Extracellular Calpains Increase Tubular Epithelial Cell Mobility

IMPLICATIONS FOR KIDNEY REPAIR AFTER ISCHEMIA

Carlos Frangié*, Wenhui Zhang, Joëlle Perez, Yi-Chun Xu Dubois, Jean-Philippe Haymann, and Laurent Baud*

From INSERM U702; Université Pierre et Marie Curie, 75020 Paris, France

Calpains are intracellular Ca$^{2+}$-dependent cysteine proteases that are released in the extracellular milieu by tubular epithelial cells following renal ischemia. Here we show that externalized calpains increase epithelial cell mobility and thus are critical for tubule repair. In vitro, exposure of human tubular epithelial cells (HK-2 cells) to $\mu$-calpain limited their adhesion to extracellular matrix and increased their mobility. Calpains acted primarily by promoting the cleavage of fibronectin, thus preventing fibronectin binding to the integrin $\alpha_\beta_3$. Analyzing downstream integrin effects, we found that the cyclic AMP-dependent protein kinase A pathway was activated in response to $\alpha_\beta_3$ disengagement and was essential for calpain-mediated increase in HK-2 cell mobility. In a murine model of ischemic acute renal failure, injection of a fragment of calpastatin, which specifically blocked calpain activity in extracellular milieu, markedly delayed tubule repair, increasing functional and histological lesions after 24 and 48 h of reperfusion. These findings suggest that externalized calpains are critical for tubule repair process in acute renal failure.

Calpains are intracellular Ca$^{2+}$-dependent cysteine proteases (1). The major isozymes, calpain 1 or $\mu$-calpain and calpain 2 or m-calpain, are distributed ubiquitously and activated

Received for publication, March 30, 2006, and in revised form, June 8, 2006 Published, JBC Papers in Press, July 5, 2006, DOI 10.1074/jbc.M603007200

*To whom correspondence should be addressed: INSERM U702, Hôpital Tenon, 4 rue de la Chine, 75020 Paris, France. Tel.: 33-1-56-01-79-51; Fax: 33-1-56-01-70-03; E-mail: laurent.baud@tnn.ap-hop-paris.fr.

**The on-line version of this article (available at http://www.jbc.org) contains Videos 1 and 2.

1 Supported by a grant from the Académie Nationale de Médecine and a fellowship from Paris-Des cartes University School of Medicine.

2 To whom correspondence should be addressed: INSERM U702, Hôpital Tenon, 4 rue de la Chine, 75020 Paris, France. Tel.: 33-1-56-01-79-51; Fax: 33-1-56-01-70-03; E-mail: laurent.baud@tnn.ap-hop-paris.fr.

The proximal straight tubule in the outer medulla of the kidney is particularly susceptible to ischemia/reperfusion injury, which remains the leading cause of acute renal failure (ARF) (10, 11). Damages to this segment are characterized initially by the disruption of tight junctions that control cell polarity (12, 13). The loss of cell polarity is responsible for the redistribution of integrin subunits from the basolateral to the apical membrane, contributing to the shedding of cells into the tubule lumen. With more sustained ischemia/reperfusion, epithelial cells of the proximal tubule undergo necrosis or apoptosis (14). Calpains are considered as a key mediator of this death. Their activation results from both a rise in cytosolic Ca$^{2+}$ through endoplasmic reticulum Ca$^{2+}$ release (15) and a caspase-dependent decrease in calpastatin activity (16). Upon activation, calpains hydrolyze the cytoskeleton-associated paxillin, talin, and vinculin, thus contributing to increased plasma membrane permeability and cell death (17). Epithelial cells that do not die participate in the regeneration of tubular epithelium and the restoration of renal function (11). They migrate into areas denuded by exfoliation, where they dedifferentiate, proliferate, and differentiate again (18). Because calpains leak out from dead tubular epithelial cells (19), the question arises as to inhibitory domains (1). By conducting limited proteolysis of intracellular substrates, calpain activity has been shown to be critical for a great diversity of cellular responses. They include rearrangement of cytoskeletal linkages to the plasma membrane during cell adhesion and mobility, modification of molecules in signal transduction pathways, degradation of enzymes controlling the cell cycle, and activation of proteolytic cascades leading to cell apoptosis or necrosis (1, 3, 4).

Recently, several groups showed that calpains may be released from cells into the extracellular environment and thus may have an extracellular role. Like other intracellular enzymes, calpains may indeed leak out from injured and dying cells such as hepatocytes exposed to toxic chemicals (5). The release of intracellular calpains from blood mononuclear cells (6), osteoblasts (7), chondrocytes (8), and parathyroid cells (9) is not due to cell death, but rather to a nonclassical pathway of secretion, which involves in certain cells the shedding of membrane vesicles (7, 9). In an extracellular Ca$^{2+}$-rich environment, activated calpains trigger plasma membrane proteins of the neighboring cells and extracellular matrix proteins.

The abbreviations used are: ARF, acute renal failure; BUN, blood urea nitrogen; PKA, protein kinase A; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.
whether externalized calpains play a role in this repair process. Thus, our study has focused on exploring the effects of extracellular calpains on tubular epithelial cells. By using an in vitro approach, we demonstrated that extracellular calpains increased the mobility of tubular epithelial cells by promoting the cleavage of fibronectin, the disengagement of αβ integrin, and thereby the activation of cyclic AMP signaling pathway. By using a model of ischemic ARF, we showed that extracellular calpains are indeed critical for tubule repair process.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human erythrocyte μ-calpain, human calpastatin peptide, calpastatin recombinant domain I, human placenta collagen IV, human fibroblast fibronectin, protein kinase C inhibitor calphostin C, Akt inhibitor, and adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine were obtained from Calbiochem. Human plasma thrombin was from Roche Diagnostics.

**Immunoprecipitation and Western Blot Analysis**—For immunoprecipitation of αβ integrin complexes, cell lysates were prepared by scraping HK-2 cells into an ice-cold protease inhibitory buffer. The lysate was centrifuged (4000 × g, 4 °C for 30 min). A portion of the supernatants was reserved for protein determination, and protein concentration in supernatants was adjusted with the protease inhibitory buffer. The lysate was centrifuged (4000 × g, 4 °C for 30 min). A portion of the supernatants was reserved for protein determination, and protein concentration in supernatant samples was adjusted with the protease inhibitory buffer. These samples (100 μl) were incubated for 20 h with A/G agarose coupled to anti-α,β integrin antibody before the complexes were washed extensively and solubilized in SDS sample buffer with reduction.

**Calpain Assay**—The calpain-like activity was determined in intact cells as described previously (21).

**Determination of Intracellular Cyclic AMP Concentrations**—HK-2 cells were exposed to μ-calpain in KRH medium supplemented with 2 mM CaCl₂ and 0.1 mM 3-isobutyl-1-methylxanthine. After 10 min, cyclic AMP was extracted with an ice-cold ethanol/formic acid mixture (85:5, v/v) and quantified using a radioimmunoassay ([125I]cAMP assay, Amersham Biosciences) according to the manufacturer’s protocol. Values were normalized to the protein concentration using a Bradford procedure and expressed as fmol/μg protein.

**Induction of Ischemic Acute Renal Failure**—The studies were conducted by following established guidelines for animal care, and all protocols were approved by the Institut National de la Santé et de la Recherche Médicale. C57BL/6 mice were anesthetized by intraperitoneal administration of avertin (Sigma) and subjected to bilateral flank incisions as described previously (22). Both renal pedicles were cross-clamped for 25 min. This time was chosen to obtain a reproducible acute renal failure, while minimizing animal mortality. After clamp removal, the kidneys were inspected for restoration of blood flow, the

**Experimental Procedures**

**Materials**—Human erythrocyte μ-calpain, human calpastatin peptide, calpastatin recombinant domain I, human placenta collagen IV, human fibroblast fibronectin, protein kinase C inhibitor calphostin C, Akt inhibitor, and adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine were obtained from Calbiochem. Human plasma thrombin was from Roche Diagnostics.

**Immunoprecipitation and Western Blot Analysis**—For immunoprecipitation of αβ integrin complexes, cell lysates were prepared by scraping HK-2 cells into an ice-cold protease inhibitory buffer. The lysate was centrifuged (4000 × g, 4 °C for 30 min). A portion of the supernatants was reserved for protein determination, and protein concentration in supernatant samples was adjusted with the protease inhibitory buffer. These samples (100 μl) were incubated for 20 h with A/G agarose coupled to anti-α,β integrin antibody before the complexes were washed extensively and solubilized in SDS sample buffer with reduction.

**Calpain Assay**—The calpain-like activity was determined in intact cells as described previously (21).

**Determination of Intracellular Cyclic AMP Concentrations**—HK-2 cells were exposed to μ-calpain in KRH medium supplemented with 2 mM CaCl₂ and 0.1 mM 3-isobutyl-1-methylxanthine. After 10 min, cyclic AMP was extracted with an ice-cold ethanol/formic acid mixture (85:5, v/v) and quantified using a radioimmunoassay ([125I]cAMP assay, Amersham Biosciences) according to the manufacturer’s protocol. Values were normalized to the protein concentration using a Bradford procedure and expressed as fmol/μg protein.

**Induction of Ischemic Acute Renal Failure**—The studies were conducted by following established guidelines for animal care, and all protocols were approved by the Institut National de la Santé et de la Recherche Médicale. C57BL/6 mice were anesthetized by intraperitoneal administration of avertin (Sigma) and subjected to bilateral flank incisions as described previously (22). Both renal pedicles were cross-clamped for 25 min. This time was chosen to obtain a reproducible acute renal failure, while minimizing animal mortality. After clamp removal, the kidneys were inspected for restoration of blood flow, the
For light microscopy, kidneys were fixed in 4% paraformaldehyde and processed for paraffin embedding. Sections of 3-μm thickness were made and stained with Masson trichromic solution. Tubular injury was scored by estimating the percentage of tubules in the cortex or the outer medulla that showed epithelial necrosis or had luminal necrotic debris and tubular dilatation as follows: 0, none; 1, <10%; 2, 10–25%; 3, 25–50%; 4, 50–75%, and 5, >75% (22). All evaluations were made on 10 fields/section and 10 sections/kidney by two different blinded observers.

For fluorescence microscopy, cryostat sections of frozen kidneys were fixed in 4% paraformaldehyde. They were stained with primary anti-fibronectin (1/100) and secondary TRITC-labeled (1/400) antibodies and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride, a nuclear stain.

**Statistical Analysis**—Results are expressed as mean ± S.E. Comparisons between groups of values were made with the Student's t test for unrelated groups. A difference between groups of \( p < 0.05 \) was considered significant.

**RESULTS**

**Extracellular \( \mu \)-Calpain Modulates the Behavior of Proximal Tubular Epithelial Cells**—To assess initially the impact of extracellular calpains on tubular epithelial cell viability, proliferation, and morphology, we exposed HK-2 cells to purified \( \mu \)-calpain. Concentrations of \( \mu \)-calpain ranged between 0.1 and 2 \( \mu \)g/ml, as such concentrations are reached in the extracellular medium of activated cells (6). Under these conditions, \( \mu \)-calpain did not affect HK-2 cell viability and growth, as judged by trypan blue exclusion and \([3H]\)thymidine uptake, respectively (Fig. 1A). By contrast, exposure of HK-2 cells to \( \mu \)-calpain resulted in a marked alteration of their morphology. Within 30 min, they adopted a more rounded mor-

**Assessment of Renal Function and Histology**—Samples of serum were collected from all the mice to measure both creatinine and blood urea nitrogen (BUN) using an autoanalyzer.

**Extracellular \( \mu \)-calpain targets fibronectin in extracellular matrix of proximal tubular epithelial cells.** A, calpain activity was measured in extracellular milieu (white bars), HK-2 cell monolayer plus extracellular milieu (black bars), and HK-2 cell monolayer alone (gray bars) after addition of the indicated concentrations of \( \mu \)-calpain to the extracellular milieu. Values represent the mean ± S.E. of three independent experiments. B, Western blot analysis of integrin expression in HK-2 cells. Lysates were prepared from HK-2 cells exposed for 45 min to the indicated concentrations of \( \mu \)-calpain. C, HK-2 cells were cultured for 24 h on the indicated extracellular matrix proteins. Phase contrast images of HK-2 cells were taken immediately before (upper panels) and 45 min after the addition of 2 \( \mu \)g/ml \( \mu \)-calpain to the incubation medium (lower panels). D, Western blot analysis of fibronectin cleavage and release into the extracellular milieu of HK-2 cells by using both monoclonal (upper panel) and polyclonal (lower panel)-specific antibodies. Extracellular milieu was harvested after a 45-min exposure of HK-2 cells to the indicated concentrations of \( \mu \)-calpain. MAB, monoclonal antibody.
Extracellular Calpains and Acute Renal Failure

**FIGURE 3.** Fibronectin cleavage by extracellular μ-calpain is responsible for the disengagement of α_3β_3 integrin. A, HK-2 cells were exposed for 45 min to the indicated concentrations of μ-calpain. Associations between α_3β_3 and fibronectin were determined by immunoprecipitation with anti-α_3β_3 antibody and probing with anti-fibronectin antibody. Relative binding levels of fibronectin to α_3β_3 integrin were determined by densitometry (*, p < 0.05; n = 4). B, fibronectin (red) and α_3β_3 integrin (green) were visualized by confocal microscopy. Scale bars, 20 μm. C, HK-2 cells were cultured in the presence of anti-α_3β_3 blocking antibody for 24 h. Phase contrast images of HK-2 cells were taken immediately before (upper panel) and 45 min after the addition of 1 μg/ml μ-calpain to the incubation medium (lower panel).

we next examined whether extracellular μ-calpain would affect HK-2 cell spreading and mobility by cleaving the extracellular domain of integrins. Western blot analysis of whole cell extracts established that HK-2 cells express α_3, α_5, β_1, and β_3 integrin subunits (Fig. 2B). Exposure of these cells to μ-calpain did not result in any detectable integrin cleavage.

Thus, we finally examined whether integrin ligands in the extracellular matrix would be a preferential target for extracellular μ-calpain. To this aim, HK-2 cells were cultured on surfaces coated with or without definite extracellular matrix proteins including fibronectin, laminin, and collagen type IV (Fig. 2C). After 24 h of culture, HK-2 cells attached equally to either peripheral, and β_3 integrin molecules were redistributed from the endoplasmic reticulum to the plasma membrane. Similar results were shown by analyzing β_3 integrin (data not shown). Together, these results demonstrate that extracellular μ-calpain induces cell migration and that this effect is associated with a redistribution of integrin-cytoskeleton linkages.

**Extracellular μ-Calpain Targets Fibronectin in Extracellular Matrix of Proximal Tubular Epithelial Cells**—Intracellular calpain activity is involved in the redistribution of integrin-cytoskeleton linkages during cell migration (24). Thus, we considered initially the possibility that extracellular μ-calpain enters the cells and/or affects intracellular calpain activity. In fact, measurements of calpain activity showed that μ-calpain, being added to the extracellular medium either continuously or transiently, did not affect calpain activity of HK-2 cells (Fig. 2A). Further, pre-exposure of HK-2 cells to cell-permeable calpastatin peptide (5 μg/ml) did not affect their response to extracellular calpain (data not shown), excluding again a role for intracellular calpain activity. Because integrins are a known substrate for calpains (3),...
conditions, fibronectin was a major substrate for μ-calpain. Western blot analysis confirmed this hypothesis. In the extracellular milieu of HK-2 cells exposed to μ-calpain both the monoclonal and the polyclonal anti-fibronectin antibodies reacted mainly with a fibronectin fragment of ~140 kDa (Fig. 2D). This breakdown product of 250-kDa fibronectin was undetectable in the extracellular milieu of control HK-2 cells. By comparison, collagen type IV was not released, and laminin β-1 was released without cleavage in the extracellular milieu of both control and μ-calpain-treated HK-2 cells (data not shown). Thus, fibronectin would be the main target of μ-calpain in extracellular matrix in vivo, other targets such as proteoglycans (8) not being excluded.

Fibronectin Cleavage by Extracellular μ-Calpain Is Responsible for the Disengament of αβ3 Integrin—The αβ3 integrin is the main fibronectin receptor in proximal tubular epithelial cells (25). Thus, to determine whether fibronectin cleavage interfered with fibronectin binding to αβ3, fibronectin-αβ3 complexes were analyzed by both immunoprecipitation and confocal microscopy. Immunoprecipitation experiments demonstrated the presence of fibronectin-αβ3 complexes in HK-2 cells, which was limited significantly upon cell exposure to μ-calpain (Fig. 3A). Double immunolabeling of HK-2 cells showed that αβ3 localized mainly to basolateral membrane, in close contact with fibronectin fibrils in extracellular matrix (Fig. 3B). Exposure of HK-2 cells to μ-calpain resulted initially in a marked fragmentation of fibronectin fibrils and later on in a notable accumulation of fibronectin in the cytoplasm, whereas αβ3 was still expressed at the basolateral membrane (Fig. 3B). Thus, these results suggest the disappearance of fibronectin-αβ3 complexes in HK-2 cells exposed to μ-calpain.

To determine further the involvement of αβ3 integrin in response to μ-calpain, HK-2 cells were cultured in the presence of anti-αβ3 blocking antibody. Anti-αβ3-treated cells attached less to extracellular matrix and showed no morphology change upon exposure to μ-calpain, i.e. no migration and aggregation into heaps (Fig. 3C). Collectively, these results indicate that fibronectin cleavage by μ-calpain is responsible for αβ3 disengagement, which is essential to modify HK-2 cell spreading and mobility.

Unligated αβ3 Integrin Induces PKA-dependent Mobility of Proximal Tubular Epithelial Cells—Integrin ligation is a potent regulator of PKA, and in turn, PKA pathway regulates actin-based migration of cells (26, 27), including tubular epithelial cells (28, 29). Thus, we tested the hypothesis that the cyclic AMP/PKA pathway would become activated following calpain-dependent disengagement of αβ3 and was involved in HK-2 cell migration. Fig. 4A shows that exposure of HK-2 cells to μ-calpain for 10 min produced a concentration-dependent increase in intracellular cyclic AMP levels. Blocking cyclic AMP accumulation and PKA activity by using the cell-permeable adenyl cyclase inhibitor 2′,5′-dideoxyadenosine (data not shown) and the PKA inhibitor H-89 (Fig. 4B and Video 2), respectively, completely prevented μ-calpain-induced HK-2 cell migration. By contrast, H-89 did not prevent μ-calpain-dependent fibronectin cleavage (Fig. 4C). This response was specific, as blocking PKB/Akt and protein kinase C activities by using 1-L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate and calphostin C, respectively, was ineffective in preventing μ-calpain-induced HK-2 cell migration (data not shown). Our results indicate that αβ3 binding to fibronectin would suppress PKA activity in HK-2 cells, and conversely, αβ3 disengagement would restore this activity, thereby inducing cell migration.

Externalized Calpains Play a Role in Tubular Repair after Renal Ischemia—Tubular epithelial cell migration is an essential feature of tubular repair. Indeed, epithelial cells surviving ischemia migrate over denuded areas of basement membrane before proliferating to replace lost cells (30). Because we demonstrated in vitro the role of extracellular calpains in tubular epithelial cell migration, we investigated in vivo their involvement in tubular repair after renal ischemia. To this aim, calpastatin domain I, a very specific non-cell-permeable calpain inhibitor, was administered intraperitoneally to mice, just after induction of ischemic ARF. This small macromolecule (14-kDa molecular mass) would cross the glomerular capillary wall readily and thus reach the tubular lumen. In vehicle-treated mice subjected to bilateral renal pedicle clamping for 25 min, serum creatinine levels increased after 24 h of reperfusion, stabilized at 48 h, and decreased progressively thereafter (Fig. 5A). BUN demonstrated a similar pattern. In mice given calpastatin,
Extracellular Calpains and Acute Renal Failure

FIGURE 5. Externalized calpains play a role in tubular repair after renal ischemia. Mice received normal saline or calpastatin domain I (6 mg/kg) intraperitoneally immediately prior to and once a day during the reperfusion period. They were then subjected to 25 min of renal ischemia followed by the indicated periods of reperfusion. A, glomerular dysfunction as reflected by increases in serum creatinine and BUN levels (*, p < 0.05 compared with untreated mice; n = 5–15 in each experimental group). B, representative Masson-stained sections of kidneys from untreated (left) and calpastatin domain I-treated (right) mice are shown (×200) with semiquantitative analysis of tubular necrosis (*, p < 0.05 compared with untreated mice; n = 5–15 in each experimental group).

the ischemia/reperfusion-dependent rise in serum creatinine and BUN was more severe and markedly prolonged (Fig. 5A). Remarkably, this worsening of renal dysfunction was associated with a significant increase in tubular injury (including necrosis, tubular dilation, and sloughing of epithelial cells) in the cortex and the outer medulla as assessed by semiquantitative analysis (Fig. 5B).

Extracellular μ-calpain degraded fibronectin in extracellular matrix of HK-2 cells (Fig. 2D). Thus, we next examined whether calpastatin administration would prevent fibronectin degradation by externalized calpains in posts ischemic kidney. Immunofluorescence studies demonstrated a faint fibronectin staining at the basal surface of tubular epithelial cells after 24 and 48 h of reperfusion (Fig. 6A). There was a marked increase in this staining in mice given calpastatin. Fibronectin was not detectable by immunoprecipitation and Western blot analysis in urine of control mice (Fig. 6B). After 24 h of reperfusion, a ~30-kDa fibronectin fragment became detectable, suggesting a release of the ~140-kDa fibronectin fragment from tubule extracellular matrix and its subsequent degradation by proteases in the urinary tract. Densitometric analysis showed that this expression decreased significantly in mice given calpastatin (8.2 ± 1.5 versus 19.5 ± 3.7 densitometry units, p < 0.05, n = 7). Therefore, externalized calpains seem to play a key role in kidney repair after an ischemic insult. This protective effect is accompanied by a degradation of extracellular matrix fibronectin.

DISCUSSION

Here, we have provided evidence that μ-calpain released from necrotic epithelial cells in ARF would play an essential role in tubule repair. Specifically, in vitro exposure of tubular epithelial cells to extracellular μ-calpain reduced their adhesion to fibronectin in extracellular matrix and thereby increased their mobility, which was critical to repair. Effective concentrations of calpain (~1 μM) were presumably reached in the fluid of proximal tubules 24 h after induction of ischemic ARF, as estimated by measuring calpain activity in urine and taking tubule fluid concentration process along the nephron into account (~4 μM).

One of the hallmarks of ischemic ARF is tubular cell necrosis, a damage leading to calpain externalization. Previous studies have demonstrated that externalized calpains cleave extracellular proteins, including the latent form of transforming growth factor-β (31) and the extracellular matrix components, vitronectin and fibronectin (32, 33). Consistent with this latter report, we found that extracellular μ-calpain hydrolyzes fibronectin. In the kidney, fibronectin is localized in interstitial matrix, being secreted in particular by proximal tubular epithelial cells. As a consequence of ischemia-reperfusion injury, interstitial matrix proteins such as fibronectin are exposed (18) and thus become a new substrate for externalized calpains. Our data indicate that fibronectin hydrolysis by μ-calpain results in the appearance of fibronectin breakdown products in the extracellular milieu both in vitro and in vivo. This process could help explain the observation that immunoreactive fibronectin is abundantly expressed in tubular lumen 3–24 h post ischemia (18). We measured the size fibronectin breakdown products by Western blot analysis. Both the monoclonal and the polyclonal anti-fibronectin antibodies reacted mainly with a fragment of ~140 kDa (Fig. 2D). Because the polyclonal
antibody is directed against the COOH terminus of fibronectin molecule, the ~140-kDa fragment contains this domain and thus results from the cleavage of fibronectin molecule at a site located between type III domains 7 and 10. This region is involved in interaction with several integrins (e.g. αβ3) (34) so that its proteolytic cleavage would impede fibronectin binding to such integrins.

In the kidney, fibronectin interacts with αβ3 and weakly or not with αβ1 at the surface of proximal tubular epithelial cells (25, 35). We indeed found a direct interaction of fibronectin with αβ3 integrin by communoprecipitation and colocalization experiments. This binding was markedly reduced in HK-2 cells exposed to μ-calpain, providing strong evidence for the hypothesis that fibronectin degradation by extracellular calpains causes αβ3 disengagement. A recent work by Akimov et al. (36) reported that tissue transglutaminase interacts directly with fibronectin in extracellular matrix and αβ3 integrin on the cell surface, thereby potentiating integrin-mediated cell adhesion and outside-in signal transduction. As calpains are known to cleave tissue transglutaminase (37), they could regulate αβ3 downstream signaling by hydrolyzing tissue transglutaminase in addition to fibronectin.

Integrin ligation activates downstream signaling pathways that are involved in the control of cell migration. We found that αβ3 disengagement in response to fibronectin hydrolysis activates cyclic AMP signaling pathway. Similarly, recent studies in endothelial cells have demonstrated that impeding αβ3 binding to extracellular matrix ligand activates PKA (38). The mechanisms whereby integrin αβ3 disengagement activates cyclic AMP signaling pathway are unknown. The increase in cyclic AMP resulted probably from the activation of adenyl cyclase, given that our measurements were performed in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. Furthermore, there have been reports that αβ3 integrin and integrin-associated protein (CD47) may combine to form a functional seven-transmembrane complex, which associates with G protein and, once activated, decreases cyclic AMP (39). Thus, αβ3-CD47-G protein complexes could be involved in the control of adenyl cyclase activity by αβ3 engagement/disengagement. However, further work is needed to assess the precise role of these complexes in the cell response to extracellular calpain.

Cyclic AMP signaling pathway is known to exert both negative and positive effects on cell migration (27). The current observation that αβ3 disengagement is associated with the accumulation of cyclic AMP in HK-2 cells and that inhibiting cyclic AMP signaling pathway impedes HK-2 cell migration provides strong evidence for the hypothesis that cyclic AMP signaling pathway plays a role in stimulating cell mobility in response to extracellular μ-calpain. Previous studies have also demonstrated that cyclic AMP signaling pathway stimulates tubular epithelial cell migration in vitro (28, 29). In addition, cyclic AMP signaling pathway has been implicated in the protection of these cells against the cytotoxic effect of ischemia and intracellular ATP depletion (40). Because adenosine is released from injured or dying cells and induces both cyclic AMP-dependent survival and migration of tubular epithelial cells (28, 29), there are at least two potential mechanisms (i.e. calpain externalization and adenosine release) whereby necrosis of epithelial cells could speed up repair of tubules in ARF.

In the present study, the concept that externalized calpains may accelerate tubule repair in ischemic ARF is supported by the demonstration of persistent lesions of tubules after administration of calpastatin domain I. The decreased level of fibronectin breakdown product in urine of mice given calpastatin indicates clearly that calpastatin reached tubule lumens and effectively targeted externalized calpains. By contrast, a recent report shows that two cell-permeable calpain inhibitors, PD 150606 and E-64, reduce the renal dysfunction and injury caused by ischemia reperfusion (41). Altogether, this is good evidence that extracellular calpains triggered by calpastatin domain I contribute to tubule repair, partly by inducing epithelial cell migration, whereas intracellular calpains triggered by PD 150606 and E-64 participate in tubule injury, partly by increasing oxidative stress. To our knowledge, only one other study has examined the role of externalized calpains in repair.

**FIGURE 6. Calpastatin administration prevents fibronectin degradation by externalized calpains in postischemic kidney.** Mice received normal saline or calpastatin domain I (6 mg/kg) intraperitoneally immediately prior to and once a day during the reperfusion period. They were subjected to 25 min of renal ischemia followed by the indicated periods of reperfusion. A, fibronectin (red) and cell nuclei (blue) were visualized by fluorescence microscopy. Representative kidney section of untreated (left) and calpastatin domain I-treated (right) mice after 48-h reperfusion is shown (×200). B, ~30-kDa fibronectin cleavage product was detected in urine of mice after 24-h reperfusion by electrophoretic separation of anti-fibronectin immunoprecipitations and immunoblotting with anti-fibronectin antibody. Double arrowheads indicate IgG heavy chain that was recognized by the secondary antibody.
process. Mehendale and Limaye (5) reported that calpains released from necrotic hepatocytes hydrolyze proteins in the plasma membrane of neighboring cells, leading to progression of injury rather than to repair. One hypothesis to explain the differences between this result and the present study is that calpain concentrations reached in the extracellular milieu would be markedly higher in liver exposed to acute toxic insult than in ischemic kidney.

The major conclusion of our work is that calpains released from necrotic cells in ischemic ARF trigger repair process. This effect is likely secondary to fibronectin cleavage, α3β1 integrin disengagement, and cyclic AMP signaling pathway activation. Thus, our study highlights the importance of extracellular calpains and intracellular cyclic AMP as targets for therapeutic intervention in ARF. It also proposes a novel evidence that necrotic cells may directly trigger the tissue repair response.

Acknowledgments—We thank Philippe Fontanges for help with the confocal microscopy.

REFERENCES
1. Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003) Physiol. Rev. 83, 731–801
2. Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z., and Davies, P. L. (2002) Cell 108, 649–660
3. Glading, A., Lauffenburger, D. A., and Wells, A. (2002) Trends Cell Biol. 12, 46–54
4. Franco, S. J., Rodgers, M. A., Perrin, B. J., Han, J., Bennin, D. A., Critchley, D. R., and Huttenlocher, A. (2004) Nat. Cell Biol. 6, 977–983
5. Mehendale, H. M., and Limaye, P. B. (2005) Trends Pharmacol. Sci. 26, 232–236
6. Deshpande, R. V., Goust, J.-M., Chakrabarti, A. K., Barbosa, E., Hogan, E. L., and Banik, N. L. (1999) J. Biol. Chem. 274, 2505–2512
7. Nishihara, H., Nakagawa, Y., Ishikawa, H., Ohba, M., Shimizu, K., and Nakamura, T. (2001) Biochem. Biophys. Res. Commun. 285, 845–853
8. Fushimi, K., Nakashima, S., Banno, Y., Akaike, A., Takigawa, M., and Shimizu, K. (2004) Osteoarthritis Cartilage 12, 895–903
9. Kifor, O., Kifor, I., Moore, F. D., Butters, R. R., and Brown, E. M. (2003) J. Biol. Chem. 278, 31167–31176
10. Schrier, R. W., Wang, W., Poole, B., and Mitra, A. (2004) J. Clin. Investig. 114, 5–14
11. Bonventre, J. V., and Weinberg, J. M. (2003) J. Am. Soc. Nephrol. 14, 2199–2210
12. Bush, K. T., Keller, S. H., and Nigam, S. K. (2000) J. Clin. Investig. 106, 621–626
13. Sheridan, A. M., and Bonventre, J. V. (2000) Curr. Opin. Nephrol. Hypertens. 9, 427–434
14. Padanilam, B. J. (2003) Am. J. Physiol. 284, F608–F627
15. Harriman, J. F., Liu, X. L., Aleo, M. D., Machaca, K., and Schnellmann, R. G. (2002) Cell Death Differ. 9, 734–741
16. Shi, Y., Meltikov, V. Y., Schrier, R. W., and Edelstein, C. L. (2000) Am. J. Physiol. 279, F509–F517
17. Liu, X., and Schnellmann, R. G. (2003) J. Pharmacol. Exp. Ther. 304, 63–70
18. Zuk, A., Bonventre, J. V., Brown, D., and Matlin, K. S. (1998) Am. J. Physiol. 275, C711–C731
19. Liu, X., Rainey, J. J., Harriman, J. F., and Schnellmann, R. G. (2001) Am. J. Physiol. 281, F728–F738
20. Leonard, M. O., Cottell, D. C., Godson, C., Brady, H. R., and Taylor, C. T. (2003) J. Biol. Chem. 278, 40296–40304
21. Bellocq, A., Doublier, S., Suberville, S., Perez, J., Escoubet, B., Fouqueray, B., Rodriguez Puyol, D., and Baud, L. (1999) J. Biol. Chem. 274, 36891–36896
22. Letavernier, E., Perez, J., Joye, E., Bellocq, A., Fouqueray, B., Haymann, J.-P., Heudes, D., Wahl, W., Desvergne, B., and Baud, L. (2005) J. Am. Soc. Nephrol. 16, 2395–2402
23. Paller, M. S. (1997) Kidney Int. 52, Suppl. 61, S52–S55
24. Huttenlocher, A., Palecek, S. P., Lu, Q., Zhang, W., Mellgren, B. R., Lauffenburger, D. A., Ginsberg, M. H., and Horwitz, A. F. (1997) J. Biol. Chem. 272, 3219–3222
25. Goligorsky, M. S., Lieberthal, W., Racusen, L., and Simon, E. E. (1993) Am. J. Physiol. 264, F1–F8
26. DeMali, K. A., Wennerberg, K., and Burridge, K. (2003) Curr. Opin. Cell Biol. 15, 572–582
27. Howe, A. K. (2004) Biochim. Biophys. Acta 1692, 159–174
28. Kartha, S., and Toback, F. G. (1992) J. Clin. Investig. 90, 288–292
29. Li, X., Li, H.-P., Amsler, K., Hyink, D., Wilson, P. D., and Burrow, C. R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9260–9265
30. Nony, P. A., and Schnellmann, R. G. (2003) J. Pharmacol. Exp. Ther. 304, 905–912
31. Abe, M., Oda, N., and Sato, Y. (1998) J. Cell Physiol. 174, 186–193
32. Seiffert, D. (1996) J. Biol. Chem. 271, 11170–11176
33. Dourdin, N., Brustis, J.-J., Balcerzak, D., Elamrani, N., Poussard, S., Cottin, P., and Ducastaing, A. (1997) Exp. Cell Res. 235, 385–394
34. Adair, B. D., Xiong, J.-P., Maddock, C., Goodman, S. L., Arnaout, M. A., and Yeager, M. (2005) J. Cell Biol. 168, 1109–1118
35. Glynne, P. A., Picot, J., and Evans, T. J. (2001) J. Am. Soc. Nephrol. 12, 2370–2383
36. Akimov, S. S., Krylov, D., Fleischman, L. F., and Belkin, A. M. (2000) J. Cell Biol. 148, 825–838
37. Lorand, L., and Graham, R. M. (2003) Nat. Rev. Mol. Cell Biol. 4, 140–156
38. Kim, S., Bakre, M., Yin, H., and Varner, J. A. (2002) J. Clin. Investig. 110, 933–941
39. McDonald, J. F., Zheleznyak, A., and Frazier, W. A. (2004) J. Biol. Chem. 279, 17301–17311
40. Lee, H. T., and Emala, C. W. (2002) J. Am. Soc. Nephrol. 13, 1121–1131