0026-895X/01/6006-1226–1234$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 60:1226–1234, 2001

Vol. 60, No. 6
1168/948517
Printed in U.S.A.

Interactions between Collagen IV and Collagen-Binding Integrins in Renal Cell Repair after Sublethal Injury

PAUL A. NONY[1] and RICK G. SCHNELLMANN[2]

Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Received June 18, 2001; accepted September 17, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Recent studies demonstrate that collagen IV selectively promotes the repair of physiological processes in sublethally injured renal proximal tubular cells (RPTC). We sought to further define the mechanisms of cell repair by measuring the effects of toxicant injury and stimulation of repair by L-ascorbic acid-2-phosphate (AscP), exogenous collagen IV, or function-stimulating integrin antibodies on the expression and subcellular localization of collagen-binding integrins (CBI) in RPTC. Expression of CBI subunits α_1, α_2, and β_1 in RPTC was not altered on day 1 after sublethal injury by *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). On day 6, expression of α_1 and β_1 subunits remained unchanged, whereas a 2.2-fold increase in α_2 expression was evident in injured RPTC. CBI localization in control RPTC was limited exclusively to the basal membrane. On day 1 after injury, RPTC exhibited a marked inhibition of active Na^+ transport and a loss of cell polarity characterized by a decrease in basal CBI localization and the appearance of CBI on the apical membrane. On day 6 after injury, RPTC still exhibited marked inhibition of active Na^+ transport and localization of CBI to the apical membrane. However, DCVC-injured RPTC cultured in pharmacological concentrations of AscP (500 μM) or exogenous collagen IV (50 μg/ml) exhibited an increase in active Na^+ transport, relocalization of CBI to the basal membrane, and the disappearance of CBI from the apical membrane on day 6. Function-stimulating antibodies to CBI β_1 did not promote basal relocalization of CBI despite stimulating the repair of Na^+/K^+-ATPase activity on day 6 after injury. These data demonstrate that DCVC disrupts integrin localization and that physiological repair stimulated by AscP or collagen IV is associated with the basal relocalization of CBI in DCVC-injured RPTC. These data also suggest that CBI-mediated repair of physiological functions may occur independently of integrin relocalization.

Cellular integrins are heterodimeric transmembrane receptors that provide a means for anchorage to extracellular substrates as well as two-way communication between the intracellular and the extracellular environment (Ruoslahti and Engvall, 1997; Molitoris and Marrs, 1999; Schoenwaelder and Burridge, 1999). Activation and clustering of integrins upon binding to extracellular matrix (ECM) proteins initiate focal adhesion formation and the activation of cytoskeletal signaling cascades involved in cell growth, proliferation, migration, differentiation, and gene expression (Molitoris and Marrs, 1999; Schoenwaelder and Burridge, 1999, Zuk et al., 1998). In addition to binding to ECM substrates and mediating cytoskeletal signaling, integrins also are known to influence the formation and composition of the ECM (Riikonen et al., 1995; Gotwals et al., 1996). In renal proximal tubular cells (RPTC), integrins and other proteins, such as Na^+/K^+-ATPases, are localized to the basal membrane where cells interact with the ECM as well as neighboring cells. This is in contrast to the apical membrane, where distinct physiological processes, such as Na^+-dependent glucose and amino acid transport, occur. The cellular polarity derived from the distinct functions carried out at separate membrane regions supports and is critical for proper renal tubular function (Bush et al., 2000).

The renal tubular basement membrane (BM) is composed mainly of collagens, laminins, and heparan sulfate proteoglycans (Furness, 1996, Miner, 1999). The most abundant type of collagen in the BM of the glomerulus and renal tubules is collagen IV, a globular, nonfibrillar protein (Furness, 1996). The combination of α and β integrin subunits that form the

This research was supported in part by National Institute of Environmental Health Sciences Grant ESO4410 (R.G.S.) and a predoctoral fellowship from the American Heart Association, Heartland Affiliate (P.A.N.). This work was included, in part, in a dissertation entitled "Mechanisms of renal cell repair following acute toxicant injury", submitted by P.A.N. to the Medical Library at the University of Arkansas for Medical Sciences, and was presented, in part, by P.A.N. at the Society of Toxicology Annual Meeting in San Francisco CA, March, 2001 [Nony PA and Schnellmann RG (2001) Role of collagen IV and collagen-binding integrins in renal cell repair following sublethal toxicant injury. *The Toxicologist* **60:**308].

[1] Current Address: Laboratory of Molecular Carcinogenesis, NIEHS, Bldg. 101, Mail Drop C2–14, 111 T.W. Alexander Drive, Research Triangle Park, NC 27709.

[2] Current Address: Department of Pharmaceutical Sciences, Medical University of South Carolina, 280 Calhoun St., POB 250140, Charleston, SC 29425

ABBREVIATIONS: ECM, extracellular matrix; RPTC; renal proximal tubular cells; BM, basement membrane; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; AscP, L-ascorbic acid-2-phosphate; CBI, collagen-binding integrins; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ERK, extracellular-signal regulated kinase.

functional heterodimer largely determine the binding of integrins to collagens and other ECM proteins. At least eight β-subunits and 17 α-subunits have been identified so far, and they associate noncovalently to form more than 20 heterodimers with various signaling and substrate binding properties (Kreidberg and Symons, 2000). Cells most often use the integrin heterodimers $\alpha_1\beta_1$ and $\alpha_2\beta_1$ to bind collagen IV, and the importance of signals derived from collagen-binding integrins (CBI) in normal cellular activities has been studied (Kuhn and Ebel, 1994; Gardner et al., 1996; Knight et al., 1998).

In cases of acute renal failure resulting from chemical exposure or ischemia, tubular epithelial cells may lose polarity as characterized by decreased localization of integrins in the basal membrane and their redistribution throughout the plasma membrane (Goligorsky and DiBona, 1993; Lieberthal et al., 1997; Zuk et al., 1998; Molitoris and Marrs, 1999). The result is cellular disorientation, decreased renal tubular function, and cell death and/or detachment from the tubular BM (Goligorsky and DiBona, 1993; Frisch and Ruoslahti, 1997; Tang et al., 1998; Molitoris and Marrs, 1999). Sublethally injured cells that do not die or become detached from the BM are thought to repair and/or dedifferentiate, proliferate, migrate to denuded areas of the tubule, differentiate, and promote the return of normal renal function (Abbate and Remuzzi, 1996; Molitoris and Marrs, 1999). The effects of cell injury on integrin localization and renal cell polarity have been investigated, but their importance in tubular regeneration after injury is not well understood (Goligorsky and DiBona, 1993; Lieberthal et al., 1997; Zuk et al., 1998; Molitoris and Marrs, 1999; Kreidberg and Symons, 2000).

Previous studies from this laboratory focused on determining the mechanisms of renal tubular cell regeneration using the model nephrotoxicant *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) to produce sublethal injury in primary cultures of rabbit RPTCs. RPTC exposure to DCVC produced approximately 50% cell death and loss caused the irreversible inhibition of key physiological functions, including mitochondrial function, active Na^+ transport, and Na^+/K^+-ATPase activity, in the remaining sublethally injured RPTC (Nowak et al., 1999). However, addition to the culture media of L-ascorbic acid-2-phosphate (AscP) at pharmacological concentrations promoted proliferation and repair of physiological functions in DCVC-injured RPTC (Nowak et al., 2000). The regeneration of DCVC-injured RPTC was associated with the stimulation of collagen IV deposition by AscP, suggesting that collagen IV deposition plays a key role in the ability of RPTC to recover from sublethal toxicant injury (Nony et al., 2001). Furthermore, the addition of exogenous collagen IV to the culture media of injured RPTC promoted the repair of physiological functions (Nony et al., 2001). Based on these findings, we hypothesized that AscP and exogenous collagen IV act to promote RPTC regeneration through the restoration of interactions between collagen IV and CBI. The specific goals of this study were 1) to determine the fate of CBI after sublethal RPTC injury with regard to expression and subcellular localization and 2) to examine the effect of AscP, exogenous collagen IV, and function-stimulating CBI antibodies on CBI expression and/or localization after sublethal injury in relation to the repair of physiological functions.

Experimental Procedures

Materials. Female New Zealand White rabbits (1.5–2.0 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). DCVC was a generous gift from Dr. T. W. Petry (Pharmacia Upjohn, Kalamazoo, MI) and was synthesized as described previously (Moore and Green, 1988). L-Ascorbic acid-2-phosphate (magnesium salt) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Ouabain was obtained from RBI/Sigma (Natick, MA). FITC-conjugated goat anti-mouse IgG and mouse monoclonal antibodies directed against human integrin subunits α_1 (clone FB12), α_2 (clone JBS2), and β_1 (clone B3B11) were purchased from Chemicon International, Inc. (Temecula, CA). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of Proximal Tubules and Culture Conditions. Rabbit renal proximal tubules were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes or 48-well cell culture clusters under improved conditions as described previously (Nowak and Schnellmann, 1995, 1996; Nony et al., 2001). The cell culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (without D-glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 μM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 or 500 μM) were added to the culture medium immediately before daily media change. AscP was used because L-ascorbic acid is unstable in culture media. AscP is stable in culture media and, after intracellular dephosphorylation, has the same effect on cultured cells as L-ascorbic acid (Hata and Senoo, 1989).

Sublethal Injury of RPTC. Confluent monolayers of RPTC (day 6 after seeding) were exposed to 200 μM DCVC (dissolved in water) for 1.75 h followed by toxicant removal and addition of fresh culture media. This method produces approximately 50% cell death and loss 24 h after the exposure. In some experiments, exogenous collagen IV (50 μg/ml), collagen I (50 μg/ml), or function-stimulating antibodies to CBI subunits α_2 or β_1 (5 μg/ml) were added daily to the culture media of DCVC-injured RPTC cultured in the absence of pharmacological concentrations of AscP. Function-stimulating antibodies to CBI α_1 are not commercially available at this time. On days 1 and 6 after DCVC exposure, active Na^+ transport or Na^+/K^+-ATPase activity, and CBI expression and/or localization in the remaining sublethally injured RPTC was determined as described below.

Active Na^+ Transport. RPTC were gently detached from culture dishes with a rubber policeman and transferred to a 37°C oxygen consumption (QO_2) chamber. QO_2 in RPTC was measured polarographically in the absence (basal QO_2) or presence of ouabain (100 μM) (ouabain-insensitive QO_2) using a Clark-type electrode as described previously (Nowak and Schnellmann, 1995). Active Na^+ transport (ouabain-sensitive QO_2) was calculated by subtracting ouabain-insensitive QO_2 from basal QO_2. Protein concentrations were determined using the bicinchoninic acid microassay according to the manufacturer's instructions (Pierce, Rockford, IL).

Na^+/K^+-ATPase Activity. Total ATPase activity was measured as previously described (Nony et al., 2001). Briefly, RPTC cultured in 48-well cell culture clusters were scraped, solubilized in dissociation buffer (5 mM HEPES, pH 7.4, 25 mM imidazole, 1% BSA, 0.065% SDS) for 10 min at room temperature, and combined with fresh ATPase assay buffer (2.54 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 5 mM HEPES, 10 U/ml lactate dehydrogenase, 7 U/ml pyruvate kinase, 2.54 mM Na_2ATP, 2.54 mM phospho(enol) pyruvate, and 0.5 mM β-NADH). ATPase activity was measured under linear conditions spectrophotometrically (340 nm) as the oxidation of β-NADH to NAD^+ at 37°C in the absence or presence of ouabain (0.1 mM). Na^+/K^+-ATPase activity was calculated as total ATPase activity minus ouabain-insensitive ATPase activity.

Expression of CBI. CBI expression was measured by flow cytometry of RPTC immunostained with monoclonal antibodies to in-

tegrin subunits α_1, α_2, and β_1. RPTC monolayers were washed three times with ice-cold PBS, gently scraped from culture dishes into cell culture media containing 5% BSA (BSA/media), and transferred to microcentrifuge tubes. RPTC were dissociated by pipetting, incubated on ice with moderate shaking for 20 min, centrifuged, and resuspended in 1% BSA/media containing 2 μg/ml of a specific anti-integrin antibody or nonspecific IgG on ice with moderate shaking for 1 h. After three washes with 1% BSA/media, RPTC were incubated for 30 min in the dark with a goat-anti mouse FITC-conjugated IgG diluted 1:100 in 1% BSA/media, followed by three washes with ice-cold PBS. Membrane expression of CBI was determined immediately by flow cytometry using a FACSCalibur four-color cell sorter/analyzer (Becton Dickinson, San Jose, CA) with a blue argon laser for detection of FITC. Specific binding was calculated as total fluorescence minus that in IgG controls.

Subcellular Localization of CBI. CBI localization was determined using confocal microscopy of RPTC monolayers stained with monoclonal antibodies to integrin subunits α_1, α_2, and β_1. RPTC monolayers were washed three times with PBS and fixed with 10% buffered formalin (4% formaldehyde) for 20 min at room temperature. After three washes with PBS, monolayers were permeabilized in PTB buffer (PBS, 0.3% Triton X-100, 0.1% BSA) for 10 min at room temperature. Monolayers were washed three times with 0.1% BSA in PBS and incubated with 8% BSA in PBS for 30 min at room temperature. BSA (1%) in PBS containing 5 μg/ml of specific integrin antibodies or nonspecific IgG was added to RPTC monolayers and incubated overnight at 4°C with moderate shaking. After three washes with PTB buffer, monolayers were incubated for 2 h in the dark at room temperature with 1% BSA in PBS containing a 1:100 dilution of a FITC-conjugated goat anti-mouse IgG. Monolayers were washed three times with PTB and glass coverslips applied after the addition of two to three drops of mounting media. Confocal microscopy was performed using a Zeiss confocal laser scanning microscope (model 410; Carl Zeiss, Inc., Thornwood, NY). Basal and apical membrane locations were determined visually in the Z-plane using light field microscopy. Two to three photomicrographs per monolayer at the basal and apical membranes were then scanned with an omnichrome laser filtered at 488 nm to detect FITC.

Statistical Analysis. RPTC isolated from one rabbit represent one experiment ($n = 1$) that consisted of data collected from one to two plates of cells. Experiments were repeated until an n of 3 to 6 was reached. Data are presented as means ± S.E.M. Significant differences between treatment groups ($p < 0.05$) were determined using SigmaStat one-way analysis of variance and Student-Newman-Keuls *post hoc* test for the comparison of multiple means (Jandel Scientific, San Rafael, CA).

Results

Effect of AscP and Exogenous Collagen IV on Active Na^+ Transport in DCVC-Injured RPTC. Exposure of uninjured RPTC to pharmacological concentrations of AscP or exogenous collagen IV had no effect on active Na^+ transport on days 1 or 6 (data not shown). On day 1 after injury, active Na^+ transport was decreased approximately 80% in injured RPTC grown in the absence or presence of pharmacological concentrations of AscP or collagen IV (Fig. 1A). On day 6, DCVC-injured RPTC cultured in the presence of exogenous collagen IV exhibited a concentration-dependent improvement in active Na^+ transport, similar to that seen in injured RPTC cultured in the presence of pharmacological concentrations of AscP (Fig. 1B).

Effects of Sublethal Injury and Exogenous Collagen IV on Total CBI Expression in RPTC. Monoclonal antibodies to the CBI subunits α_1, α_2, and β_1 and flow cytometry were used to measure total plasma membrane expression of CBI on days 1 and 6 after DCVC exposure. Figure 2 demonstrates the fluorescence-shift observed in response to incubation of rabbit RPTC with the anti-integrin antibodies for individual CBI subunits. Exposure of uninjured RPTC to pharmacological concentrations of AscP or exogenous collagen IV did not affect CBI α_1, α_2, and β_1 expression (data not shown). After exposure to DCVC, levels of expression of CBI subunits α_1, α_2, and β_1 in injured RPTC were unchanged on day 1 compared with control (Fig. 3, a, c, and e). On day 6 after injury, expression of CBI subunits α_1 and β_1 was unchanged in DCVC-injured RPTC compared with control (Fig. 3, b and f). However, membrane expression of CBI subunit α_2 was increased approximately 2.2-fold in DCVC-injured RPTC (Fig. 3d). The presence of pharmacological concentrations of AscP or exogenous collagen IV did not affect the expression of CBI subunits α_1, α_2, or β_1 in sublethally injured RPTC.

Effects of Sublethal Injury and Exogenous Collagen IV on the Subcellular Localization of CBI in RPTC. On day 1 after DCVC exposure, the intensity of CBI α_1, α_2, and β_1 fluorescent staining at the basal membrane was decreased compared with control (A–D in Figs. 4, 6, and 8). For comparison with injured RPTC on day 6, uninjured, subconfluent (80%) RPTC cultures were used as controls for basal localization of CBI. As opposed to uninjured RPTC in day 12 of culture (6 days of growth to confluence plus the 6 experimen-

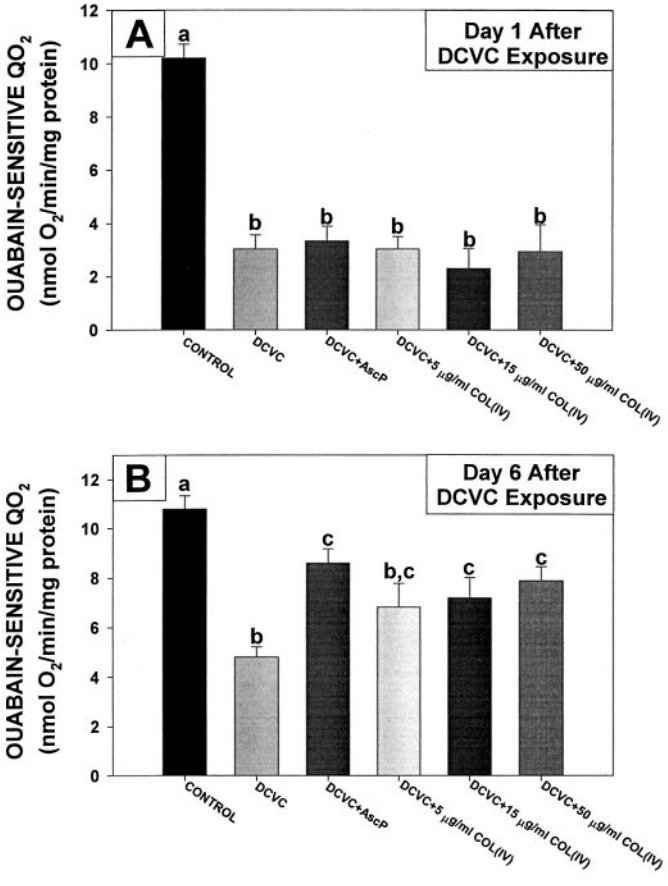

Fig. 1. Active Na^+ transport measured as ouabain-sensitive QO_2 in RPTC sublethally injured by DCVC and cultured in the absence or presence of exogenous collagen IV (0, 5, 15, and 50 μg/ml). Ouabain sensitive QO_2 was measured on days 1 and 6 after DCVC injury. Data are presented as means ± S.E.M., $n = 4$ to 5 separate experiments. Bars labeled with different letter symbols are significantly different from each other ($P < 0.05$).

tation days), subconfluent RPTC cultures exhibit morphology and cell density more like that of sublethally injured RPTC cultures. On day 6 after injury, basal localization of CBI subunits α_1, α_2, and β_1 in RPTC cultured in the absence of AscP or exogenous collagen IV was still decreased compared with subconfluent controls (F in Figs. 4, 6, and 8). In contrast, sublethally injured RPTC cultured in the presence of either pharmacological concentrations of AscP or exogenous collagen IV exhibited a return to control levels of basal localiza-

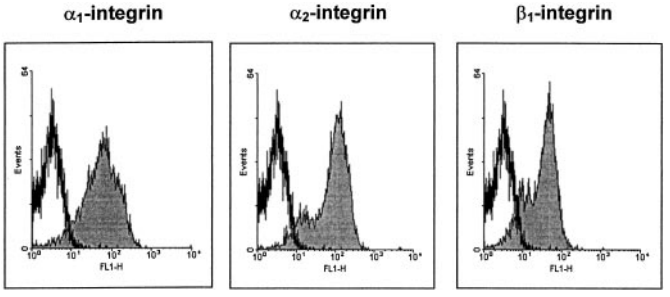

Fig. 2. Binding of monoclonal antibodies to CBI subunits. Untreated rabbit RPTC were gently scraped from culture dishes and incubated with nonspecific IgG or primary monoclonal antibodies to CBI subunits α_1, α_2, or β_1. Fluorescence intensity of FITC-conjugated goat anti-mouse secondary antibodies was measured by flow cytometry. FL1-H (x-axis) displays fluorescence intensity on a logarithmic scale. Shaded histograms represent fluorescence intensity of RPTC labeled with primary antibodies to CBI subunits. Open histograms represent RPTC incubated with a nonspecific IgG.

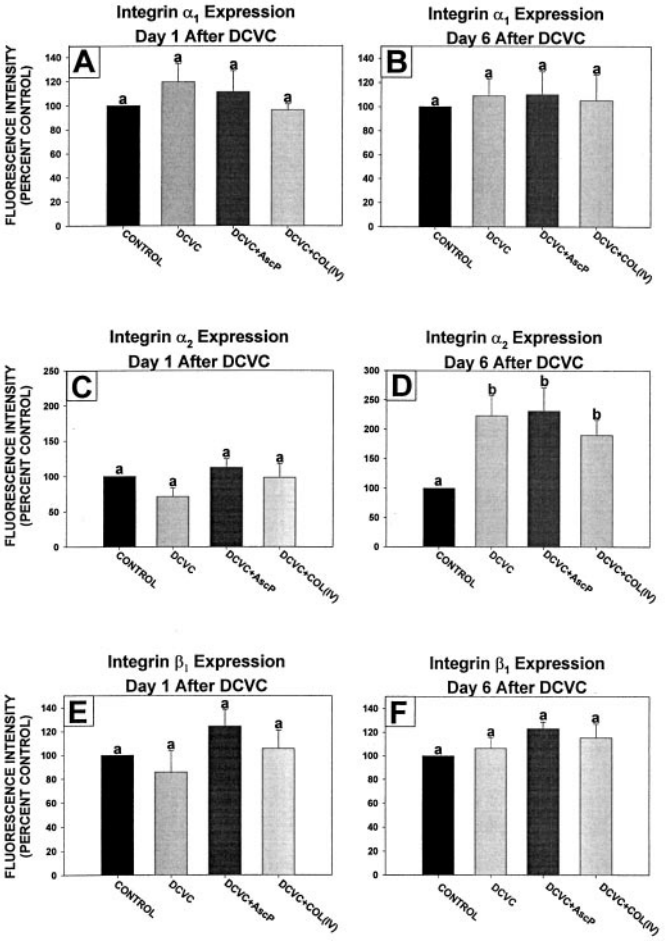

Fig. 3. Expression of CBI subunits α_1, α_2, and β_1 in RPTC sublethally injured by DCVC and cultured in the absence or presence of pharmacological concentrations of AscP (500 μM) or exogenous collagen IV (50 μg/ml). On days 1 (A, C, and E) and 6 (B, D, and F) after DCVC injury, plasma membrane expression of CBI was analyzed by flow cytometry. Fluorescence intensity is expressed as a percent of control ± S.E.M. after subtraction of fluorescence attributed to nonspecific IgG binding (n = 4–6). Bars labeled with different letter symbols are significantly different from each other ($P < 0.05$).

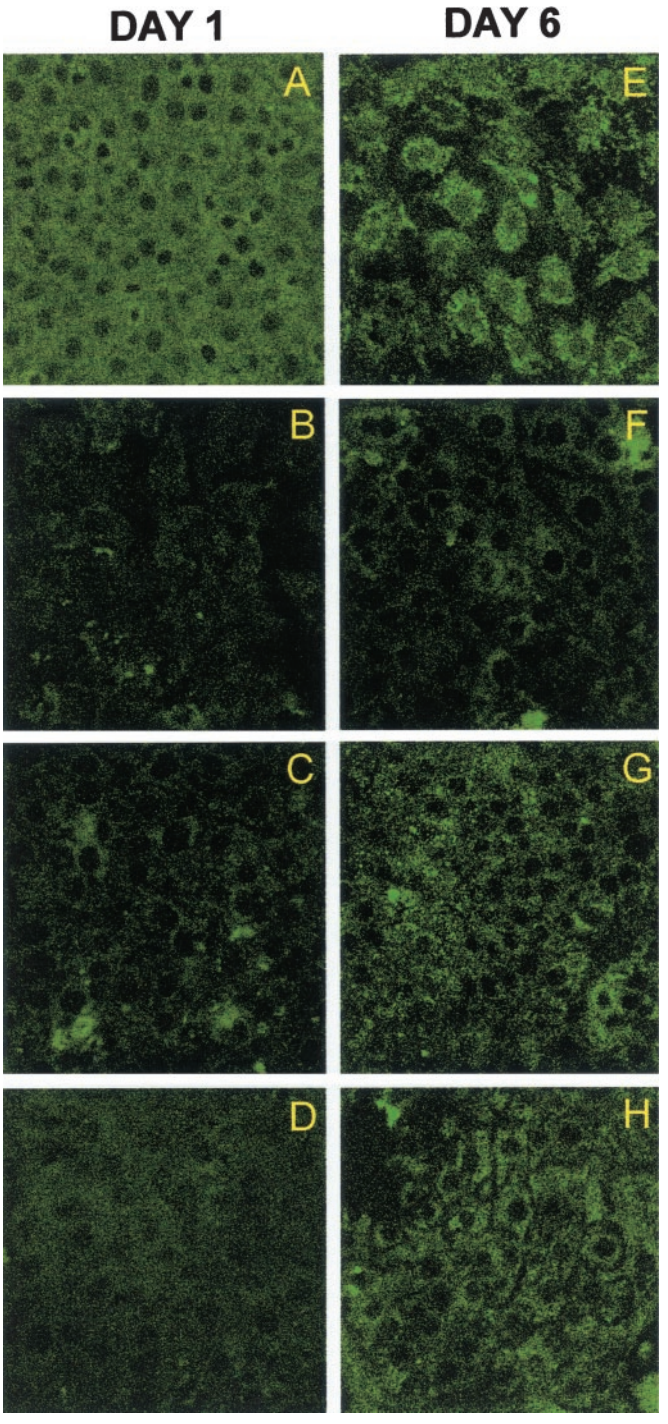

Fig. 4. Basal membrane localization of CBI subunit α_1 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative confocal photomicrographs from three to four separate experiments (magnification, 400×).

tion of CBI subunits α_1, α_2, and β_1 (G and H in Figs. 4, 6, and 8). With respect to the apical membrane, uninjured control animals showed no CBI staining, whereas CBI subunits α_1, α_2, and β_1 were partially redistributed to the apical membrane in sublethally injured RPTC on day 1 after injury (D in Figs. 5, 7, and 9). On day 6, sublethally injured RPTC continued to exhibit CBI distributed to the apical membrane (F in Figs. 5, 7, and 9). However, injured RPTC cultured in the presence of pharmacological concentrations of AscP or exogenous collagen IV revealed a complete disappearance of CBI from the apical membrane by day 6 (G and H in Figs. 5, 7, and 9). These data show that sustained redistribution of CBI characterized by decreased basal localization and the appear-

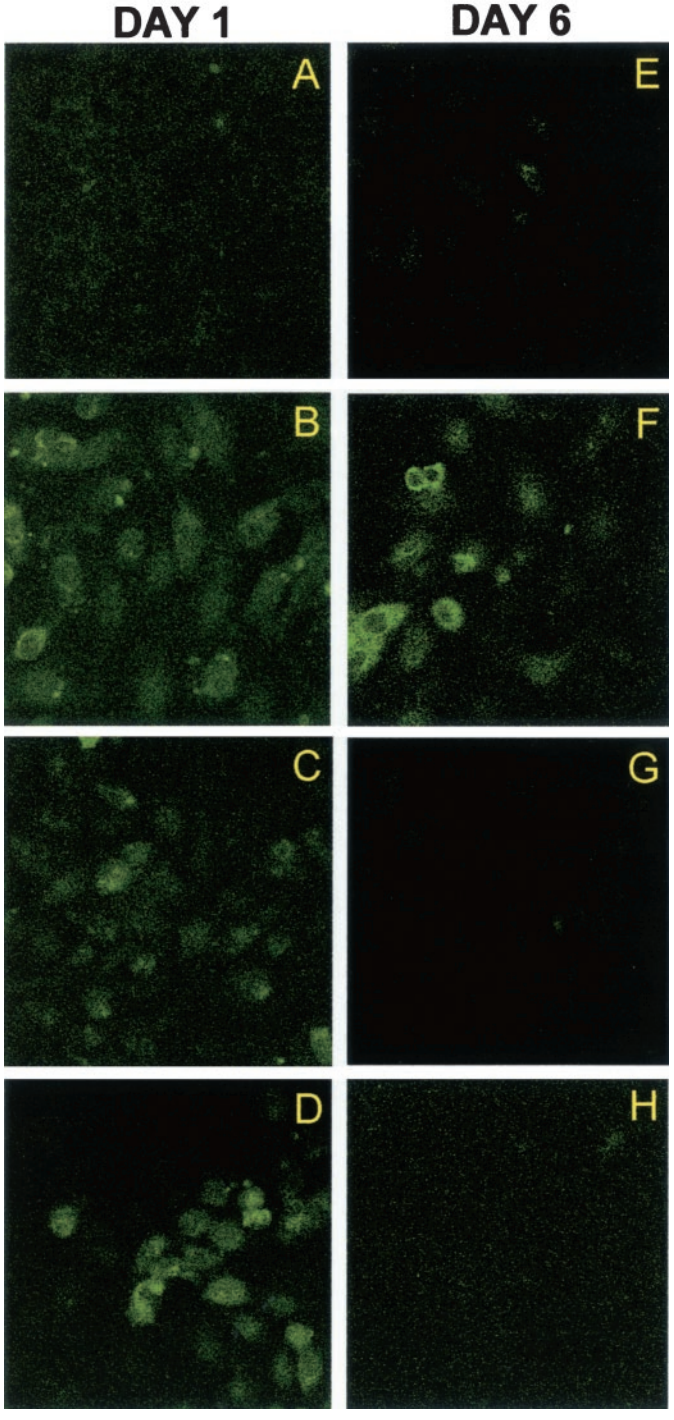

Fig. 5. Apical membrane localization of CBI subunit α_1 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative photomicrographs from three to four separate experiments (magnification, 400×).

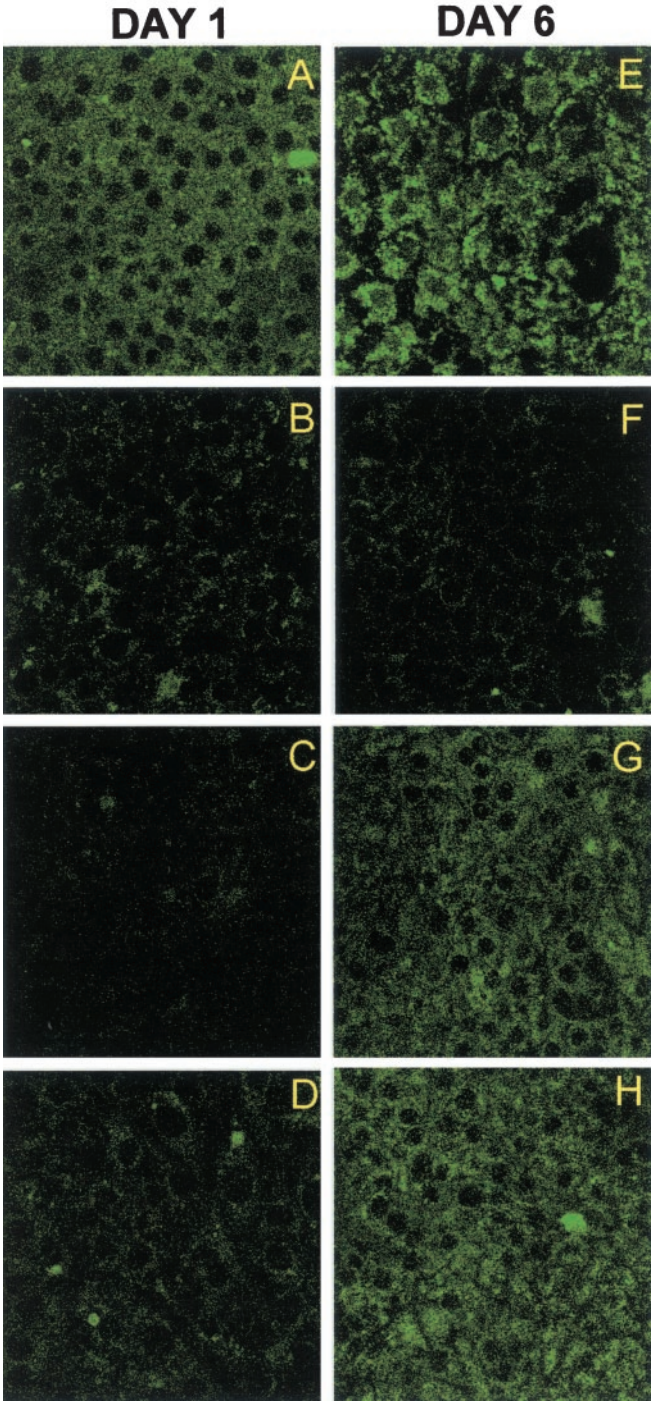

Fig. 6. Basal membrane localization of CBI subunit α_2 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative confocal photomicrographs from three to four separate experiments (magnification, 400×).

ance of CBI on the apical membrane of RPTC is a consequence of sublethal injury by DCVC. In addition, sublethally injured RPTC cultured in the presence of pharmacological concentrations of AscP or exogenous collagen IV are able to reorient CBI to the basal membrane.

Effect of Function-Stimulating Antibodies to CBI on Na^+/K^+-ATPase Activity and the Subcellular Localization of CBI in RPTC. Function-stimulating antibodies to CBI subunits α_2 and β_1 were added to the culture media of DCVC-injured RPTC. The function-stimulating antibodies to CBI subunits α_2 and β_1 did not affect the degree of DCVC-induced RPTC injury on day 1 after exposure (data not shown). On day 6 after injury, the CBI β_1-stimulating anti-

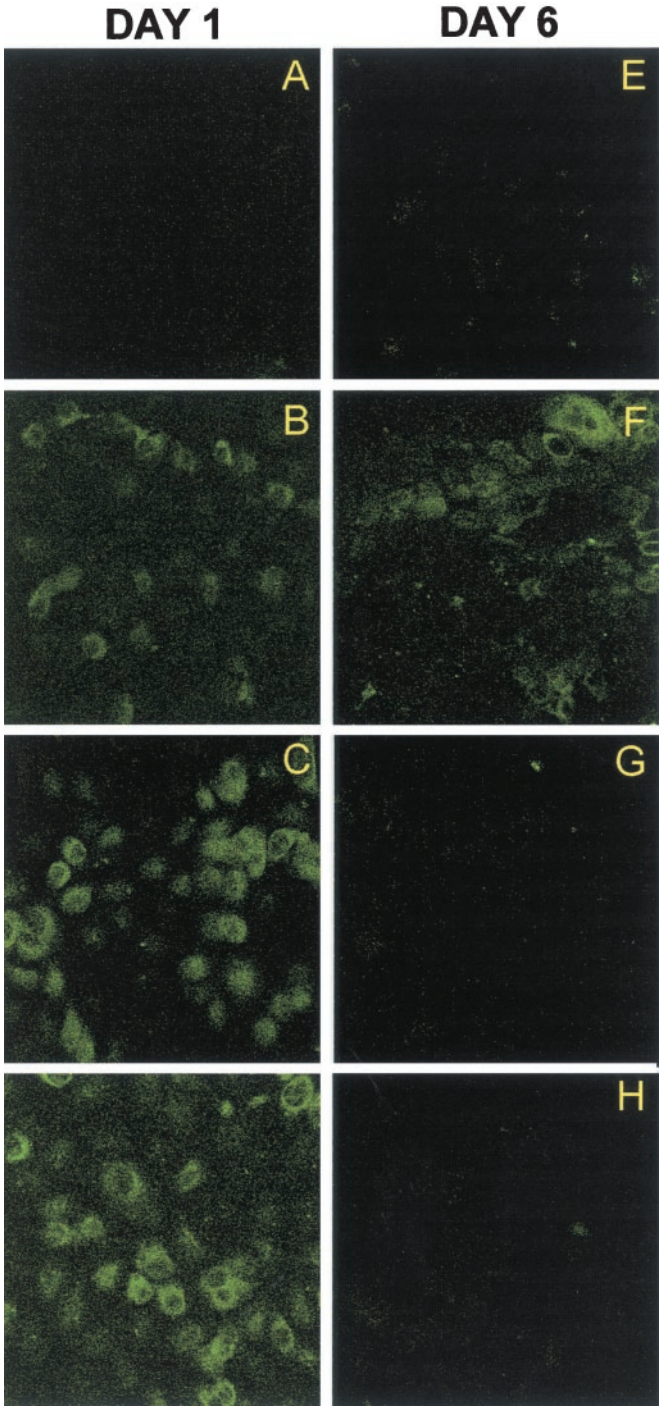

Fig. 7. Apical membrane localization of CBI subunit α_2 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative photomicrographs from three to four separate experiments (magnification, 400×).

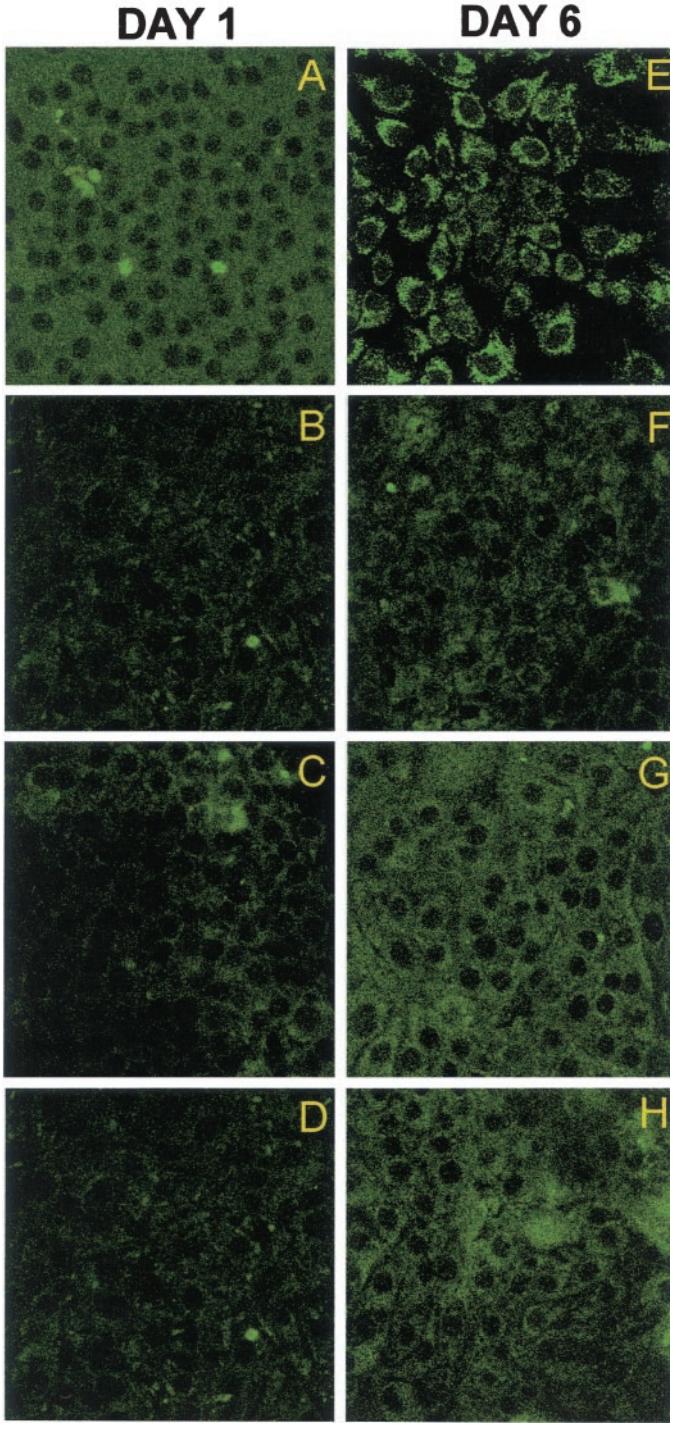

Fig. 8. Basal membrane localization of CBI subunit β_1 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative confocal photomicrographs from three to four separate experiments (magnification, 400×).

body, but not the CBI α_2-stimulating antibody, promoted the repair of Na^+/K^+-ATPase activity in DCVC-injured RPTC (Fig. 10). The addition to the culture media of function-stimulating antibodies to CBI subunits α_2 or β_1 did not prevent basal delocalization or partial apical redistribution of CBI α_2 or β_1 on day 1 after DCVC exposure (data not shown). Despite the repair of Na^+/K^+-ATPase activity on day 6, CBI subunit β_1 remained delocalized and partially redistributed to the apical membrane in RPTC cultured in the presence of the β_1-stimulating antibody (Fig. 11).

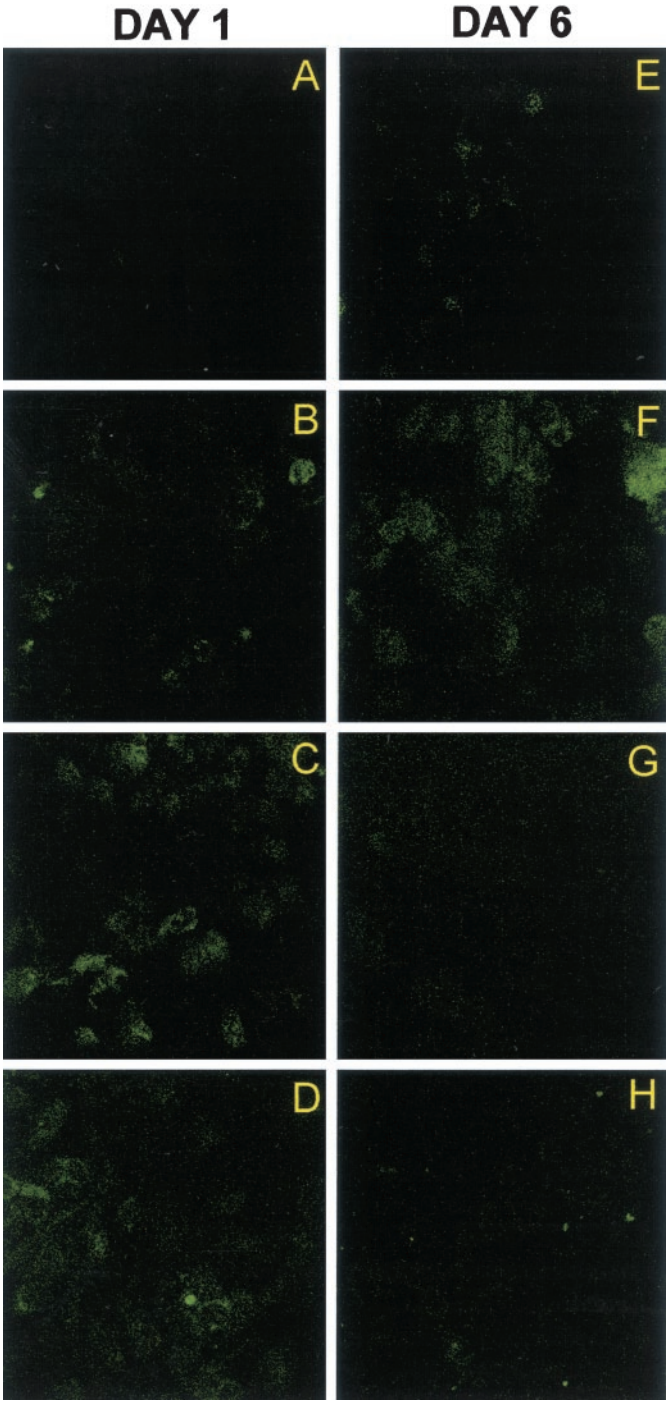

Fig. 9. Apical membrane localization of CBI subunit β_1 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative photomicrographs from three to four separate experiments (magnification, 400×).

Discussion

Anchorage-dependent cell growth, proliferation, migration, and differentiation depend on the ability of the cell to recognize anchoring substrates in the ECM. Localization of ECM-binding integrins to the point of contact provides a strong but dynamic interaction that supports not only cellular attachment but also communication between the cell and the ECM. Given the importance of these interactions and the maintenance of cell polarity, the loss of integrin-ECM interactions and cell polarity plays an essential role in cell injury. Likewise, the restoration of integrin-ECM interactions and cell polarity probably plays an equally important role in the return of normal cell function after injury.

Loss of renal epithelial cell polarity caused by partial integrin redistribution throughout the plasma membrane has been shown to be a key event in renal dysfunction after acute chemical exposure or ischemia (Goligorsky and DiBona, 1993; Lieberthal et al., 1997; Zuk et al., 1998; Molitoris and Marrs, 1999). The resulting cellular disorientation and dysfunction with cell death and/or detachment from the BM leads to decreased renal tubular function (Goligorsky and DiBona, 1993; Frisch and Ruoslahti, 1997; Tang et al., 1998; Molitoris and Marrs, 1999). Despite evidence demonstrating the loss of integrin polarity during renal cell injury, the importance of the restoration of integrin localization and cell polarity in tubular regeneration after injury is not well understood (Goligorsky and DiBona, 1993; Lieberthal et al., 1997; Kreidberg and Symons, 2000; Molitoris and Marrs, 1999; Zuk et al., 1998). Previous studies in our laboratory demonstrated that the ability of injured RPTC to deposit collagen IV is associated with the repair of inhibited physiological functions after DCVC injury (Nony et al., 2001). Furthermore, exogenous collagen IV added to the culture media

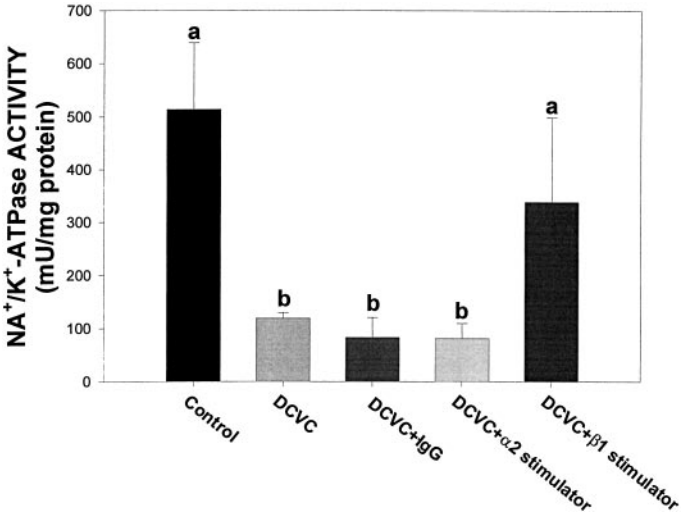

Fig. 10. Na^+/K^+-ATPase activity in RPTC sublethally injured by DCVC and cultured in the absence or presence of function-stimulating antibodies to CBI subunits α_2 or β_1 (5 μg/ml). Na^+/K^+-ATPase activity was measured on days 1 and 6 after injury. Data are presented as means ± S.E.M., $n = 3$ to 5 separate experiments. Bars labeled with different letter symbols are significantly different from each other ($P < 0.05$).

of injured RPTC promoted repair of physiological functions, providing the first evidence that a key ECM protein in the renal proximal tubule is involved in cell repair after injury (Nony et al., 2001). In contrast, collagen I, laminin, and fibronectin did not promote cell repair after injury. Because renal epithelial cells interact with collagen IV through CBI, our data suggested that physiological repair in injured RPTC may involve an effect of collagen IV on the expression, localization, and/or function of CBI. As mentioned above, sublethally injured cells may experience a loss in cell polarity because of decreased localization of certain proteins to specific areas of the plasma membrane. Two potential reasons for a decrease in basal membrane protein localization include a decrease in membrane expression of those proteins because of translational effects or receptor internalization or the redistribution of those proteins to other areas of the plasma membrane. After DCVC-induced sublethal injury to RPTC, no changes in total membrane expression of CBI were evident on day 1. However, confocal microscopy showed that CBI localization to the basal membrane was decreased and accompanied by the appearance of CBI on the apical membrane. These observations show that sublethal toxicant injury produces a decrease in basal CBI localization because of redistribution of CBI as opposed to decreased overall expression of CBI on the plasma membrane. On day 6 after injury, confocal microscopy demonstrated that all CBI were still redistributed to the apical membrane, suggesting that DCVC-injured RPTCs remain disoriented with lost cellular polarity. These novel findings associate sustained integrin redistribution with the lack of repair of physiological functions after DCVC injury.

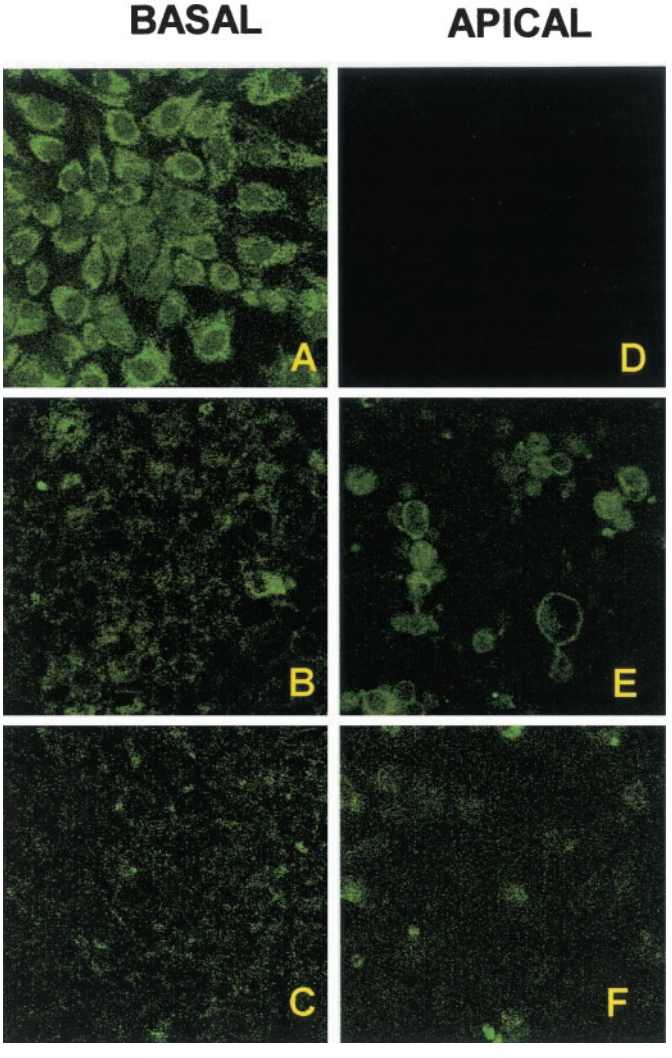

Fig. 11. Basal (left) and apical (right) localization of CBI subunit β_1 on day 6 after DCVC exposure in RPTC cultured in the absence or presence of CBI subunit β_1-stimulating antibodies. A and D, control RPTC; B and E, DCVC-injured RPTC; C and F, DCVC-injured RPTC+β_1-stimulating antibody. Shown are representative confocal photomicrographs from two separate experiments (magnification, 400×).

Because pharmacological concentrations of AscP and exogenous collagen IV stimulated repair of physiological functions after DCVC exposure, we determined the effects of AscP and exogenous collagen IV on the expression and localization of CBI. Addition of pharmacological concentrations of AscP or exogenous collagen IV to culture media of injured RPTC produced no changes in total membrane expression of CBI after sublethal injury. However, exposure to AscP and exogenous collagen IV resulted in the return of basal membrane localization of CBI with the loss of CBI from the apical membrane. These observations suggest that extracellular collagen IV, whether stimulated by AscP or added exogenously, promotes the return of cellular polarity characterized by the basal re-orientation of CBI and Na^+/K^+-ATPase (Nowak et al., 2000), and the return of Na^+/K^+-ATPase activity. Concerning cell repair, these data suggest that an important step in the repair of physiological functions stimulated by extracellular collagen IV is the reorientation of CBI to the basal membrane and the restoration of cellular polarity.

On day 6 after DCVC-injury, RPTC exhibited a significant increase in the membrane expression of the CBI subunit α_2 in the absence or presence of pharmacological concentrations of AscP or exogenous collagen IV. However, the basal membrane localization of CBI subunit α_2 on day 6 after injury correlated well with that of CBI subunits α_1 and β_1, although membrane expression of those subunits did not change. The qualitative assessment of CBI localization used in this study allows for the comparison of spatially distinct membrane regions with regard to the absence or presence of CBI. Our data do not permit an accurate quantification of CBI density at the observed regions of the plasma membrane, nor does it account for CBI localized to lateral membrane regions between the apical and basal membranes. Therefore, the physiological or pathological relevance of an increase in membrane expression of CBI subunit α_2 in this study is not clear.

Because interactions between collagen IV and CBI seemed to be associated with the promotion of physiological repair, we hypothesized that repair could be stimulated by activating CBI in the absence of collagen IV or AscP. To test this, injured RPTC were cultured in the presence of function-stimulating antibodies to CBI subunits α_2 or β_1. Indeed, the CBI subunit β_1 antibody, but not the subunit α_2 antibody, promoted the return of Na^+/K^+-ATPase activity in injured RPTC, suggesting that signaling through the β_1 integrin is linked to the repair of physiological functions. However, it is not known which α subunit is associated with β_1 under these conditions. Although it seems that the α_2 subunit is not associated with β_1, it cannot be excluded that the α_2 antibody can bind to α_2 but does not act as a stimulating antibody in this model. It is also possible that α_1 or another unknown

subunit is associated with β_1. Although an α_1-stimulating antibody was not available, the development of additional function stimulating or blocking antibodies would advance this line of research.

Despite the stimulation of repair, decreased CBI localization in the basal membrane and partial apical redistribution in injured RPTC were not reversed in response to the α_2 and β_1-stimulating integrin antibodies. This result suggests that activation of β_1 integrins promotes repair through a mechanism that is independent of CBI relocalization after injury. Furthermore, the repair and relocalization effects of collagen IV may be through two different CBI. Alternatively, the difference observed between the β_1 antibody response and collagen IV may occur downstream of the CBI binding. For example, outside-in signaling cascades mediated by integrins have been shown to stimulate the activation of extracellular-signal regulated kinases (ERKs) (Schlaepfer et al., 1994; Boudreau and Jones, 1999). In addition, integrin-mediated ERK activation has been shown to proceed through distinct pathways depending upon what type of integrin is involved (Lin et al., 1997; Barberis et al., 2000; Tian et al., 2000). At this time, the role of ERK activation in RPTC repair is unknown. Therefore, linking repair and the activation of integrin β_1 independent of integrin-ECM interactions in RPTC requires further study.

In conclusion, our data show that the total expression of CBI in sublethally injured RPTC is not altered on day 1 after injury, but that CBI are decreased in the basal membrane and partially redistributed to the apical membrane. On day 6 after injury, DCVC-treated RPTC that do not repair physiological functions still exhibit decreased CBI and redistribution. In contrast, the presence of pharmacological concentrations of AscP or exogenous collagen IV in the culture media of DCVC-injured RPTC promotes the disappearance of CBI from the apical membrane and basal membrane reorientation of CBI by day 6 after injury. This study demonstrates for the first time that AscP- or collagen IV-mediated relocalization of CBI is related to the repair of physiological functions. In addition, antibody addition experiments suggest that integrin-mediated repair of physiological functions may proceed through multiple pathways. These novel findings suggest that there is a specific role for collagen IV to promote physiological repair, in part, through the restoration of CBI localization and cellular polarity, shedding new light on the mechanisms of renal cell repair after chemical-induced injury.

Acknowledgments

We thank Dr. Thomas W. Petry (Upjohn Pharmacia, Kalamazoo, MI) for his generous gift of *S*-(1,2-dichlorovinyl)-L-cysteine.

References

Abbate M and Remuzzi G (1996) Acceleration of recovery in acute renal failure: from cellular mechanisms of tubular repair to innovative targeted therapies. *Ren Fail* **18:**377–388.

Barberis L, Wary KK, Fiucci G, Liu F, Hirsch E, Brancaccio M, Altruda F, Tarone G, and Giancotti F (2000) Distinct roles of the adaptor protein shc and focal adhesion kinase in integrin signaling to ERK. *J Biol Chem* **275:**36532–36540.

Boudreau NJ and Jones PL (1999) Extracellular matrix and integrin signaling: the shape of things to come. *Biochem J* **339:**481–488.

Bush KT, Keller SK, and Nigam SK (2000) Genesis and reversal of the ischemic phenotype in epithelial cells. *J Clin Invest* **106:**621–626.

Frisch SM and Ruoslahti E (1997) Integrins and anoikis. *Curr Opin Cell Biol* **9:**701–705.

Furness PN (1996) Extracellular matrix and the kidney. *J Clin Pathol* **49:**355–359.

Gardner H, Kreidberg J, Koteliansky V, and Jaenisch R (1996) Deletion of integrin alpha 1 by homologous recombination permits normal murine development but gives rise to a specific deficit in cell adhesion. *Dev Biol* **175:**301–313.

Goligorsky MS and DiBona GF (1993) Pathogenetic role of arg-gly-asp-recognizing integrins in acute renal failure. *Proc Natl Acad Sci USA* **90:**5700–5704.

Gotwals PJ, Chi-Rosso G, Lindner V, Yang J, Ling L, Fawell SE, and Koteliansky VE (1996) The $\alpha 1\beta 1$ integrin is expressed during neointima formation in rat arteries and mediates collagen matrix reorganization. *J Clin Invest* **97:**2469–2477.

Hata RI and Senoo H (1989) L-Ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissue-like substance by skin fibroblasts. *J Cell Physiol* **138:**8–16.

Knight CG, Morton LF, Onley DJ, Peachey AR, Messent AJ, Smethurst PA, Tuckwell DS, Farndale RW, and Barnes MJ (1998) Identification in collagen type I of an integrin $\alpha_2\beta_1$-binding site containing an essential GER sequence. *J Biol Chem* **273:**33287–33294.

Kreidberg JA and Symons JM (2000) Integrins in kidney development, function and disease. *Am J Physiol* **279:**F233–F242.

Kuhn K and Ebel J (1994) The structural bases of integrin-ligand interactions. *Trends Cell Biol* **4:**256–261.

Lieberthal W, McKenney JB, Kiefer CR, Snyder LM, Kroshian VM, and Sjaastad MD (1997) Beta1 integrin-mediated adhesion between renal tubular cells after anoxic injury. *J Am Soc Nephrol* **8:**175–183.

Lin TH, Aplin AE, Shen Y, Chen Q, Schaller M, Romer L, Aukhil I, and Juliano RL (1997) Integrin-mediated activation of MAP kinase is independent of FAK: Evidence for dual integrin signaling pathways in fibroblasts. *J Cell Biol* **136:**1385–1395.

Miner JH (1999) Renal basement membrane components. *Kidney Int* **56:**2016–2024.

Molitoris BA and Marrs J (1999) The role of cell adhesion molecules in ischemic acute renal failure. *Am J Med* **106:**583–592.

Moore RB and Green T (1988) The synthesis of nephrotoxin conjugates of glutathione and cysteine. *Toxicol Environ Chem* **17:**153–162.

Nony PA, Nowak G, and Schnellman RG (2001) Collagen IV promotes repair of renal cell physiological functions after toxicant injury. *Am J Physiol* **281:**F443–F453.

Nowak G, Carter CA, and Schnellmann RG (2000) Ascorbic acid promotes recovery of cellular functions following toxicant-induced injury. *Toxicol Appl Pharmacol* **167:**37–45.

Nowak G, Keasler KB, McKeller DE, and Schnellmann RG (1999) Differential effects of EGF on repair of cellular functions after dichlorovinyl-L-cysteine-induced injury. *Am J Physiol* **276:**F228–F236.

Nowak G and Schnellmann RG (1995) Integrative effects of EGF on metabolism and proliferation in renal proximal tubular cells. *Am J Physiol* **269:**C1317–C1325.

Nowak G and Schnellmann RG (1996) L-Ascorbic acid regulates growth and metabolism of renal cells: improvements in cell culture. *Am J Physiol* **271:**C2072–C2080.

Riikonen T, Westermarck J, Koivisto L, Broberg A, Kahari V, and Heino J (1995) Integrin $\alpha 2\beta 1$ is a putative regulator of collagense (MMP-1) and collagen $\alpha 1(1)$ gene expression. *J Biol Chem* **270:**13548–13552.

Ruoslahti E and Engvall E (1997) Integrins and vascular extracellular matrix assembly. *J Clin Invest* **100:**S53–S56.

Schlaepfer DD, Hanks SK, Hunter T, and van der Geer P (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature (Lond)* **372:**786–790.

Schoenwaelder SM and Burridge K (1999) Bidirectional signaling between the cytoskeleton and integrins. *Curr Opin Cell Biol* **11:**274–286.

Tang M, Hu J, Lin H, Chiu W, and Jiang S (1998) Collagen gel overlay induces apoptosis of polarized cells in cultures: disoriented cell death. *Am J Physiol* **275:**C921–C931.

Tian W, Zhang Z, and Cohen SM (2000) MAPK signaling and the kidney. *Am J Physiol Renal Physiol* **279:**F593–F604.

Zuk A, Bonventre JV, Brown D, and Matlin KS (1998) Polarity, integrin, and extracellular matrix dynamics in the postischemic rat kidney. *Am J Physiol* **275:**C711–C731.

Address correspondence to: Rick G. Schnellmann, Ph.D., Department of Pharmaceutical Sciences, Medical University of South Carolina, 280 Calhoun Street, POB 250140, Charleston, SC 29425. E-mail: schnell@musc.edu