Collagen IV promotes repair of renal cell physiological functions after toxicant injury

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Nony, Paul A., Grazyna Nowak, and Rick G. Schnellmann. Collagen IV promotes repair of renal cell physiological functions after toxicant injury. Am J Physiol Renal Physiol 281: F443–F453, 2001.—Collagen IV is found in the renal proximal tubular cell (RPTC) basement membrane and is a mediator of renal development and function. Pharmacological concentrations of l-ascorbic acid phosphate (AscP) promote the repair of physiological functions in RPTC sublethally injured by S-(1,2-dichlorovinyl)-L-cysteine (DCVC). We hypothesized that AscP promotes RPTC repair by stimulating collagen IV synthesis and/or deposition. RPTC exhibited increased synthesis but decreased deposition of collagen IV after DCVC exposure. In contrast, RPTC cultured in pharmacological concentrations of AscP maintained collagen IV deposition. The activity of prolyl hydroxylase was decreased in RPTC after DCVC injury, an effect that was partially attenuated in injured RPTC cultured in pharmacological concentrations of AscP. The addition of exogenous collagen IV to the culture media of DCVC-injured RPTC promoted the repair of mitochondrial function and Na+/K+-ATPase activity. However, neither collagen I, laminin, nor fibronectin promoted cell repair. These data demonstrate an association between AscP-stimulated deposition of collagen IV and exogenous collagen IV and repair of physiological functions, suggesting that collagen IV plays a specific role in RPTC repair after sublethal injury.

cell injury; regeneration; extracellular matrix; collagen synthesis and deposition; prolyl hydroxylase

THE MESHLIKE BASEMENT MEMBRANE (BM) provides structural support and influences the growth, function, and survival of many cell types in most organ systems (15). Collagens are extracellular matrix (ECM) proteins that form the renal tubular BM with other ECM proteins such as laminin and heparan sulfate proteoglycans (29). The most abundant type of collagen in the BM of the glomerulus and renal tubules is collagen IV, a globular, nonfibrillar protein. This characteristic distinguishes it from collagen I, the major fibrillar component of connective tissues and the second most abundant ECM protein in the proximal tubular BM (12, 21). Collagen IV forms a triple-helical monomer that consists most often of two \( \alpha(1) \) chains and one \( \alpha(2) \) chain or three \( \alpha(1) \) chains (14, 42). The collagen IV chains \( \alpha(3), \alpha(4), \alpha(5), \) and \( \alpha(6) \) have been identified and can associate in various combinations (15, 20). However, these isoforms have not been detected in the human proximal tubule or in primary cultures of rabbit renal proximal tubular cells (12, 29). Except in rodents, their renal expression appears to be limited to the distal tubular BM and the glomerular BM, where they have been implicated in the development of Goodpasture and Alport syndromes and diffuse leiomyomatosis (11, 15, 19, 20, 27, 29). By using functional analyses of cell-matrix interactions, collagen IV has been shown to play a crucial role in tubular function and kidney development (31). Because collagen IV is an important anchorage substrate for many cell types, especially in the kidney, the regulation of collagen IV synthesis and degradation plays an important role in cell function, growth, migration, and organ remodeling (15).

Under conditions of ischemia or after acute chemical exposures, renal epithelial cells may die or detach from the ECM and slough into the tubular lumen. Here, they may aggregate with other sloughed cells, forming casts that cause tubular obstruction. Cells that do not die or that become detached from the ECM are thought to dedifferentiate, proliferate, and migrate to denuded areas of the tubule, thus replacing the sloughed cells. The cells of the newly lined tubule may then differentiate, promoting the return of normal tubular function and overall renal function (1). The roles of collagens and other ECM proteins in renal cell survival, migration, and function have been examined (4). Surprisingly, few reports exist regarding the role of collagens in cellular repair and regeneration, although proliferation, migration, and return of normal function do contribute to renal regeneration after injury (49).

Ascorbic acid is known to prevent the effects of scurvy, a disease characterized by defective connective tissue resulting from decreased collagen synthesis (40). In posttranslational processing mechanisms, ascorbate acts as an essential iron-reducing cofactor in the production of collagens, specifically in the hydroxylation of susceptible proline and lysine residues in procollagen \( \alpha \) chains. These hydroxylation reactions are catalyzed by prolyl and lysyl hydroxylases, respectively, and are

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necessary for the proper folding of procollagen triple helices as well as other posttranslational modifications, including glycosylation and monomer cross-linking (9, 24). Insufficiently hydroxylated procollagens have been shown to accumulate intracellularly, be deposited much more slowly, and be targeted for rapid degradation both intracellularly and extracellularly (17, 18, 42). Ascorbic acid also is known to promote the synthesis of both fibrillar and nonfibrillar collagen types in an array of cell types in vitro (10, 13, 33, 45). In addition, ascorbate has been suggested to act pretranslationally by stimulating mRNA expression of multiple collagen types in various culture systems, independent of its role as an enzymatic cofactor (6, 14, 32, 41, 46). Ascorbic acid has been implicated as an important mediator of cell growth and differentiation in a variety of cell types, through its effects on collagen synthesis and deposition (2). Through mechanisms unrelated to ECM production, ascorbic acid has been shown to both stimulate and inhibit cell proliferation depending on ascorbate concentration and cell type (7, 16, 48). Previous studies from our laboratory demonstrated that ascorbic acid promotes increased cell growth and density and improvement of key physiological functions, including brush-border enzyme activity, basal oxygen consumption (QO2), and Na+–K+–ATPase activity in primary cultures of rabbit renal proximal tubular cells (RPTC) (39).

The halocarbon conjugate S-(1,2-dichlorovinyl)-L-cysteine (DCVC) is a model toxicant that causes RPTC necrosis and acute renal failure (23). We previously showed that primary cultures of rabbit RPTC sublethally injured by DCVC neither proliferate nor repair physiological functions (37). In those experiments, RPTC were grown under physiological concentrations of all culture media supplements including 50 μM L-ascorbic acid 2-phosphate (AscP). However, on addition of pharmacological concentrations of AscP (500 μM), RPTC exposed to DCVC were able to proliferate and repair physiological functions, although AscP provided no protective effect during injury. In addition, pharmacological concentrations of ascorbic acid were shown to stimulate collagen IV synthesis and deposition in uninjured RPTC (36). Therefore, the goals of this study were 1) to examine the effect of DCVC on the synthesis, deposition, and proline hydroxylation of collagen IV in DCVC-injured RPTC, 2) to determine whether AscP-stimulated synthesis and/or deposition of collagen IV is associated with AscP-stimulated repair of physiological functions in DCVC-injured RPTC, and 3) to characterize the specific role of collagen IV and other ECM proteins in RPTC repair.

MATERIALS AND METHODS

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 previously described (30). AscP (magnesium salt) was purchased from Wako Chemicals (Richmond, VA). Ouabain was obtained from RBI/Sigma (Natick, MA). L-[35S]-methionine (>1,000 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). L-[14C]-proline (0.275 Ci/mmol) and L-[4,5-3H]-proline (24 Ci/mmol) were purchased from New England Nuclear Life Science Products (Boston, MA). The mouse anti-collagen IV monoclonal antibody M3F7, developed by Dr. Heinz Furthmayr, was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Hyperfilm enhanced chemiluminescence was purchased from Amersham Life Science (Cleveland, OH). Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and AscP (50 or 500 μM) were added to fresh culture medium immediately before daily change of media. AscP was used because L-ascorbic acid is unstable in culture media. AscP is stable in culture media for ≥7 days at 37°C and, after intracellular dephosphorylation, has the same effect on cultured cells as L-ascorbic acid (13).

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 ~50% cell death and loss 24 h after exposure. On days 1, 4, and 6 after DCVC exposure, the ability of the remaining RPTC to regenerate and repair physiological function was monitored as described below. In some experiments, immediately after DCVC exposure and before daily change of media during day 6 after injury, collagen I (0, 5, 15, or 50 μg/ml), collagen IV (0, 5, 15, or 50 μg/ml), laminin (50 μg/ml), or cellular fibronectin (50 μg/ml) was added to the culture media of uninjured and DCVC-injured RPTC grown in physiological concentrations of AscP. Exogenous collagens I and IV used in this study were triple helical and contained only α1 and α2 chains in conformations similar to those found in renal BM (3, 25). Laminin was a mixture of biologically active laminin chains found in most epithelial tissues (8, 50), and cellular fibronectin was composed of functional dimers (51).

Cell density. Measurement of monolayer protein content over time was used to estimate cell density. On days 1, 4, and 6 after sublethal DCVC injury, RPTC monolayers were washed with PBS and solubilized in Triton buffer (0.05% Triton X-100, 100 mM Tris-base, 150 mM NaCl, pH 7.5). Samples were sonicated, and protein concentrations were determined by using the method of Lowry (26) or the bicinchoninic acid microassay according to the manufacturer’s instructions (Pierce, Rockford, IL).

Basal oxygen consumption. On days 1, 4, and 6 after sublethal DCVC injury, RPTC were grown in 35-mm tissue culture dishes or 48-well cell culture clusters under improved conditions as previously described (38, 39). The cell culture medium was a 1:1 mixture of DMEM/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 AscP (50 or 500 μM) were added to fresh culture medium immediately before daily change of media. AscP was used because L-ascorbic acid is unstable in culture media. AscP is stable in culture media for ≥7 days at 37°C and, after intracellular dephosphorylation, has the same effect on cultured cells as L-ascorbic acid (13).
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Na⁺-K⁺-ATPase activity. On days 1 and 6 after sublethal DCVC injury, total ATPase activity was measured using a modification of a previously described procedure (44). Briefly, RPTC cultured in 48-well cell culture clusters were scraped and incubated in dissociation buffer [5 mM HEPES (pH 7.4), 25 mM imidazole, 1% BSA, 0.065% SDS] for 10 min at room temperature and placed on ice. The dissociated RPTC were then dialyzed fivefold with an additional dissociation buffer minus SDS. Aliquots of dissociated RPTC were combined with fresh ATPase assay buffer ([in mM] 2.54 MgCl₂, 100 NaCl, 10 KCl, 5 HEPES, 2.54 Na₄ATP, 2.54 phosphoenolpyruvate, and 0.5 β-NADH), as well as 10 μM lactate dehydrogenase and 7 U/ml pyruvate kinase. ATPase activity was measured spectrophotometrically under linear conditions as the oxidation of β-NADH to NAD⁺ at 37°C in the absence or presence of ouabain (0.1 mM) at a wavelength of 340 nm. Na⁺-K⁺-ATPase activity was calculated as total ATPase activity minus ouabain-insensitive ATPase activity.

Immunoprecipitation of synthesized and deposited collagen IV. Immuno precipitation of newly synthesized and deposited collagen IV was performed by using the method of Yiki et al. (34), with some modifications. On days 1, 4, and 6 after sublethal DCVC injury, RPTC were metabolically labeled for 24 h with 25 μCi/ml of [³⁵S]methionine. Cell monolayers were washed with PBS, solubilized for 30 min in lysis buffer [20 mM Tris-HCl (pH 8.8), 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM N-ethylmaleimide, 1% Triton X-100, 1% sodium deoxycholic acid, 150 mM NaCl, and 1 mM EGTA] containing protease inhibitors (25 μg/ml pepstatin A, 12.5 μg/ml leupeptin, 12.5 μg/ml aprotinin), and centrifuged at 15,000 g for 10 min at 4°C. The resulting supernatants (soluble fraction, cell-associated collagen) were transferred to fresh tubes, snap-frozen in liquid N₂, and stored at −80°C. Protein concentration was determined using a Coomassie protein assay (Pierce) with BSA as the standard. Sample aliquots equaling 0.5 mg of total protein were diluted threefold in immunoprecipitation buffer ([in mM] 20 Tris-HCl (pH 8.8), 2 EDTA, 0.2 PMSF, 10 N-ethylmaleimide, and 10 diithiothreitol, as well as 1% SDS), sonicated, boiled for 5 min, and incubated in the presence of iodoacetamide (25 mM) for 30 min at 37°C with shaking. Samples were centrifuged at 15,000 g for 10 min at 4°C. Supernatants were then transferred to fresh microcentrifuge tubes, snap-frozen in liquid N₂, and stored at −80°C.

Protein concentration in cell-associated and ECM-associated fractions was determined using a Coomassie protein microassay (Pierce) with BSA as the standard. Sample aliquots equaling 0.5 mg of total protein were diluted threefold in immunoprecipitation buffer ([in mM] 20 Tris-HCl (pH 8.8), 2 EDTA, 0.2 PMSF, 10 N-ethylmaleimide, and 10 diithiothreitol, as well as 1% SDS), sonicated, boiled for 5 min, and incubated in the presence of iodoacetamide (25 mM) for 30 min at 37°C with shaking. Samples were centrifuged at 15,000 g for 10 min at 4°C. Supernatants were then transferred to fresh microcentrifuge tubes, snap-frozen in liquid N₂, and stored at −80°C.

RESULTS

Cell density. Monolayer protein content was used to measure cell density over a 6-day recovery period in RPTC sublethally injured by DCVC. There were no significant differences at any time point in monolayer protein content between untreated RPTC grown in 50 or 500 μM AscP. One day after DCVC exposure (200 μM), RPTC cultured in 50 μM AscP exhibited a 56% decrease in monolayer protein content compared with control, representing cell death and loss (Fig. 1). RPTC cultured in the presence of 500 μM AscP sustained the same degree of cell death and loss (55%) on day 1. Monolayer protein content 4 days after DCVC exposure remained at day 1 levels in RPTC incubated in 50 or 500 μM AscP. However, 6 days after DCVC exposure, monolayer protein content in DCVC-treated RPTC that was cultured in 500 μM AscP was 34% greater than that of DCVC-treated RPTC grown in 50 μM AscP. These data show that pharmacological concen-
trations of ascorbic acid do not protect against DCVC-induced cell injury and loss but do promote an increase in cell density over time in RPTC sublethally injured by DCVC.

Basal QO2 and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. Basal QO2 (a measure of mitochondrial function) and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity were used as measures of physiological functions in RPTC over the 6-day recovery period after DCVC exposure. Basal QO2 and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in untreated RPTC grown in 50 and 500 μM AscP were equivalent at all time points. One day after DCVC exposure (200 μM), RPTC grown in both 50 and 500 μM AscP exhibited decreases in QO2 and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity of 50 and 65%, respectively (Fig. 2, A and B). QO2 remained at day 1 levels on day 4 after DCVC exposure in RPTC incubated in 50 or 500 μM AscP. On day 6, QO2 in RPTC grown in 500 μM AscP was 49% higher than that in RPTC grown in 50 μM AscP and equal to that in controls. Similarly, Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity on day 6 was 50% higher in DCVC-injured RPTC cultured in 500 μM AscP than in injured RPTC cultured in physiological concentrations of AscP. These data show that pharmacological concentrations of ascorbic acid do not prevent the inhibition of physiological functions caused by DCVC exposure but do stimulate physiological repair in RPTC sublethally injured by DCVC.

Synthesis of collagen IV. Figure 3 shows a representative autoradiograph of newly synthesized but not deposited \textsuperscript{35}S-labeled collagen IV in RPTC after exposure to DCVC. Densitometric analysis of the 206-kDa collagen IV band was performed on scanned images of individual autoradiographs from four separate experiments (Fig. 4). To illustrate changes in collagen IV synthesis over time in each group, values are expressed as a percentage of day 1 controls grown in 50 μM AscP. Collagen IV synthesis on day 1 was decreased in uninjured RPTC grown in 500 μM AscP compared with those grown in 50 μM AscP. One day after DCVC exposure, there was an equivalent 1.8-fold increase in collagen IV synthesis in RPTC grown in both 50 and 500 μM AscP. Levels of collagen IV synthesis at days 4 and 6 after DCVC exposure and in controls were reduced compared with day 1 in RPTC grown in both 50 and 500 μM AscP. No significant differences between groups were found on days 4 and 6. These findings demonstrate that RPTC sublethally injured by DCVC increase collagen IV synthesis 1 day after the injury. However, this stimulation is independent of the concentration of ascorbic acid.
Deposition of collagen IV. Figure 5 shows a representative autoradiograph of newly deposited 35S-labeled collagen IV in RPTC after exposure to DCVC. Densitometric analysis of the 206-kDa collagen IV band was performed on scanned images of individual autoradiographs from three separate experiments (Fig. 6). To illustrate changes in collagen IV deposition over time in each group, values are expressed as a percentage of day 1 controls grown in 50 μM AscP. There was a numerical increase in collagen IV deposition by uninjured RPTC cultured in pharmacological concentrations of AscP compared with RPTC cultured in physiological concentrations of AscP. However, this numerical increase was not statistically significant. One day after DCVC exposure, collagen IV deposition was significantly inhibited in cells grown in the presence of 50 μM AscP. Collagen IV deposition was numerically decreased ~50% compared with controls in DCVC-injured RPTC grown in physiological concentrations of AscP on day 4 after injury, although this decrease was not statistically significant. However, DCVC-injured RPTC grown in 500 μM AscP maintained collagen IV deposition at levels equal to that of controls throughout the experiment. Compared with day 1, on days 4 and 6 a decrease in collagen IV deposition was seen in all groups. On day 6, there were no differences in collagen IV deposition among any treatment groups. These findings demonstrate that RPTC sublethally injured by DCVC in the presence of physiological concentrations of ascorbic acid decrease collagen IV deposition after the injury. However, in the presence of pharmacological concentrations of ascorbic acid, sublethally injured RPTC maintain collagen IV deposition at levels equal to controls.

Proline hydroxylation in collagen IV. Loss of 3H due to hydroxylation of the 4-trans position of proline in newly synthesized collagen IV was used as a marker of prolyl hydroxylase activity. RPTC grown in 50 and 500 μM AscP were dual labeled with L-[4-3H]proline and L-[14C]proline over a 24-h period after DCVC exposure, and the loss of 3H was measured as a decrease in the ratio of L-[4-3H]proline to L-[14C]proline. DCVC-injured RPTC grown in 50 μM AscP had a ratio of L-[4-3H]proline to L-[14C]proline that was ~80% greater than controls on day 1 after injury, indicating a significant decrease in proline hydroxylation (Fig. 7). The L-[4-3H]proline-to-L-[14C]proline ratio in DCVC-injured RPTC grown in 500 μM AscP was ~45% greater than controls on day 1 after injury. These data suggest that DCVC exposure inhibits prolyl hydroxylase activity and that decreased proline hydroxylation may contribute to the inhibition of collagen IV deposition in DCVC-injured RPTC grown in 50 μM AscP. In contrast, the degree of proline hydroxylation in newly synthesized collagen IV is higher in DCVC-injured RPTC grown in the presence of 500 μM AscP, suggesting that ascorbic acid-stimulated prolyl hydroxylase activity may contribute in part to the deposition of collagen IV in DCVC-injured RPTC.

Effect of exogenous ECM proteins on cell repair. Because DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP maintain collagen IV deposition and are capable of physiological repair, we determined whether exogenous renal tubular ECM proteins promoted cell repair in DCVC-injured RPTC.
jured RPTC. DCVC-injured RPTC grown in physiological concentrations of AscP were exposed to exogenous collagen I or collagen IV (0, 5, 15, 50 μg/ml) immediately after DCVC exposure and continuously through day 6 after injury. Basal QO2 and monolayer cell density were measured on days 1 and 6 after injury induced by DCVC. Exposure of untreated RPTC to collagen I or collagen IV had no effect on basal QO2 or monolayer cell density on days 1 or 6 (data not shown).

On day 1 after injury, monolayer cell density and basal QO2 were decreased; 50% in injured RPTC grown in the absence or presence of collagen IV (Fig. 8, top, and Fig. 9, top) and collagen I (data not shown). On day 6, cell density in injured RPTC cultured in the presence of collagen IV remained 50% of uninjured RPTC and equal to the cell density in DCVC-injured RPTC grown in the absence of collagen IV (Fig. 8, bottom). However, DCVC-injured RPTC cultured in the presence of exogenous collagen IV recovered basal QO2 by day 6 after injury, with a complete repair in RPTC grown in the presence of 50 μg/ml collagen IV (Fig. 9, bottom). DCVC-injured RPTC cultured in the presence of collagen I exhibited neither increased cell density nor repair of basal QO2 by day 6 after injury (Fig. 10).

We determined the effects of fibronectin and laminin on cell density and the effects of collagen I, collagen IV, laminin, and fibronectin on the repair of Na-K-ATPase activity in sublethally injured RPTC. DCVC-injured RPTC grown in physiological concentrations of AscP were exposed to exogenous collagen I, collagen IV, laminin, or fibronectin immediately after DCVC exposure and through day 6 after injury. Exogenous proteins were added directly to culture media at a concentration of 50 μg/ml because this concentration of collagen IV was found to promote complete repair (Fig. 9). The addition of exogenous collagen I, collagen IV, fibronectin, or laminin to injured RPTC produced no change in monolayer protein content on day 1 (data not shown) or day 6 (Fig. 11, top). These results suggest that, like collagen I and collagen IV, exogenous laminin or fibronectin does not stimulate injured RPTC to proliferate. Na-K-ATPase activity was significantly increased in injured RPTC cultured in the presence of collagen IV on day 6 after DCVC injury (Fig. 11, bottom). Na-K-ATPase activity remained inhibited on day 6 after injury in RPTC cultured in the presence of exogenous collagen I, fibronectin, or laminin (Fig. 11, bottom). These results and those illustrated in Fig. 9, suggest that repair of physiological functions in DCVC-

Fig. 5. Representative autoradiograph of newly deposited collagen IV in RPTC sublethally injured by DCVC in the presence of either 50 or 500 μM AscP. RPTC were exposed to DCVC (200 μM) for 1.75 h and metabolically labeled with L-[35S]methionine for 24 h on days 1, 4, and 6 after injury. Collagen IV immunoprecipitates were subjected to SDS-PAGE and exposed to film for 3 wk. The molecular mass of collagen IV is 206 kDa. Abbreviations are as defined in Fig. 3.
injured RPTC is a process mediated specifically by extracellular collagen IV.

DISCUSSION

RPTC that do not die or that become detached from the ECM after ischemic or chemical injury are thought to undergo repair or dedifferentiate, proliferate, and migrate to denuded areas of the tubules. The cells of the newly lined tubule may then differentiate, promoting the return of normal tubular function and overall renal function. During our studies of the mechanisms of RPTC repair and regeneration after toxicant exposure, we observed that RPTC sublethally injured by DCVC neither proliferated nor repaired physiological functions (37). In contrast, RPTC sublethally injured by the oxidant t-butyl hydroperoxide proliferated and repaired physiological functions (35). However, when RPTC sublethally injured by DCVC were cultured in the presence of pharmacological concentrations of AscP, they proliferated and repaired physiological functions (36). Thus one goal of this study was to determine whether one of the mechanisms of action of pharmacological concentrations of ascorbic acid in promoting RPTC repair and regeneration is through the stimulation of the synthesis and deposition of collagen IV.

AscP is known to promote collagen synthesis and deposition in cultured cells (13, 33). Collagen IV is the most abundant component of the proximal tubular BM, and the regulation of collagen IV synthesis and degradation plays an important role in cell function, growth, migration, and organ remodeling in many tissues (15). Furthermore, collagen IV synthesis and deposition are increased in control RPTC exposed to AscP (36). We examined collagen IV synthesis and deposition in

injured RPTC.

Fig. 9. Basal QO2 in RPTC sublethally injured by DCVC and cultured in the presence of collagen IV. RPTC were exposed to DCVC (200 μM) for 1.75 h, and collagen IV was added immediately after DCVC exposure and after daily media change at the indicated concentrations. QO2 was measured on days 1 (top) and 6 (bottom) after injury. Data are presented as means ± SE, n = 4–5 separate experiments. Bars labeled with different letter symbols are significantly different from each other (P < 0.05).
RPTC sublethally injured by DCVC. One day after DCVC exposure, collagen IV synthesis increased in RPTC grown in the presence of physiological concentrations of Ascp and decreased to control levels on days 4 and 6. In contrast, collagen IV deposition into the ECM was inhibited by DCVC 1 day after exposure, a trend that was still evident on day 4, although the statistical significance of this decrease was not conclusive due to a significant decrease in collagen IV deposition at control levels. These data suggest that the maintenance of collagen IV deposition after DCVC exposure promotes RPTC repair and regeneration by day 6 after injury, thus creating an association between the ability of injured RPTC to deposit collagen IV and to repair physiological functions.

Fig. 10. Total protein content and basal QO2 in RPTC sublethally injured by DCVC and cultured in the presence of exogenous ECM proteins. RPTC were exposed to DCVC (200 μM) for 1.75 h, and collagen I was added immediately after DCVC exposure and after daily change in media at the indicated concentrations. Total protein (top) and QO2 (bottom) measured on day 6 after injury are shown. Data are presented as means ± SE (n = 4–5 separate experiments). COL(I), collagen I. Bars labeled with different letter symbols are significantly different from each other (P < 0.05).

Fig. 11. Total protein content and Na+–K+-ATPase activity in RPTC sublethally injured by DCVC and cultured in the presence of exogenous ECM proteins. RPTC were exposed to DCVC (200 μM) for 1.75 h, and collagen I, collagen IV, laminin, or fibronectin was added immediately after DCVC exposure and after daily change in media at the indicated concentrations. Total protein (top) and Na+–K+-ATPase activity (bottom) measured on day 6 after injury are shown. Data are presented as means ± SE (n = 3–4 separate experiments). FN, fibronectin; LAM, laminin. Bars labeled with different letter symbols are significantly different from each other (P < 0.05).
One potential explanation for the lack of collagen IV deposition in DCVC-injured RPTC grown in physiological concentrations of AscP is that newly synthesized procollagen α chains are not being properly hydroxylated at susceptible proline residues. Underhydroxylated procollagen α chains will not fold into triple helical monomers adequately, and most are targeted for degradation intracellularly rather than being secreted into the ECM. Prolyl hydroxylase is the microsomal enzyme responsible for proline hydroxylation of procollagen α chains, and ascorbic acid is the preferred iron-reducing cofactor for prolyl hydroxylase activity. Approximately 45–50% of susceptible collagen proline residues are hydroxylated in normal vertebrate tissues; however, when the degree of proline hydroxylation is decreased, so is the amount of collagen deposited into the ECM. In our study, proline hydroxylation in newly synthesized collagen IV was decreased after DCVC exposure in RPTC grown in the presence of physiological concentrations of AscP. However, proline hydroxylation in injured RPTC grown in pharmacological concentrations of AscP was greater compared with injured RPTC grown in physiological concentrations of AscP, suggesting that these cells retain some ability to hydroxylate susceptible proline residues. Although we have no evidence of a direct interaction between DCVC and prolyl hydroxylase, these results suggest that insufficient proline hydroxylation contributes to decreased collagen IV deposition in DCVC-injured RPTC grown in physiological concentrations of AscP. In addition, pharmacological concentrations of Asp increase the degree of proline hydroxylation in collagen IV after DCVC injury and may promote the maintenance of collagen IV deposition into the ECM. These results further support our conclusion that the deposition of collagen IV is associated with RPTC repair after sublethal injury.

Figure 4 shows that uninjured RPTC grown in pharmacological concentrations of AscP synthesize less collagen IV on day 1 after confluence than RPTC grown in physiological concentrations of AscP. This finding can be explained by the idea that RPTC cultures synthesize less collagen over time, especially after reaching confluence, as the cultures begin to quiesce. This hypothesis is supported by previous studies in regenerating tissues demonstrating upregulated ECM protein expression that returns to basal levels when tissue structure is restored. Because ascorbate is known to enhance cellular growth, RPTC grown in pharmacological AscP concentrations may decrease collagen synthesis to basal levels sooner than RPTC grown in physiological concentrations of AscP. Further evidence of this effect is observed on day 4 when RPTC cultured in physiological AscP concentrations exhibit collagen IV synthesis that is decreased to levels of RPTC cultured in pharmacological concentrations of AscP.

The next goal of this study was to determine the effect of exogenous collagens I and IV, laminin, and fibronectin on cell repair in RPTC sublethally injured by DCVC. On the basis of the correlation between cell repair and collagen IV deposition, we hypothesized that adding exogenous collagen IV to the culture media would promote cell repair and/or proliferation in DCVC-injured RPTC. In addition, by determining the effects of collagen I, laminin, or fibronectin on cell repair, we would determine whether any effect of collagen IV to stimulate repair is specific to collagen IV or is a general effect of ECM proteins. Collagen IV, but not collagen I, laminin, or fibronectin, promoted repair of mitochondrial oxygen consumption (basal $Q_{O_2}$) and active Na$^+$ transport (Na$^+$-K$^+$-ATPase activity) after injury, implicating collagen IV as an important ECM protein involved in the repair of physiological functions in RPTC. However, DCVC-injured RPTC cultured in the presence of collagen IV did not exhibit increased cell density on day 6 after DCVC exposure. This finding suggests that collagen IV is not involved in the proliferation of RPTC as observed in the presence of pharmacological concentrations of AscP and that ascorbic acid plays other important roles in renal recovery not related to ECM production. These data provide strong evidence of a specific role for collagen IV in cell repair, but not proliferation, after sublethal injury.

Despite the prevalence of theories implicating collagen IV as a mediator of renal tubular development and function, the nonpathological role of collagen IV in renal injury, repair, and restoration of tubular function is relatively unknown. The novel findings presented here suggest that extracellular collagen IV specifically promotes the repair of physiological functions in injured RPTC. The ECM proteins collagen I, laminin, and fibronectin did not promote repair of physiological functions. DCVC-injured RPTC exhibited decreased collagen IV hydroxylation and deposition and were unable to repair physiological functions. In contrast, RPTC grown in pharmacological concentrations of AscP maintained collagen IV hydroxylation and deposition and were able to repair physiological functions. This suggests that extracellular collagen IV is a mediator of renal tubular repair and deposition in injured RPTC.

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