical membrane fragments and a detached cell (∗), thinning of proximal tubular cells to maintain monolayer tubule integrity (arrowhead), and dividing cells and accumulation of white cells within the microvascular space in the peritubular area (arrow). The patient required renal replacement therapy but did regain complete renal function.

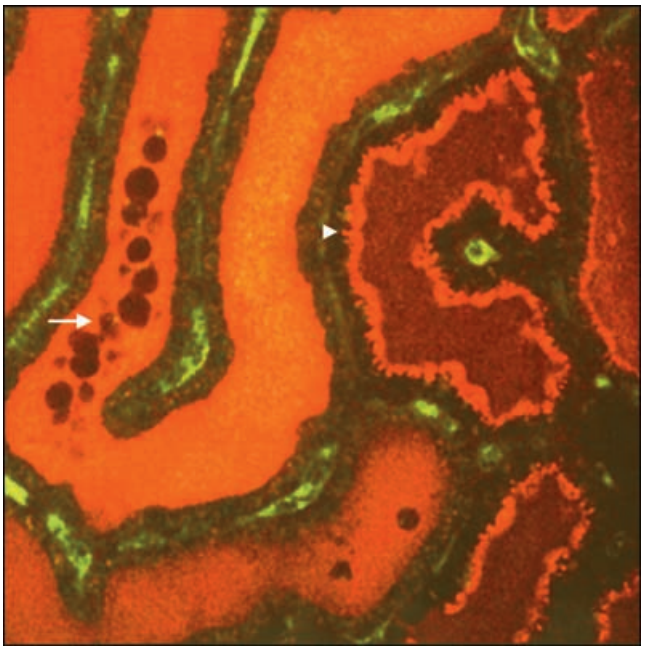

Fig. 3. Urinary cast formation following clamp ischemia in a rat model. Intravital two-photon microscopy of a rat cortex following 45 minutes of renal ischemia and intravenous infusion of a 3000 molcular weight Texas red dextran and a 500,000 molcular weight fluorescein isothiocyanate (FITC) dextran. The tubule lumen appears red as the small red dextran is freely filtered across the glomerulus. The intraluminal vesicular blebs appear as dark spheres as they do not contain the dextran material. The large green dextran outlines the peritubular microvasculature and dark figures within this microvasculature represent cells moving through it. A cast (arrow) is shown in a distal tubule when distal tubule cells can be identified by lack of cellular endocytosis of red dextran [81]. Shown on the right is a proximal tubule with endocytic uptake of the red dextran (arrowhead). For a movie showing the dynamic process, go to *http://www.blackwellpublishing.com/products/journals/suppmat/ki66/ki66-2_818/ki66-2.mov*.

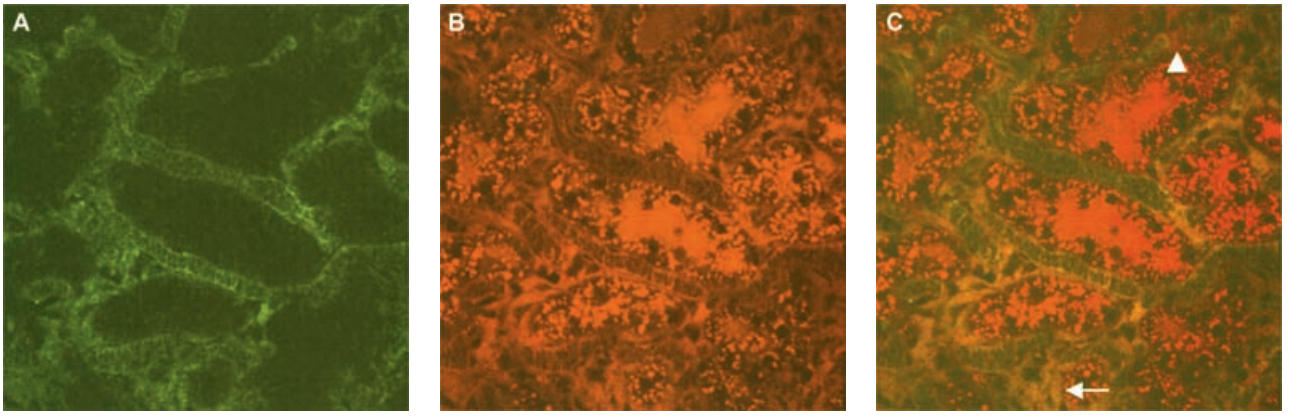

Fig. 4. Microvascular permeability defect following ischemic injury. A 3000 D rhodamine-labeled dextran and a 500,000 D fluorescein isothiocyanate (FITC)-labeled dextran were injected via the tail vein into a rat following 24 hours of reperfusion after 45 minutes of ischemia and were viewed using intravital two-photon microscopy [63, 81]. Images obtained from the same field. (*A*) The extent of microvascular extravasation of the high-molecular-weight dextran. (*B*) The extent of microvascular extravasation of the low-molecular-weight dextran. (*C*) A color overlay of (A and B), with the low-molecular-weight dextran in red and the high-molecular-weight dextran in green. The large arrow in (C) indicates an area of diminished microvascular flow and extravasation of both dextrans. The arrowhead shows only the small red dextran moving into the interstitial space.

Nucleation proteins mediate and enhance actin filament formation. The coordinated interactions of actin-binding proteins result in the continual dynamic remodeling of the cytoskeleton and allow for rapid responses to external stimuli and internal signaling cascades [3, 4, 11].

Pathology

Severe reductions in renal blood flow produce characteristic, rapidly occurring, and duration-dependent effects on the actin cytoskeleton of numerous renal cell types. Although best described for epithelial cells, documentation of effects on endothelial and smooth muscle cells now suggests that these alterations are independent of the cell type [6–9, 12, 13]. Even very short ischemic insults provoke cellular changes in surface membrane polarity and junctional complexes mediated by the actin cytoskeleton. The apical microvillar region of PTCs is particularly sensitive to ischemic insults, and rapid and severe but repairable damage occurs [13]. Within 5 minutes of ischemia, the microvillar F-actin core and its overlying plasma membrane show signs of degeneration [13, 14]. The plasma membrane no longer maintains its finger-like microvillar structure, but becomes more amorphous, while the internal F-actin core begins to deteriorate. With longer ischemic insults, the plasma membrane and microvillar F-actin cores degenerate further. This is easily seen in Figure 2, which indicates that similar events occur as early events in clinical ischemic ATN. The reason for this extreme sensitivity, compared to other tubular epithelial cells, might relate to the recently documented role of actin in endocytosis [15, 16]. Since PTC apical membranes carry out extensive endocytic events [17], the rate of actin polymerization must be very rapid, rendering it highly susceptibile to ischemic injury as ATP depletion occurs. Proximal tubule cell substrate requirements and energy utilization also render it particularly susceptible [18].

The actin-binding protein family of ADF and cofilin proteins plays a critical role in the apical microvilli breakdown in response to ischemia [19–21]. ADF, an important regulator of F-actin dynamics, binds F-actin in a pH-dependent manner and mediates F-actin severing and pointed-end depolymerization [22, 23]. The phosphorylated form of ADF is inactive and cannot bind actin [24–26]. LIM kinases, activated by Rho GTPases and testicular protein kinases, phosphorylate ADF/cofilin specifically at Ser-3 and thereby inhibit its function [22]. "Slingshot" is a recently described family of phosphates that dephosphorylate ADF/cofilin and thereby activate it [27]. Renal ischemia induces rapid dephosphorylation/activation and relocalization of ADF in PTCs [20, 21] from a diffuse cytoplasmic pattern to the surface membrane and to plasma-membrane-bound vesicles that have been shed into the PTC lumen [20, 21]. The mechanism of ADF penetration into the apical microvillar region involves ischemia-induced dissociation of tropomyosins from the terminal web [28]. Tropomyosins, a family of F-actin binding and stabilizing proteins, are localized to the terminal web, a region where the ADP-actin-rich microvilli microfilament pointed ends are located [29, 30]. Several in vitro biochemical and structural studies have demonstrated that ADF competes with tropomyosin for F-actin binding [31, 32]. In vitro studies also suggest that localization of tropomyosin at the terminal web not only stabilizes microfilaments but protects the filaments from binding by active ADF/cofilin proteins, thus preventing filament severing and depolymerization. In addition, ezrin, a phosphorylated protein that mediates microvillar F-actin to surface membrane binding, dissociates during ischemia and frees the surface microvillar membrane from its cytoskeletal attachments [33]. Therefore, ischemia-induced tropomyosin and ezrin dissociation from the terminal web and microvillar microfilaments allows for the rapid binding and destabilization of the apical membrane domain by ADF/cofilin proteins through disruption of microvilli microfilament cores [28, 33]. Finally, pharmacologic disruption of PTC F-actin induces apoptosis [34], and cofilin activation and mitochondrial translocation are necessary for apoptosis [35]. Therefore, cofilin and ADF activation play multiple roles in cell injury and cell death.

In PTCs, these apical microvillar alterations form membrane-bound, free-floating extracellular vesicles termed "blebs" that either undergo internalization or are released into the tubular lumen [20, 21, 36–39]. These transformations substantially reduce apical membrane surface area, thus decreasing the effective reabsorption area (Fig. 2). In addition, the membrane blebs and cytoplasmic contents shed into the tubule lumen often aggregate and cause tubular obstruction. That similar processes hold for human ischemic ATN is suggested by the staining pattern of the cast shown in Figure 1. F-actin, ADF, and cofilin were identified within the cast and as free-floating vesicular components within the urine, as was seen following ischemic injury in rats [20, 21, 36]. Both loss of surface membrane and tubular obstruction decrease the glomerular filtration rate (GFR), a hallmark of ischemic ARF [39]. Obstruction of the tubular lumen eliminates glomerular filtration within that nephron by increasing intratubular pressures to levels inconsistent with filtration. The formation of such a cast was recently documented in a rat following renal artery clamp ischemia, using intravital multiphoton microscopy (Fig. 3) (Quick Time movie is at the URL *www.nephology.iupui.edu/kiforum3*). Numerous surface membrane vesicles/blebs and other intralumenal material aggregate within the distal tubular lumen and then move as a unit. Casts can be passed into the urine or remain within the tubule and cause obstruction.

Two additional PTC-related events decrease GFR following ischemic injury. Reductions in proximal tubule ion and water reabsorption result in high distal delivery of Na^+, K^+, Cl^- and high distal flow rates. This, in turn, via tubular glomerular feedback (TGF), produces afferent arteriole constriction and its attendant reduction in GFR. Finally, opening of tight and occluded junctions results in the unregulated paracellular movement of ions and water, thereby causing increased backleak, the third mechanism of reduced GFR following ischemic injury [39]. In an eloquent series of experiments in transplant ATN, the laboratory of Brian Myers showed that afferent vasoconstriction secondary to activation of TGF, tubular obstruction, and backleak across open tight junctions are responsible for the decrease in GFR [40–42]. Therefore, ischemia-induced alterations in PTCs in large part account for the reduction in GFR occurring following ischemic injury.

In addition to the apical membrane changes, cellular junctional complex dissociation allows membrane lipids and integral and peripheral membrane proteins to diffuse laterally into the alternate surface membrane domain [12, 43, 44]. This loss of membrane polarity creates a more homogenous surface membrane without unique apical or basolateral membrane regions. Dissociation of cellular junctions also reduces cell-to-cell contacts and causes unregulated paracellular diffusion of water, ions, and macromolecules [12, 44, 45]. The now nonpolarized renal epithelial cells can no longer reabsorb and transport appropriately.

Both microvillar breakdown and loss of cellular polarity during ischemia are preceded by structural alterations in the actin microfilament network [13, 14, 43, 46]. These cytoskeletal changes reflect the severity and duration of the ischemic injury. The microvillar actin cores, the cytoskeletal meshwork of the terminal web, and the cortical actin of the epithelial cell are disrupted. The concentration of F-actin in the cell increases with the formation of F-actin aggregates in the cytoplasm [13]. We recently elucidated the mechanism responsible for unregulated actin polymerization and aggregation during ischemia [47]. With cellular ATP depletion, generation of ATP-G-actin from ADP-G-actin is not possible. Since thymosins, far and away the most quantitatively important actin-sequestering proteins in mammalian cells, cannot bind ADP-G-actin, it accumulates and, after exceeding its critical concentration, polymerizes. However, instead of polymerizing in a regulated fashion, large cytosolic aggregates accumulate, especially in the perinuclear region [6, 43, 48] and also near the junctional complexes and basolateral membrane [49]. The lack of active Rho A GTPase might be in part responsible for this abnormal F-actin distribution [50]. The well-known activation of ADF/cofilin proteins during ATP depletion accentuates this process by increasing F-actin depolymerization and thereby providing a source of more ADP-G-actin that cannot be sequestered and neutralized. Finally, the rapid depletion of cellular ATP-G-actin during cellular ATP depletion implies its rapid utilization [47].

Microvascular dysfunction

Recent studies indicate a central role for microvascular injury with attendant disruption of the endothelial cell actin cytoskeleton in the pathophysiology of ischemic ARF [51–53]. In particular, endothelial cell injury, including cell swelling, altered cell-cell attachment, and reduced endothelial cell-basement membrane attachment are some of the alterations that have been observed following ischemic injury [52, 54]. Functional consequences of these morphologic alterations include reduced blood flow to affected microvascular beds secondary to altered vascular reactivity, increased leukocyte adherence and extravasation, altered coagulation due to endothelial dysfunction, and increased interstitial edema secondary to increased endothelial permeability [55].

Specialized cellular junctions maintain endothelial cell-cell contacts. Tight junctions are more prominent in "tight" vascular beds such as between the endothelial cells of the cerebral vasculature forming the blood-brain barrier, whereas they are sparse and simplified in "leaky" vascular beds such as post-capillary venules [56]. Cadherin-containing adherens junctions are ubiquitous between endothelial cells throughout the vasculature [56]. Recent studies highlighting differences in the molecular composition of junctional complexes within the kidney provide insight into the potential functional differences of the cellular junctions in these vascular beds [57–59]. Disruption of endothelial adherens junctions in vivo, by the use of an inhibitory antibody to VE-cadherin, induces gaps between endothelial cells, increases endothelial permeability, and promotes the accumulation of inflammatory cells in coronary and pulmonary vascular beds [60]. Furthermore, in vitro studies suggest that the interaction of endothelial cell-cell junctions with the actin cytoskeleton plays an important role in regulating endothelial paracellular transport [61]. Data also indicate that microvascular congestion and localized interstitial edema follow renal ischemia [55, 62, 63]. However, a direct role for altered junctional function in increased permeability following ischemic injury is lacking.

The finding that circulating von Willebrand factor (vWF) increased following renal ischemic injury further suggests that the renal vascular endothelium is injured [63]. In other organ systems, release of vWF from the endothelium occurs in a biphasic fashion in response to ischemia and reperfusion [64, 65]. These studies suggest that hypoxia, as well as reperfusion, mediate endothelial release of vWF via potentially different mechanisms. Furthermore, inflammatory processes can contribute to

endothelial vWF release [66]. The peak elevation of circulating vWF following renal clamp ischemia was monophasic and occurred 24 hours after the ischemic insult. Therefore, continued hypoxia, reperfusion injury, and/or inflammatory processes all could be playing a role in vWF release [63]. The utility of such a finding might lie in the ability to characterize the extent of the underlying injury by circulating markers of endothelial injury and thus provide a useful clinical correlate for disease severity, prognosis, and therapeutic intervention.

Examination of the morphologic alterations in renal microvascular endothelial cells revealed that the normal structure of the actin cytoskeleton is disrupted during ischemia [63]. Alterations of the normal actin cytoskeleton of endothelial cells in cell culture have been demonstrated with ATP depletion and with H_2O_2 as a model of oxidant-mediated reperfusion injury. ATP depletion rapidly and reversibly disrupts the normal cortical and basal F-actin structures in endothelial cells and results in F-actin aggregation and polymerization [8, 67, 68]. Oxidant-mediated endothelial cell injury also disrupts the cortical actin band in cultured endothelial cells [69–71]. The actin cytoskeleton in endothelial cells of the renal microvasculature subjected to ischemic injury in vivo was most prominent immediately following the ischemic insult [63]. There was an increase in F-actin polymerization and aggregation at the basal and basolateral aspects of endothelial cells following ischemia. This F-actin polymerization and aggregation were reminiscent of the alterations observed following ischemia in proximal tubular epithelial cells [6, 43, 48] and renal vascular smooth muscle cells [6].

Alterations in the actin cytoskeleton of renal microvascular endothelial cells preceded alterations in VE-cadherin staining at endothelial cell-cell junctions and increases in endothelial permeability [63]. These findings are consistent with a mechanism by which loss of integrity of the actin cytoskeleton contributes to breakdown of the actin-associated adherens junctions and contributes to the concomitant permeability defect. Much of the current knowledge regarding the mechanisms regulating endothelial cell-cell interactions during ischemic and oxidant injury has come from in vitro models utilizing cultured endothelial cells. ATP depletion of endothelial cell monolayers and exposure of endothelial monolayers to oxidants such as H_2O_2 have both been demonstrated to increase endothelial permeability and intercellular gap formation [72, 73]. Increased endothelial permeability in these models also has been associated with internalization of VE-cadherin from endothelial adherens junctions [74].

Vascular reactivity following ischemic injury might also in part be due to actin cytoskeletal alterations. Studies evaluating the effect of ischemia on vascular smooth muscle cells revealed disruption and aggregation of F-actin in all renal vessels studied [6]. Furthermore, the effects worsened with reflow in the corticomedullary vasa recta area. The authors [6] postulated that the lack of organized F-actin and increased F-actin polymerization could in part be responsible for the known loss of autoregulation of renal blood flow following ischemic injury [75].

Additional data implicate actin in the regulation of vascular tone with a potential causative role in reduced renal blood flow after ischemic injury. Endothelial nitric oxide synthase (eNOS) is directly regulated by the actin cytoskeleton with increases in F-actin associated with reduced eNOS activity [76]. This, taken in conjunction with data indicating that eNOS activity was necessary for minimizing ischemic ARF with endothelial cell infusion [52], suggests that endothelial NO generation might be reduced because of endothelial cell actin polymerization during ischemia. This could be particularly true in the cortical medullary region, where continued hypoxia exists for prolonged periods secondary to poor reperfusion [77].

These problems are compounded by the more rapid and complete reperfusion of the renal cortical area as compared to limited reflow of the cortical medullary area [55]. This rapid reperfusion of the cortex leads to rapid PTC repair, unlike the continued hypoxia in the cortical medullary area that results in progressive cellular injury of endothelial, smooth muscle, and epithelial cells. Since most pathology specimens of human ATN come from either autopsy specimens or a renal biopsy taken from nonrecovering ATN, it is quite possible that the early phase of injury to cortical PTC is not apparent. Therefore, the lack of renal biopsies within the first 24 hours following the initiation of ARF has limited our ability to judge the utility of animal models [78, 79].

The development of Tie2 mice [80] and the utilization of two-photon intravital microscopy [81] for visualizing the permeability defect in the renal microvasculature following ischemia gave us the ability to study renal microvascular endothelial injury (Fig. 4). The power of this imaging technique was demonstrated by the ability to simultaneously evaluate the disparity in the permeability defect of two different-sized fluorescent probes, and observe a correlation, in a timed image series, between alterations in blood flow and severity of the permeability defect [63]. These data imply a direct relationship between the extent and duration of continued reduced blood flow and the increased permeability defects between endothelial cells. While these observations were limited to the cortical area of rats because of technical considerations, the permeability defect in the corticomedullary area presumably could be even more pronounced than that observed in the cortical microvasculature [55, 77]. Implications for leakage of plasma from the vascular space and increased interstitial edema, especially in the corticomedullary area, include further

diminishment of the compromised medullary blood flow by extrinsic compression of peritubular capillaries [82] and by hemoconcentration, as observed in other organs [83].

QUESTIONS AND ANSWERS

DR. JOHN T. HARRINGTON (*Division of Nephrology, Tufts-New England Medical Center, Boston, Massachusetts*): Could you tell us a bit more about the normal structure and function of actin depolymerizing factor and what regulates it?

DR. MOLITORIS: ADF/cofilin is a noncapping, calcium-independent, F-actin binding/severing protein that also sequesters G-actin and is regulated by pH_i and phosphorylation [22]. It contains a nuclear localization sequence [84] and binds to PI and polyphosphoinositides [85]. At a low pH_i (<7.1), it binds to F-actin and forms 1:1 complexes with individual subunits [86]. As pH_i increases, it becomes destructive, severing F-actin and leaving uncapped barbed ends. Phosphorylated (serine-3) ADF accounts for 15% to 60% of total ADF [25] and is an active severing protein only in the dephosphorylated form. Two protein kinase families, LIMK1/2 and TESK1/2, are tissue-specific kinases that specifically phosphorylate and inactivate ADF/cofilin at the N-terminal serine residue [87, 88]. The LIM kinase families are activated through phosphorylation mediated by p21-activated kinase-1 (PAK1), p21-activated kinase-4 (PAK4), or Rho-associated kinase (ROCK) [89]. In turn PAK1 and PAK4 are activated through interactions with Rac/Cdc42, and ROCK is activated through Rho. TESK protein kinases are stimulated by an integrin-mediated signaling mechanism. The phosphatase protein family, slingshot, dephosphorylates ADF/cofilin and activates ADF/cofilin activity [90]. Very recent information indicates that the ADF/cofilin family also is involved in mediating receptor-mediated endocytosis and apoptosis. During ischemia, rapid dephosphorylation of ADF increases its activity and directly leads to F-actin destruction. Being able to prevent this activation would minimize morphologic changes in epithelial, endothelial, and smooth muscle cells. However, at present there is no known inhibitor of slingshot, and the kinases apparently have minimal function under conditions of ATP depletion.

DR. HARRINGTON: In the early 1970s, Leaf and colleagues at the Massachusetts General Hospital described microvascular injury with endothelial cell swelling as a critical finding in ischemic renal injury [54]. Do your observations fit with those earlier observations?

DR. MOLITORIS: It is unfortunate that the early observations by Leaf and colleagues were not extended. Recent information from several laboratories indicate that, indeed, endothelial cell injury and involvement in ischemic ARF are of critical importance. Using the Tie2 mouse model, Sutton et al showed early duration-dependent alterations in endothelial cell actin cytoskeleton that lead to permeability changes [63]. Goligorsky's laboratory showed the importance of eNOS in the protection provided by infused endothelial cells [52]. Still unstudied are the microvascular alterations of the cortical medullary region, a region of minimal reflow where continued epithelial cell injury occurs during the reperfusion period.

DR. RONALD PERRONE (*Division of Nephrology, Tufts-New England Medical Center*): You beautifully described how renal ischemia affects multiple subcellular pathways. I would like to know your thoughts regarding potential therapeutic interventions including interventions targeting more than one pathophysiologic event.

DR. MOLITORIS: The pathophysiology of ischemic and septic ARF is multifactorial in nature. Vasoconstriction, inflammation, coagulation, apoptosis, and cytoskeletal alterations are several of the broad categories involved. Within each category several areas also must be considered. For example, vasoconstriction occurs at the macrovascular level and is mediated by many factors such as endothelin. At the microvascular level, it is mediated by many factors including the lack of NO. Therapeutic interventions must take into account all these factors, so I do not believe a "silver bullet" exists. Several years ago we and other investigators suggested considering a multifaceted approach, such as the present-day approach to cancer therapy [91].

DR. PERRONE: In your opinion, what is the potential time course for intervention?

DR. MOLITORIS: The earlier the better, beginning with preventive measures in high-risk patients and extending through rapid treatment of newly diagnosed ARF. We recently reviewed the rationale behind early diagnosis and therapy [77]. We have postulated that an "extension phase" exists in ischemic ARF that is mediated by ongoing endothelial injury and lack of reperfusion, especially to the cortical medullary region. The existence of this phase would make early treatment of paramount importance. However, before we can make progress in this area, we must deal with several obstacles that currently limit our overall approach to the patient with ARF. First, we must realize that ARF is an independent risk factor for mortality, as has now been documented in several clinical studies, and convince our clinical colleagues that our early involvement in patient care is beneficial. Then we must develop criteria that will allow us to identify high-risk patients so that adequate preventive measures can be employed [92]. Finally, we must develop surveillance methods that allow for early diagnosis and ways to quantify the extent of initial injury. Fortunately several groups of investigators are working diligently in these last two areas to provide the information necessary. Also, the

American Society of Nephrology (ASN) has established a task force in ARF that is discussing many of these issues and is very interested in establishing an ARF clinical trials network to hasten progress in the treatment of ARF. Specifically, the identification of biomarkers of ARF is an urgent issue for this group. Qualitative and later quantitative markers will allow for rapid progress. At present, I am a believer in measuring serum creatinine more frequently (until a better marker is available) to help with the early diagnosis. Measuring the serum creatinine level only once every 24 hours in a high-risk patient, such as a postoperative surgical patient with preexisting chronic renal disease, is just not frequent enough. Perhaps other markers, such as cystatin C, will provide earlier detection in our patients [93].

DR. PERRONE: The role of the primary epithelial cell cilium has been extensively evaluated in polycystic kidney disease. Have you evaluated the role of cilia in ATN?

DR. MOLITORIS: To my knowledge, there have been no reports evaluating a role for the cilia in ATN.

DR. BERTRAND JABER (*Division of Nephrology, Tufts-New England Medical Center*): Circulating endothelial cells have been detected in patients with chronic kidney disease [94]. Are circulating endothelial cells detectable in sepsis and ARF? They could be surrogate markers for acute endothelial injury.

DR. MOLITORIS: Yes, circulating endothelial cells have been isolated from patients with sepsis [66, 95]. They have not been isolated from patients with ATN alone, but I am not sure anyone has tried to do this. I am hopeful that investigations are under way that will identify unique serum markers of ATN, and endothelial cell proteins might be a logical target.

DR. JABER: In experimental ARF, the administration of high-dose recombinant human erythropoietin (rHuEpo) accelerates recovery of renal function [96]. Can you speculate on the role of rHuEpo as a potential growth factor for tubular cell regeneration?

DR. MOLITORIS: As you know, many growth factors have a positive effect on GFR in ischemic models of ARF. Part of this effect is mediated by hemodynamic forces that favor increased filtration regardless of the baseline GFR. However, other effects, such as minimizing apoptosis, might be playing an important role. It has always been hard for me to conceive of how a growth factor can influence the GFR within the first 24 hours primarily by increasing tubular cell regeneration.

DR. HARRINGTON: We know that Epo receptors exist in all renal tubular cells and that Epo expression is abolished within a few hours of inducing ischemic ARF in rats. At the 2003 meeting of the ASN, abstracts showed that Epo protects the kidney via nuclear factor-κB (NF-κB [abstract; Westenfelder C, et al, *J Am Soc Nephrol* 14:272A, 2003] and by inhibiting the caspase cascade [abstract; Sharples EJ, et al, *J Am Soc Nephrol* 14:273A, 2003]. Could you comment on those papers or simply tell us about Epo's protective effects?

DR. MOLITORIS: Dr. Christof Westenfelder has established a link among erythropoietin, inflammation, and apoptosis [97]. His data indicate that cytokine-mediated activation of NFkB decreases erythropoietin transcription. This decrease in turn results in loss of Epo's permissive and active effects on inhibiting apoptosis. These data, along with the recent work from Kelly's and Dagher's laboratories [98], suggest an important role for apoptosis in the pathophysiology of ATN. The renal community has experience with erythropoietin administration in numerous types of patients, so Epo will be a likely candidate for the "growth factor" aspect of combination therapy in the treatment or prevention of ARF.

DR. JABER: The use of high-dose rHuEpo in ARF has to be carefully analyzed because this drug can increase endothelin production, and this increase can lead to resistance to the vasodilating action of NO [99]. Would you agree that these biologic effects can be deleterious in critically ill patients with sepsis, patients who have an already compromised microvasculature?

DR. MOLITORIS: Most therapies come with trade-offs and potential side effects. Erythropoietin increases endothelin production and can potentially increase vasoconstriction within the kidney. However, we do know that Epo receptors within the kidney are rapidly up-regulated in ATN. Therefore we should be thinking about more physiologic doses, and not the "industrial" amounts we use to enhance red blood cell production. Furthermore, preconditioning with Epo protects against subsequent ischemic injury in the kidney [100].

DR. IRA M. HERMAN (*Professor, Department of Physiology, Tufts University School of Medicine*): Have you thought about why the renal epithelial cells' apical actin cytoskeleton, associated with brush border, is so particularly sensitive to disassembly? Wouldn't you also expect that the actin network associated with the adherens junctions, that is, the belt of actin associated with the terminal web, would also be similarly affected?

DR. MOLITORIS: The sensitivity of the actin cytoskeleton to ATP depletion is related to the rate of actin turnover. Apical membrane microfilaments have a very rapid turnover secondary to the rapid rate of endocytosis [15, 16]. We have shown that the junctional complex also undergoes rapid alterations with ischemic injury [12, 44], but for this Forum I have concentrated on the microvilli because of their role in loss of apical membrane surface area and tubule obstruction.

DR. HERMAN: The apical actin reorganization observed in the renal epithelial cell during acute renal failure is reminiscent of the apical cytoskeletal response that occurs in the gastrointestinal system following enteropathogenic *Escherichia coli* challenge. That is, one observes an effacing lesion, and the actin filaments in the

microvilli are disassembled and then re-assembled as a pedestal downstream of the bacterial type III secretion system. In this case, as might be the case in the renal epithelium, the disassembly is mediated by a calcium-dependent protease and ezrin-mediated reorganization [101].

DR. MOLITORIS: Ezrin has clearly been shown to undergo dephosphorylation during ischemia and dissociate from the surface membrane; this alteration destabilizes the microvillar membrane [33]. However, there is no evidence for calpain-mediated actin alterations within this domain.

DR. HERMAN: Bruce, have you considered whether the anomalies in flow are related to the mural cells associating with the peritubular capillaries? It has been well studied from the time of our first characterization [102] that pericytes are smooth-muscle-like contractile cells capable of regulating microvascular flow. Local constriction of pericytes could control capillary blood flow, and the pericyte response to endothelin-1 could be causally linked to the observable anomalies.

DR. MOLITORIS: Pericytes have not been studied in ischemia within the kidney. They are known to be located around the renal microvasculature in a sparse fashion. Dr. Connie Temm is currently looking at the interconversion/activation of renal pericytes into smooth muscle cells following ischemia. She uses the expression of alpha actin as the distinguishing feature of smooth muscle cells (personal communication).

DR. HERMAN: There are numerous examples that pericyte foot processes cover many, many endothelial cells, and there are as many examples revealing the circumferential disposition of these cells, which share the basement membrane with the capillary endothelium. The beautiful images that you showed (Fig. 4) are possibly graphic representations of the focally constricted nature of the capillary wall, which might be related to endothelin-mediated pericyte contraction and capillary constriction.

DR. HARRINGTON: Could you comment on the new so-called "micro-MRI" methods that estimate tubular function and interstitial inflammation in experimental animals [103]?

DR. MOLITORIS: I would refer you to the excellent review in which Dr. Robert Star discusses the use of MRI methods to advance the understanding of ischemic ATN [104]. He has used these techniques to study PTC function and inflammation in rats and mice. There is the possibility that these techniques will soon be available for clinical studies.

DR. V. S. BALAKRISHNAN (*Division of Nephrology, Tufts-New England Medical Center*): Is there much information on the transcriptional regulation of ADF? If so, could this be a potential target?

DR. MOLITORIS: Actin depolymerizing factor transcription is under the control of cytosolic G-actin levels [22]. I think the target in minimizing ADF activation during ischemia will be transient inhibition of the SSH (slingshot) phosphatase responsible for its activation.

DR. DILIP K. PAHARI (*Fellow, Division of Nephrology, Tufts-New England Medical Center*): Heat-shock proteins (HSP) hasten recovery of acute renal failure. Has anyone studied the effect of HSP on depolymerization of actin filaments?

DR. MOLITORIS: Dr. N. Siegel's laboratory has shown an association between HSPs and F-actin during the reperfusion phase in renal ischemia [105]. Eric Shelden has shown that HSP-27 associates with the basolateral boundaries of ATP-depleted cells [9]. However, there is no apparent direct association between HSPs and the regulation of actin polymerization.

DR. HARRINGTON: It appears that a great deal of the damage from renal ischemia occurs during the reperfusion phase, just as in other organs. What specifically causes the microvascular problems during reperfusion?

DR. MOLITORIS: The defect in microvascular flow following ischemic/septic injury is multifactorial. Endothelial cell swelling, increased permeability, thrombin activation/generation leading to coagulation, and the inflammatory cascade including white blood cell activation and cytokine generation, endothelin-mediated vasoconstriction, and reactive oxygen species mediated several effects, including decreases in NO-mediated vasculature vasodilatation. This is why I believe we must consider a multifaceted approach to therapy if we are going to be successful. However, some potential therapeutic agents have multiple effects. For instance activated protein C is known to be anti-apoptotic and anti-inflammatory, and it prevents thrombotic events as it inactivates thrombin. Finally, delivery of any agent to the involved area depends on microvascular flow. Therefore, maintaining or re-establishing this flow is of paramount importance. This is why preventive or early therapy is essential.

DR. JABER: How far are we from a new blood or urinary marker of renal injury that would be more sensitive than the serum creatinine, which rises later in the process?

DR. MOLITORIS: We are entering a new phase of exploration for the "troponin" equivalent for ischemic/septic ATN. Several investigators are now using proteomic approaches on both blood and urine. Cystatin C is one available serum marker recently shown to be superior to creatinine in diagnosing early ARF [93]. Other markers remain investigational like the proteins recently described from the laboratories of Star and Bonventre [106, 107].

DR. ANDREW S. LEVEY (*Division of Nephrology, Tufts-New England Medical Center*): Do we know anything about the actin cytoskeleton in chronic kidney disease?

DR. MOLITORIS: To date I know of no actin cytoskeletal abnormalities directly related to the pathophysiology of

chronic kidney disease. This will be an interesting area of research, especially at the golmerular cell level.

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Reprint requests to Dr. Bruce A. Molitoris, Indiana University School of Medicine, Nephrology Division, 950 W. Walnut Street, R2-202C, Indianapolis, IN 46202.
E-mail: bmolitor@iupui.edu

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