Regulation of Dedifferentiation and Redifferentiation in Renal Proximal Tubular Cells by the Epidermal Growth Factor Receptor

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ABSTRACT

Repair of injured renal epithelium is thought to be mediated by surviving renal proximal tubular cells (RPTC) that must dedifferentiate to allow the proliferation and migration necessary for epithelial regeneration. RPTC then redifferentiate to restore tubular structure and function. Current models suggest that epidermal growth factor receptor (EGFR) activation is required for dedifferentiation characterized by enhanced vimentin expression, decreased N-cadherin expression, spindle morphology, and loss of apical-basal polarity after injury. Because an in vitro model of RPTC redifferentiation has not been reported, and the mechanism(s) of redifferentiation has not been determined, we used rabbit RPTC in primary cultures to address these issues. H₂O₂ induced the dedifferentiated phenotype that persisted >48 h; redifferentiation occurred spontaneously in the absence of exogenous growth factors after 72 to 120 h. Phosphorylation of two tyrosine residues of EGFR increased 12 to 24 h, peaked at 24 h, and declined to basal levels by 48 h after injury. EGFR inhibition during dedifferentiation restored epithelial morphology and apical-basal polarity, and it decreased vimentin expression to control levels 24 h later. In contrast, exogenous epidermal growth factor addition increased vimentin expression and potentiated spindle morphology. p38 mitogen-activated protein kinase (MAPK) and transforming growth factor (TGF)-β receptor inhibitors did not affect redifferentiation after H₂O₂ injury. Similar results were observed in a mechanical injury model. These experiments represent a new model for the investigation of RPTC redifferentiation after acute injury and identify a key regulator of redifferentiation: EGFR, independent of p38 MAPK and the TGF-β receptor.

The physiologic role of renal proximal tubular cells (RPTC) engenders their susceptibility to ischemic and toxic injury. However, the proximal tubular epithelium is capable of regeneration, and full recovery is often observed (Cuppage et al., 1972; Houghton et al., 1976). Regeneration is initiated when surviving RPTC sequentially dedifferentiate, migrate, and proliferate to restore monolayer confluence (Toback, 1992; Toback et al., 1993; Abbate and Remuzzi, 1996; Molitoris and Marrs, 1999). Finally, regenerated cells redifferentiate to restore RPTC function and structure (Nony and Schnellmann, 2003; Zhuang et al., 2004; Rasbach and Schnellmann, 2007).

After RPTC injury, dedifferentiation and redifferentiation processes resemble epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition, respectively (Brabletz et al., 2005; Zhuang et al., 2005). The terms EMT and mesenchymal-epithelial transition indicate the direction of transdifferentiation of cells between the epithelial and mesenchymal phenotypes (Brabletz et al., 2005). RPTC are columnar and cuboidal in nature, with adherens junctions that contain, in part, N-cadherin, which are supported by cortical actin rings and contribute to apical-basal polarity (Takeichi, 1991; Prozialeck et al., 2004). During dedifferentiation, epithelial cells lose apical-basal polarity, which is characterized by a flattened and elongated morphology, a lack of tight junctions, and reorganization of actin into stress fibers (Hay, 2005). Furthermore, expression of cadherins specific to epithelial differentiation de-

AABBREVIATIONS: RPTC, renal proximal tubular cell(s); EMT, epithelial-mesenchymal transition; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; Src, Rous sarcoma virus homolog gene product; TGF, transforming growth factor; AG1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SU6656, 2-oxo-3-(4,5,6,7-tetrahydro-1H-indol-2-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide; SB231542, 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridyl)-1H-imidazol-2-yl]benzamide; EGF, epidermal growth factor; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
creases, and the intermediate filament, vimentin, is expressed (Molitoris et al., 1997; Korsching et al., 2005; Zhuang et al., 2005; Zeineldin et al., 2006). During redifferentiation, these mesenchymal markers are lost, epithelial markers return, and physiologic function is restored (Fujigaki et al., 2006).

Unlike many cell lines, primary cultures of rabbit RPTC, cultured under improved conditions (Nowak and Schnellmann, 1995), exhibit aerobic metabolism, are gluconeogenic, and display markers of differentiation similar to those observed in vivo. RPTC exhibit cuboidal morphology, are joined by tight junctions, are polarized with β1-integrins and Na⁺/K⁺ ATPase localized to the basolateral membrane, and exhibit low levels of vimentin (Nowak et al., 2000; Nony et al., 2001; Zhuang et al., 2005). Furthermore, cultured in the absence of serum, RPTC recover from various forms of oxidative, toxic, and mechanical injury without the addition of exogenous growth factors (Nowak et al., 1998; Zhuang et al., 2004; Rasbach and Schnellmann, 2007). Thus, they are an excellent model to study autocrine/paracrine-mediated RPTC differentiation and regeneration in vitro.

Using this in vitro model, we determined that epidermal growth factor receptor (EGFR) activation is a critical step in dedifferentiation after oxidant injury. EGFR was activated during the initial 30 min of oxidant exposure, and it required p38 mitogen-activated protein kinase (p38 MAPK) activity (Zhuang et al., 2005; Rasbach and Schnellmann, 2007). Inhibition of either the Rous sarcoma virus homolog gene product (Src), p38 MAPK, or EGFR prevented vimentin expression and fusiform morphology, indicating that sequential activation of Src, p38 MAPK, and EGFR mediates dedifferentiation of RPTC (Zhuang et al., 2005). These results reveal that EGFR is required for RPTC dedifferentiation subsequent to oxidant injury (Okada et al., 1997; Docherty et al., 2006). Transforming growth factor (TGF-β) is a potent stimulus of EMT in RPTC (Okada et al., 1997), and there is evidence that it cooperates with the EGFR axis to regulate differentiation (Okada et al., 1997; Uchiyama-Tanaka et al., 2001; Docherty et al., 2006). It is noteworthy that EGF exacerbates TGF-β-induced EMT in RPTC (Okada et al., 1997), and there is evidence that EGF increases after oxidant injury (Okada et al., 1997; Docherty et al., 2006). We have shown that production of TGF-β increases after oxidant exposure in cultured RPTC (Nowak and Schnellmann, 1997), and other studies indicate that TGF-β causes shedding of EGFR agonists from the membrane (Uchiyama-Tanaka et al., 2001). These studies raise the possibility for TGF-β-induced autocrine activation of the EGFR.

Although the EGFR plays a critical role in dedifferentiation in regenerating RPTC, the signaling events that trigger redifferentiation are poorly understood because models that allow exploration of the mechanisms of redifferentiation do not exist. In this study, we describe, for the first time, a novel model to study RPTC redifferentiation. Furthermore, the use of this model suggests that the primary mechanism for regulating redifferentiation is decreasing EGFR activation.

Materials and Methods

Reagents. AG1478, SB203580, SU6656, and SB431542 were purchased from Calbiochem (San Diego, CA). Recombinant human EGF and recombinant human TGF-β1 were obtained from R&D Systems (Minneapolis, MN). Rhodamine-phalloidin was purchased from Cytoskeleton (Denver, CO). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies to phospho-EGFR residues (Y845, Y1068, and Y1173) were purchased from Cell Signaling Technology Inc. (Danvers, MA). Antibodies to EGFR, vimentin (clone V9), N-cadherin, glyceraldehyde-3-phosphate dehydrogenase, and Na⁺/K⁺ ATPase were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Sigma-Aldrich, BD Biosciences (San Jose, CA), RDI (Concord, CA), and Millipore Corporation (Billerica, MA), respectively.

Isolation and Culture of Renal Proximal Tubules. Female New Zealand White rabbits were purchased from Myrtle’s Rabbitry (Thomson Station, TN). RPTC were isolated using the iron oxide perfusion method and were grown in six-well or 35-mm tissue culture dishes under improved conditions as described previously (Nowak and Schnellmann, 1996b). The culture medium was a 1:1 mixture of Dulbecco’s modified Eagle’s medium/Ham’s F-12 (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM l-glutamine, 1 mM 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 μM) were added to fresh culture medium.

Injury Models. Confluent RPTC were used for all experiments. RPTC monolayers were injured with 1 mM H₂O₂ for 5 h and then washed twice with culture medium as described previously (Zhuang and Schnellmann, 2004). The completion of this wash step was considered time 0 h for all experiments. Where noted, RPTC were cultured for 24 h after H₂O₂ exposure to allow dedifferentiation to occur before the addition of the EGFR inhibitor AG1478 (10 μM), the p38 MAPK inhibitor SB203580 (20 μM), EGF (10 ng/ml), the TGF-β receptor inhibitor SB431542 (5 μM), TGF-β (0.2 ng/ml), or diluent (1% DMSO) for an additional 24 h (48 h after injury). SB431542 was added for a 30 min preincubation before the addition of TGF-β at 24 h after injury. For some experiments, RPTC were injured with 1 mM H₂O₂ for 5 h. At 4 and 12 h after H₂O₂ removal, RPTC were treated with the Src inhibitor SU6656 (10 μM) or diluent (0.1% DMSO) and cells were either lysed or fixed for immunoblot and immunocytochemical analysis. Cell morphology was observed by light microscopy.

Confluent RPTC were swiped with a comb to produce four 2-mm-wide linear wounds as described previously (Zhuang et al., 2004). Sham controls were produced by dragging a pick through the media without touching the cell monolayer. RPTC were washed and incubated for 24 h, after which AG1478 (10 μM), EGF (10 ng/ml), or diluent (0.1% DMSO) was added, and cells were incubated for an additional 24 h. Morphology and vimentin expression were evaluated by light microscopy and immunocytochemistry, respectively, 24 and 48 h after wounding.

Immunoblot Analysis. RPTC were washed twice with ice-cold phosphate-buffered saline (PBS), scraped into boiling lysis buffer (1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, and 10 mM Tris, pH 7.4), and then boiled for 5 min. Cell membranes were disrupted by sonication for 30 s. Equal amounts of lysate protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk or bovine serum albumin (BSA) overnight at 4°C, incubated 1 h with various primary antibodies (1:1000), and washed thoroughly with Tris-buffered saline/Tween 20 (5 mM Tris base, 15 mM sodium chloride, 0.1%Tween 20). Secondary detection of primary antibodies was achieved using an appropriate horseradish peroxidase-conjugated secondary antibody (Pierce Chemical, Rockford, IL) for 1 h. Chemiluminescence of bound secondary antibody was detected using a Chemilumer (Danvers, MA). Densitometry was performed using AlphaEase software (Alpha Innotech).
nostics, Indianapolis, IN), and washed with PBS. Proteins were eluted by boiling in SDS-polyacrylamide gel electrophoresis loading buffer. Immunoblot analysis followed as described above.

Microscopy. RPTC were fixed with methanol at −20°C (confocal microscopy) or 10% formalin (fluorescence microscopy), permeabilized, blocked with 8% BSA, and then incubated with primary antibody or nonspecific IgG control. An Alexa Fluor 488-conjugated secondary antibody was added, and RPTC were visualized by fluorescence or confocal microscopy. To examine actin, cells were fixed in 10% formalin, permeabilized with 0.1% Triton X-100 in PBS, and then blocked with 1% BSA in PBS. Cells were then incubated with phalloidin-rhodamine diluted in blocking solution for 20 min to label F-actin before observation by fluorescent microscopy.

Statistical Analysis. All data are presented as the mean ± S.E.M. Cells isolated from the same rabbit represent one experiment (n = 1). Means were compared by ANOVA followed by a Student-Newman-Keuls post hoc analysis. p < 0.05 was considered statistically significant.

Results

We monitored RPTC differentiation status after \( \text{H}_2\text{O}_2 \)-induced injury using immunoblot analysis to measure N-cadherin and vimentin expression, and light microscopy to observe morphology (Figs. 1 and 2). As reported previously, RPTC were approximately 50 to 75% confluent 5 h after \( \text{H}_2\text{O}_2 \)-induced injury (time 0) (Zhuang et al., 2005). At this time, bare areas of plastic were exposed at sites of cell sloughing; however, cells surrounding these bare areas maintained their cuboidal morphology. During the next 24 to 48 h, single cells with fusiform morphology and prominent filopodia began to migrate into the bare areas. This morphological pattern remained until 72 to 96 h after injury at which time the monolayer had nearly reformed, and the repopulating cells became less elongated and more cuboidal. The cell morphology of injured and control cells was similar at 96 and 144 h after injury. These studies reveal that RPTC display mesenchymal morphology 24 and 48 h after oxidant injury and that epithelial morphology returns gradually between 96 and 144 h.

Vimentin expression in RPTC exposed to \( \text{H}_2\text{O}_2 \) was elevated at 24 h, it remained elevated at 48 h, it decreased at 72 h, and then it returned to control levels at 120 h (Fig. 2). Vimentin in control RPTC did not change over time. N-Cadherin expression was present in control RPTC, it decreased significantly at 24 h after \( \text{H}_2\text{O}_2 \) exposure, and it remained decreased over the experimental period. In sum-
mary, phenotypic markers of dedifferentiation, specifically vimentin expression and loss of N-cadherin, occurred at 24 and 48 h coincidentally with the appearance of mesenchymal morphology. Over time, vimentin decreased and epithelial morphology returned, indicating redifferentiation.

Apical-basal polarity is a characteristic of differentiated epithelial cells. To determine the effects of H$_2$O$_2$-induced dedifferentiation on cell polarity after injury and during regeneration, the basolateral expression of Na$^+$/K$^+$ ATPase in RPTC was measured using confocal microscopy. Na$^+$/K$^+$ ATPase staining was localized to the basolateral membrane in control RPTC cultures as described previously (Fig. 3A) (Nowak et al., 2000). At 24 and 48 h after injury, basolateral Na$^+$/K$^+$ ATPase staining decreased, and it was diffuse (Fig. 3, B and C). By 72 h after injury, Na$^+$/K$^+$ ATPase expression increased, and it began to relocalize to the basolateral membrane (Fig. 3D). These results reveal that apical-basal polarity is disrupted during increased vimentin staining and mesenchymal RPTC morphology, and it returns during the loss of vimentin expression and return of cuboidal morphology.

In previous studies using this model, we showed that the EGFR mediates dedifferentiation after H$_2$O$_2$ injury to RPTC (Zhuang et al., 2005). However, the mechanism by which redifferentiation occurs has not been determined. To ascertain whether EGFR activation maintains the dedifferentiated state, EGFR was inhibited using the EGFR pharmacological inhibitor AG1478. RPTC were treated with H$_2$O$_2$ as described above, and dedifferentiation was established at 24 h when AG1478 or diluent was added. Elevated vimentin expression and spindle morphology persisted in injured cells treated with oxidant and diluent 24 h later (Fig. 4). In contrast, RPTC treated with AG1478 during dedifferentiation displayed cuboidal morphology and decreased vimentin expression, similar to uninjured cells. In contrast, RPTC treated with exogenous EGF subsequent to H$_2$O$_2$-induced dedifferentiation exhibited enhanced vimentin expression, increased numbers of filopodia, and elongated morphology 24 h later. Diluent and AG1478 alone had no effect on morphology or vimentin expression of uninjured cells. These data reveal that inhibition of EGFR in dedifferentiated RPTC decreases vimentin expression and increases cuboidal morphology (redifferentiation) and that EGF exposure of RPTC in a dedifferentiated state enhances and extends vimentin expression and elongated morphology. We suggest that EGFR maintains dedifferentiation, that the termination of EGFR activation initiates redifferentiation, and that further EGFR activation enhances and extends dedifferentiation of RPTC.

We previously showed that p38 MAPK activation is required for EGFR activation and RPTC dedifferentiation after oxidative injury (Zhuang et al., 2005; Rasbach and Schnellmann, 2007). There also is evidence that EGFR mediates TGF-β-induced dedifferentiation (Okada et al., 1997; Uchiyama-Tanaka et al., 2001; Uchiyama-Tanaka et al., 2002; Docherty et al., 2006). Thus, we repeated the above-mentioned experiment using a p38 MAPK inhibitor, SB203580, a TGF-β receptor inhibitor, SB431542, and TGF-β$_1$. Treating dedifferentiated RPTC with TGF-β$_1$ for 24 h enhanced spindle morphology and vimentin expression compared with dedifferentiated cells treated with diluent. These TGF-β$_1$-induced effects were abrogated to levels similar to dedifferentiated cells after treatment with SB431542. We have reported previously that 20 μM SB203580 is sufficient to prevent p38 MAPK activation after oxidant injury (Zhuang et al., 2004, 2005; Rasbach and Schnellmann, 2007). Dedifferentiated RPTC treated with SB203580 or SB431542 under the same conditions exhibited vimentin and fusiform morphology similar to dedifferentiated RPTC treated with diluent (Fig. 4). SB203580, SB431542, or TGF-β$_1$ alone had no effect on uninjured RPTC. These data reveal that exogenous TGF-β$_1$ enhances oxidant-induced dedifferentiation, whereas inhibition of p38 MAPK or TGF-β receptor activity had no effect on dedifferentiated RPTC.

As shown above, Na$^+$/K$^+$ ATPase expression is localized to
the basolateral membrane in differentiated RPTC, and its expression is decreased and diffuse during dedifferentiation. Treatment of RPTC with the EGFR receptor inhibitor AG1478, after H2O2-induced dedifferentiation increased basolateral membrane localization of Na+/K+ ATPase compared with injured RPTC treated with diluent (Fig. 5). Dedifferentiated cells treated with EGF for 24 h retained depleted basolateral Na+/K+ ATPase expression, similar to cells treated with diluent. Neither AG1478, EGF, nor diluent altered Na+/K+ ATPase expression in uninjured cells. Consistent with the findings of decreased vimentin staining and increased cuboidal morphology, EGFR inhibition initiated the return of the apical-basal polarity in dedifferentiated RPTC.

Cytoskeletal morphology during dedifferentiation and re-differentiation was observed using rhodamine-phalloidin for F-actin staining and fluorescence microscopy. In uninjured cells, actin was arranged in cortical ring structures (Fig. 6). Dedifferentiated RPTC treated with diluent or EGF at 24 h had stress fibers and lacked cortical rings at 48 h; however, AG1478 treatment at 24 h restored cortical ring structure at 48 h. These results reveal that EGFR inhibition restores epithelial actin morphology in dedifferentiated cells, which is consistent with the increased cuboidal morphology and apical-basal polarity and the decreased vimentin staining observed in the above-mentioned experiments.

To determine the role of EGFR activation in redifferentiation, EGFR was immunoprecipitated from cell lysates obtained at several time points after H2O2 treatment, and it was immunoblotted using antibodies specific for EGFR phosphorylation at tyrosine residues Y845, Y1068, and Y1173. No appreciable change in total EGFR expression was detected under these conditions (Fig. 7). Phosphorylation at all three residues of the EGFR was similar to uninjured cells immediately after treatment with H2O2. EGFR became increas-
ingly phosphorylated at Y845 and Y1068, and it reached a maximum approximately 24 h subsequent to injury before returning to control levels at 48 h (Fig. 7, A–C). These experiments reveal that an increase in EGFR phosphorylation occurs at 24 h when the mesenchymal phenotype predominates. The phosphorylation was transient, falling to control levels at 48 h, and it preceded redifferentiation.

We previously reported that EGFR activation occurred within 10 min of H$_2$O$_2$-induced injury and required Src activation (Zhuang et al., 2005). To determine whether Src mediates EGFR activation and the persistence of the dedifferentiated phenotype at 48 h when the mesenchymal phenotype predominates. The phosphorylation was transient, falling to control levels at 48 h, and it preceded redifferentiation.

We previously reported that EGFR activation occurred within 10 min of H$_2$O$_2$-induced injury and required Src activation (Zhuang et al., 2005). To determine whether Src mediates EGFR activation and the persistence of the dedifferentiated phenotype at 48 h, we evaluated EGFR activation, vimentin expression, and cortical actin staining after the addition of a specific Src inhibitor, SU6656. SU6656 had no effect on EGFR phosphorylation at 24 h (Fig. 8A) or on vimentin expression at 48 h (Fig. 8B) after oxidant injury. Injured cells treated with SU6656 displayed fewer cellular processes, somewhat less elongation (Fig. 8B), and increased cortical actin staining (Fig. 9C) compared with diluent-treated dedifferentiated cells at 48 h. These results reveal that EGFR activation and dedifferentiation at 48 h does not require Src. However, between 24 and 48 h, Src seems to play a role in cellular morphology and actin reorganization.

To determine whether the dedifferentiation/redifferentiation observed in the oxidant model occurred in another injury model in the absence of an exogenous oxidant, a mechanical injury model was used as described previously by our laboratory (Counts et al., 1995; Zhuang et al., 2005). At 24 h after mechanical injury, RPTC adjacent to the wound edge expressed vimentin and elongated morphology (Fig. 9). However, cells peripheral to the wound edge retained epithelial morphology, and they have no appreciable vimentin expression (Fig. 9). These results reveal that dedifferentiation does not require decreased cell density or exogenous oxidants and that it is a common response to both mechanical and oxidant injuries.

The effects of EGFR inhibition on vimentin expression after mechanical injury was examined by adding AG1478 or diluent to RPTC 24 h after wounding. Vimentin labeling was elevated 24 h after mechanical injury, and it remained elevated at 48 h, albeit to a lesser degree (Fig. 10). Vimentin staining 48 h after mechanical injury was less than that observed 48 h after oxidant injury (Fig. 10). In contrast to diluent-treated controls, RPTC treated with AG1478 24 h after mechanical injury did not express vimentin or elongated morphology 24 h later. In contrast, RPTC treated with exogenous EGF 24 h after mechanical injury had elevated vimentin staining and increased cell scattering and elongation 24 h later compared with diluent-treated controls. These experiments reveal that mechanical injury results in reversible dedifferentiation of RPTC near the wound, that exogenous EGFR stimulation increases dedifferentiation, and that inhibition of EGFR stimulates redifferentiation.

Fig. 7. EGFR phosphorylation in regenerating RPTC. Confluent RPTC were treated with 1 mM H$_2$O$_2$ or diluent for 5 h, and then they were incubated for 0, 12, 24, 36, and 48 h. EGFR was immunoprecipitated from lysates collected at the indicated times. Phosphorylation of EGFR Y845 (A), Y1068 (B), and Y1173 (C) was measured by immunoblot analysis. Representative blots are shown along with densitometric analysis of EGFR phosphorylation at 24 h. * $p < 0.05$, statistically significant difference from control RPTC ($n = 3$).
Discussion

Current evidence suggests that the regeneration of noninjured and sublethally injured RPTC is a major mechanism of recovery from acute kidney injury (Lin et al., 2005). Furthermore, various in vivo and in vitro studies, including our own, have identified the EGFR as the primary mediator of proliferation and migration in regenerating RPTC after various mechanisms of injury (Humes et al., 1989; Coimbra et al., 1990; Nony and Schnellmann, 2003; Zhuang et al., 2004). In addition, we recently reported that the EGFR is also necessary for dedifferentiation of RPTC after H2O2-induced injury (Zhuang et al., 2005). A critical gap in the literature is studies to address the mechanism(s) of redifferentiation that results in the return of tubular structure and function. Here, we used an H2O2-injury model and a model of mechanical injury to demonstrate redifferentiation of RPTC after injury and to reveal a critical role for the EGFR in redifferentiation.

The lack of an in vitro model of renal redifferentiation has significantly hindered our understanding of RPTC repair and regeneration. With our H2O2-induced RPTC de-differentiation model, we observed that dedifferentiation persisted beyond 24 to 48 h and that redifferentiation began spontaneously at 72 h after the removal of H2O2 in the absence of exogenous growth factors. The time frame of dedifferentiation and redifferentiation was consistent with EGFR activation and inactivation, respectively. Dedifferentiation was characterized using multiple markers, including vimentin expression, cell elongation, and the loss of N-cadherin and apical-basal polarity. As cells redifferentiated, epithelial morphology returned, and it was characterized by compact, round, cobblestone morphology. Closer inspection revealed restoration of the cortical actin ring and the return of Na+/K+ ATPase to the basolateral membrane. Decrease of vimentin expression to control levels is a phenotypic marker of redifferentiation, and it was consistent with the morphologic markers. To ensure that these observations were not specific to oxidant-induced dedifferentiation or extensive cell loss, a mechanical injury model was used and the same dedifferentiation and redifferentiation results were observed. Thus, RPTC undergo dedifferentiation and redifferentiation after different injuries through an autocrine/paracrine pathway.

Unlike numerous epithelial cells, N-cadherin predominates in the proximal tubule of humans and several other species, including the rabbit (Nouwen et al., 1993; Prozialeck et al., 2004). Cadherins are an integral part of the adherens junctions that allow membrane compartmentalization and apical-basal polarity (Takeichi, 1991). Despite the return of apical-basal polarity as the RPTC redifferentiated and became confluent, N-cadherin expression did not return during the 144 h observed. It is possible that N-cadherin expression eventually recovers, but additional studies are needed to understand N-cadherin regulation under these conditions.

The persistence of the dedifferentiated phenotype between 24 and 48 h allowed us to explore the role of several potential signaling pathways in redifferentiation. We previously observed increased EGFR activation within minutes of H2O2 addition that was sustained for 2 h (Zhuang et al., 2005). Here, we reveal a second phase of EGFR activation, which

Fig. 8. EGFR phosphorylation and vimentin expression in dedifferentiated RPTC after Src inhibition. Confluent RPTC were treated with diluent or 1 mM H2O2 for 5 h. SU6656 (10 μM) or diluent (0.1% DMSO) was added at 12 h after the completion of oxidant injury. A, some cells were lysed at 24 h, and lysates were enriched for EGFR by immunoprecipitation and then immunoblotted. Remaining cells were fixed at 48 h for microscopy. B, vimentin was labeled with fluorescein isothiocyanate by immunocytochemistry. C, F-actin was labeled with phalloidin-rhodamine.

Fig. 9. Vimentin expression in mechanically injured RPTC. Confluent RPTC were scraped, washed, and incubated for 24 h. Vimentin was labeled by immunocytochemistry and then examined by fluorescent microscopy. Representative micrographs from three experiments.
peaks at 24 h and returns to control levels at 48 h after the removal of H₂O₂. We hypothesize that this second phase of EGFR activation maintains the dedifferentiated phenotype, based upon our observation that EGFR phosphorylation returned to basal levels before redifferentiation. Adding an EGFR inhibitor at 24 h initiated redifferentiation that occurred at 48 h, further supporting this hypothesis. In contrast, the addition of exogenous EGF during dedifferentiation enhanced and extended dedifferentiation. Therefore, we suggest that dedifferentiation is initiated and maintained by EGFR activation and that redifferentiation is initiated by EGFR inactivation.

The mechanism responsible for the second phase of EGFR activation is unknown. Src-mediated p38 MAPK activation within the first several minutes of oxidant injury is responsible for activating the EGFR to initiate dedifferentiation in this model (Zhuang et al., 2005). In contrast, inhibition of Src or p38 MAPK did not stimulate redifferentiation. Therefore, the mechanism of the second phase of EGFR activation differs from the mechanism that initiates it, and neither Src nor p38 MAPK acts downstream of the EGFR to initiate redifferentiation after oxidant injury.

Several possibilities exist for the initiation of the second phase of EGFR activation. For example, EGFR activation may result from the autocrine/paracrine release of EGF or other EGFR ligands (Liu and Arment, 2004; Schäfer et al., 2004; Thomas et al., 2006; Xu and Yu, 2007). It is also possible that the EGFR is transactivated by a mechanism that is independent of EGFR ligand production. Finally, several protein tyrosine phosphatases (i.e., protein tyrosine phosphatase 1B and TC-protein tyrosine phosphatase) can negatively regulate EGFR activation (Ostman et al., 2006). Inactivation of these phosphatases could also contribute to increased EGFR activity during dedifferentiation.

TGF-β is a known stimulus of dedifferentiation in many models of epithelial EMT, and its effects are potentiated by EGF (Okada et al., 1997; Docherty et al., 2006). Previous studies indicate that RPTC produce TGF-β after oxidant injury, and at a concentration of 0.2 ng/ml exogenous TGF-β induces glycolytic metabolism, decreased Na⁺/K⁺ ATPase activity, and cell rounding and detachment (Nowak and Schnellmann, 1996a; Nowak and Schnellmann, 1997). Whereas the current experiments revealed that exogenous TGF-β enhances dedifferentiation, TGF-β receptor inhibition had no effect on redifferentiation. Therefore, we suggest that autocrine/paracrine TGF-β signaling does not maintain dedifferentiation or mediate redifferentiation under these conditions.

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References


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