EGFR activity is required for renal tubular cell dedifferentiation and proliferation in a murine model of folic acid-induced acute kidney injury

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Acute kidney injury (AKI) is a serious clinical problem with a mortality of ~50% (6, 12). It can arise in a variety of pathological conditions such as ischemia and exposure to nephrotoxins (17, 27). After injury, some of renal tubular cells die by apoptosis and necrosis and some sublethally injured cells can be detached from the tubular basement membrane, leaving denuded areas of the basement membrane. Over time, injured tubules are repaired by repopulation of remaining survival epithelial cells through proliferation if the injury is mild (2, 10, 25). Recent studies have demonstrated that the cells to proliferate within the proximal tubule after injury are those that are injured and dedifferentiated rather than uninjured bystanders and stem cells (8, 14, 15). Thus understanding the mechanism by which injured tubular cells are converted to dedifferentiated phenotype would be beneficial to develop the approach to promote renal repair and regeneration after AKI.

Currently, the molecular basis responsible for renal epithelial cell dedifferentiation in response to injury remains unclear. In adult mature kidney, renal tubular cells are well differentiated and do not express some molecules like vimentin and neural cell adhesion molecule (NCAM) that are only expressed in mesenchymal cells (1, 10). However, a large number of cells expressing these molecules are found in proximal tubules after ischemia/reperfusion injury (1, 2, 10), suggesting that autocrine and paracrine growth factors produced at the tubular sites of injury might drive renal tubular cell dedifferentiation and proliferation (28). In this regard, epidermal growth factor receptor (EGFR) has been reported to be implicated in renal repair and regeneration. For example, EGFR and its ligands epidermal growth factor (EGF), heparin binding epidermal growth factor-like growth factor (HB-EGF), and transforming growth factor-α (TGF-α) are upregulated after AKI (22, 28). Administration of exogenous EGF enhanced renal functional recovery and renal regeneration (13). Furthermore, mice with reduced EGFR tyrosine kinase or with conditional deletion of EGFR in proximal tubular cells exhibited a decrease in cell proliferation and delayed in functional recovery after acute toxic or ischemic injury (4, 26). Finally, our in vitro studies revealed that EGFR activation is required for dedifferentiation of renal proximal tubular cells in primary culture following oxidant injury or plating (32, 36). Despite these studies suggesting the importance of EGFR in regulating renal repair and functional recovery in vivo as well as renal epithelial cell dedifferentiation in vitro, the role of EGFR in renal tubular cell dedifferentiation after AKI remains unclear.

In this study, we investigated the role of EGFR in renal epithelial cell dedifferentiation and proliferation in a murine model of AKI induced by folic acid (FA) using waved-2 mice, which have reduced tyrosine kinase activity of EGFR (20), and mice treated with gefitinib, a specific EGFR inhibitor. Administration of FA for 48 h induced EGFR phosphorylation in the kidney of wild-type mice, but this was inhibited in waved-2 mice and wild-type mice given gefitinib. Compared with wild-type mice, waved-2 mice and wild-type mice treated with gefitinib had increased renal dysfunction, histologic damage, and tubular cell apoptosis after FA administration. PAX2, a dedifferentiation marker, and proliferating cell nuclear antigen, a proliferating marker, were highly expressed in renal tubular cells in wild-type mice; however, their expression was largely inhibited in the kidney of waved-2 mice. Inhibition of EGFR with gefitinib also blocked FA-induced expression of these two proteins in wild-type mice. Moreover, FA exposure resulted in phosphorylation of AKT, a downstream signaling molecule of the phosphatidylinositol 3-kinases (PI3K), which has been recognized to be the major cellular event that contributes to renal repair after acute kidney injury (AKI). However, the underlying mechanism that initiates renal tubular cell dedifferentiation in vivo remains unexplored. Here we investigated whether epidermal growth factor receptor (EGFR) mediates this process in a murine model of folic acid (FA)-induced AKI using waved-2 mice with reduced tyrosine kinase activity of EGFR and gefitinib, a specific EGFR inhibitor. Administration of FA for 48 h induced EGFR phosphorylation in the kidney of wild-type mice, but this was inhibited in waved-2 mice and wild-type mice given gefitinib. Moreover, mice with reduced EGFR tyrosine kinase or with conditional deletion of EGFR in proximal tubular cells exhibited a decrease in cell proliferation and delayed in functional recovery after acute toxic or ischemic injury (4, 26). Finally, our in vitro studies revealed that EGFR activation is required for dedifferentiation of renal proximal tubular cells in primary culture following oxidant injury or plating (32, 36). Despite these studies suggesting the importance of EGFR in regulating renal repair and functional recovery in vivo as well as renal epithelial cell dedifferentiation in vitro, the role of EGFR in renal tubular cell dedifferentiation after AKI remains unclear.

MATERIALS AND METHODS

Chemicals and antibodies. Antibodies to vimentin, phospho-EGFR, phospho-AKT, and AKT were purchased from Cell Signaling Technol-
Antibodies to EGFR, and proliferating cell nuclear antigen (PCNA), GAPDH were purchased from Santa Cruz Biotechnology. Pax-2 was purchased from Life Technology (Carlsbad, CA). Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) antibodies were purchased from R&D Systems (Minneapolis, MN). The terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay kit was obtained from Roche (Nutley, NJ). Gefitinib was purchased from AstraZeneca (Macclesfield, England). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Murine model of FA injury and waved-2 mice. Waved-2 (maintained on a C57BL/6JexC3H/HeSnJ background) and their wild-type littermates that weighed 20–25 g (Jackson Laboratory, Bar Harbor, ME) were used for this study. Mice were injected (ip) with FA at 240 mg/kg body wt. Sodium bicarbonate (0.3 M NaHCO₃, the vehicle used for FA administration) alone was used as controls. To examine the efficacy of gefitinib after FA injury, gefitinib at 100 mg/kg in 50 μl of DMSO was given via intraperitoneally immediately after FA injection and then administered daily. DMSO-treated animals were used as controls. At 48 h after FA administration, the animals were killed and the kidneys were collected for protein analysis and histological examination. Five mice were used in each group. Animal experiments were performed in accordance with the policies of the Institutional Animal Care and Use Committee at Rhode Island Hospital.

Assessment of renal function. Renal function was monitored by measuring serum creatinine using a colorimetric kit (Sigma Diagnostics) based on the protocol provided by the manufacture.

Fig. 1. Epidermal growth factor receptor (EGFR) activation in the kidney following folic acid (FA) administration in mice treated with gefitinib, wild-type (WT), and waved-2 (Wa-2) mice. Kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against phospho-EGFR (p-EGFR) and EGFR in WT mice on day 2 after FA administration (A and D). Expression levels of p-EGFR and EGFR were quantified by densitometry and normalized with EGFR (C and F), respectively. Data are represented as the means ± SE (n = 5). Bars with different superscript letters are significantly different from one another (P < 0.05).
Immunoblot analysis. Immunoblot analysis for tissue samples was carried out according to our previous protocols. The densitometry analysis of immunoblot results was conducted by using NIH Image software (National Institutes of Health, Bethesda, MD).

Periodic acid-Schiff and immunofluorescence stainings. Renal tissues were fixed in 4.5% buffered formalin, dehydrated, and embedded in paraffin. Sections were stained with periodic acid-Schiff. For immunofluorescent staining, primary antibodies against PCNA (1:500), Pax-2 (1:500), NGAL (1:200) and fluorescent-conjugated secondary antibodies (1:500) were applied to the sections. Examination and scoring of sections from each kidney (n = 3–5 for each condition) were carried out in a blinded fashion. Morphological damage (epithelial necrosis, luminal necrotic debris, and tubular dilation) in 3–4 sections per kidney and 10–12 fields per section were quantified using the following scale: none = 0; <10% = 1; 11–25% = 2; 26–75% = 3; and >75% = 4.

In situ TUNEL assays. TUNEL staining kit was used to detect DNA strand breaks according to the instructions provided by the manufacturer. The number of TUNEL-positive nuclei per field was evaluated in five fields per section and five sections per kidney.

Statistical analysis. All the experiments were conducted at least three times. Data depicted in graphs represent the means ± SE for each group. Multiple means were compared using Tukey’s test. The differences between two groups were determined by Student’s t-test. Statistical significant difference between mean values was marked in each graph. P < 0.05 is considered to be significant.

RESULTS

EGFR activation is reduced in waved-2 mice and mice given gefitinib after FA administration. As a first step toward understanding the role of EGFR in renal tubular cell dedifferentiation in vivo, we examined EGFR activation in a murine model of FA-induced AKI in waved-2 mice and mice treated with gefitinib. As shown in Fig. 1, the basal level of total EGFR, but not phospho-EGFR, was detectable in vehicle-treated kidneys. FA administration induced EGFR phosphorylation in the kidney of wild-type mice, but it was largely suppressed in waved-2 mice and mice treated with gefitinib. The expression levels of total EGFR were also slightly increased in the kidney of wild-type mice after FA exposure compared with that in vehicle-treated kidneys, whereas EGFR expression in FA-treated waved-2 mice and mice treated with FA and gefitinib was not altered. These data suggest that renal EGFR activation is induced in wild-type mice after FA administration, and it is reduced in waved-2 mice and wild-type mice given gefitinib.

Inhibition of EGFR activation aggravates renal function and kidney damage after FA injection. To understand the change of renal function in both wild-type and waved-2 mice after FA administration, we measured serum creatinine levels over time after FA injection. As shown in Fig. 2, A and B, mice developed AKI at 24 h after FA administration as indicated by increased serum creatinine levels, which were similar in both wild-type and waved-2 mice. However, at 2 days of FA administration, serum creatinine levels were significantly higher in waved-2 mice than that in vehicle-treated wild-type animals. Serum creatinine levels were also increased significantly in mice receiving gefitinib relative to those in vehicle-treated wild-type mice at 48 h but not 24 h after FA administration. The baseline serum creatinine levels were identical in wild-type and waved-2 mice, ~0.3–0.7 mg/dl (Fig. 2). The pathologic changes as revealed by tubular dilatation, necrosis, and luminal congestion were predominantly seen in the cortical and outer medullary regions of the kidney at 48 h after FA administration (Fig. 3). These changes were more severe in wild-type mice treated with gefitinib and waved-2 mice. No pathologic changes were observed in vehicle-treated wild-type and waved-2 mice. These data indicated reduced EGFR activity...
delayed renal functional and morphologic recovery after toxic injury.

Inhibition of EGFR activity enhances expression of KIM1 and NGAL, two acute kidney injury markers, after FA injury. Numerous studies have indicated that expression levels of both NGAL and KIM-1 are markedly increased in AKI, which has been identified as valuable biomarkers for AKI diagnosis (3, 7, 24). To elucidate whether EGFR activation is associated with their expression in the kidney after FA administration, we examined their levels in the kidney of wild-type mice treated with/without gefitinib and waved-2, at day 2 after FA injury by immunostaining and immunoblot analysis. As shown in Fig. 4, renal expression levels of KIM-1 and NGAL were barely observed in vehicle-treated mice, and FA administration resulted in increased levels of both proteins in the kidney of wild-type mice. Their expression levels were further elevated in the FA injured kidneys in mice treated by gefitinib or waved-2 mice. To confirm this observation, we also examined NGAL expression using fluorescent microscopy. As shown in Fig. 4, C–E, increased expression of NGAL was observed in the FA-injured kidney tubules of waved-2 or mice treated with gefitinib compared with those in wild-type mice. These data illustrated that waved-2 exhibit a greater extent of renal tubular damage.

Inhibition of EGFR activation enhances tubular cell apoptosis in the kidney after FA exposure. As apoptosis has been implicated in tubular damage after AKI (2), we further examined the effect of EGFR inhibition on renal tubular apoptosis after FA administration using the TUNEL assay. Figure 5 demonstrates that TUNEL-positive tubular cells were not detected in the kidney of sham-operated wild-type mice; this population of cells was clearly observed in the renal tubular
cells in wild-type mice receiving FA. Administration of gefitinib led to their increase in the kidney of wild-type mice. The similar observation was also made in the kidney of waved-2 with FA injection. These results indicated that EGFR activation protects renal tubular cells against apoptosis after AKI. EGFR activity is required for renal tubular cell dedifferentiation in the kidney after FA exposure.

It is generally thought that after acute injury, renal repair and regeneration are concurrently initiated with renal tubular injury (1, 2). Dedifferentiation of renal tubular cell after injury is the first step of regenerative responses (2). Previously, we have shown that EGFR activation is required for dedifferentiation of renal tubular cells in the in vitro culture system (36), implying the role of EGFR in mediating this process in vivo. To confirm this mechanism in vivo, we further examined the effect of EGFR inhibition on renal tubular cell dedifferentiation using vimentin and PAX2 as markers in the kidney after FA exposure. As shown in Fig. 6, A and B, vimentin was not clearly detected in the kidney of vehicle-treated mice, but its expression levels were significantly increased after FA administration. Waved-2 mice and mice given gefitinib exhibited much less expression of this molecule (Fig. 6, A and B). Densitometry analysis of immunoblot results showed a significant increase in the expression of vimentin in the kidney of wild-type mice, compared with that in vehicle-treated kidneys. Interestingly, expression of renal vimentin was totally blocked in waved-2 mice or mice treated by gefitinib (Fig. 6, C and D). Consistent with this observation, immunofluorescent microscopy also revealed an increase in PAX2-positive tubular cells in the FA injured kidney of wild-type but not of wild-type mice treated with gefitinib or waved-2 mice (Fig. 6, E–G). Taken together, we suggest that EGFR plays an important role in initiating renal tubular cell dedifferentiation after acute toxic injury.

EGFR activity is required for renal tubular cell proliferation after FA exposure. To investigate whether EGFR is also involved in renal tubular cell proliferation after AKI, we first examined the expression levels of PCNA, a marker of cell proliferation, in the
kidney after FA injury by immunoblot analysis. Although PCNA expression was not detected in the kidney of sham-operated animal kidneys, FA administration induced its expression in the kidney of wild-type mice but not in that of waved-2 mice and mice given gefitinib (Fig. 7, A–D). Immunofluorescent microscopy also displayed increased PCNA-positive tubule epithelial cells in FA-injured kidney in wild type. Proliferation of renal tubular cells in the injured kidney was greatly suppressed in waved-2 mice and mice treated with gefitinib (Fig. 7, E–G). These data, together with the role of EGFR in mediating Pax2 and vimentin expression, suggest that EGFR is critically involved in renal regenerative responses, in particular, tubular dedifferentiation and proliferation, after AKI.

**EGFR mediates Akt phosphorylation in the kidney after FA exposure.** Our studies have shown that activation of the phosphatidylinositol 3-kinases (PI3K)/Akt pathway is necessary for renal tubular dedifferentiation and proliferation in primary cultures of renal proximal tubular cells (32). To investigate whether this pathway is activated and subjected to EGFR regulation in vivo, we conducted immunoblot analysis by using antibodies that recognize phosphorylated Akt (p-Akt; a target of PI3K) and total AKT. As shown in Fig. 8, the basal level of total AKT, but not p-AKT, was observed in vehicle-treated kidneys. FA injury induced AKT phosphorylation in the kidney of wild-type mice. However, the level of renal Akt phosphorylation was significantly reduced in waved-2 mice administered with FA. Similarly, treat-
ment with gefitinib also inhibited FA-induced Akt phosphorylation in the kidney of wild-type mice. These data indicated that the PI3/AKT pathway is activated downstream of EGFR in FA injured kidneys, suggesting the importance of this signaling pathway in moving from EGFR activation to renal tubular cell survival and regeneration.

**DISCUSSION**

Dedifferentiation is the process by which polarized renal epithelial cells reverse to a less developed state in which they are able to proliferate and migrate (1). Our recent in vitro studies revealed that EGFR plays an essential role in regulating renal tubular cell dedifferentiation and proliferation in primary cultures of renal proximal tubular cells (32, 36). Here we further demonstrated that genetic or pharmacological inhibition of EGFR activation results in decreased renal tubular cell dedifferentiation and proliferation in a murine model of FA-induced AKI. These results extend our in vitro observations and reveal that EGFR is a key tyrosine kinase receptor that mediates regulation of multiple renal regenerative responses, including dedifferentiation and proliferation after acute injury.

The mechanism responsible for the EGFR activation after AKI is not clear but may be associated with production and release of one or multiple EGFR ligands. It has been reported that EGF, HB-EGF, and TGF-α are expressed in the kidney (11, 28). EGF is upregulated in ischemia and toxicant-induced injury and is capable of stimulating proliferation of kidney epithelial and progenitor cells through the EGFR (28). Under physiological condi-
tions, Hb-EGF and TGFβ are expressed in the kidney and implicated in the development of the kidney and tubule branching (28). HB-EGF can be produced in the early phase of renal regeneration after acute injury (22). Furthermore, administration of exogenous EGF promotes renal tubular cell proliferation and enhances renal functional and structural recovery (13). Additional studies are needed to identify the role of individual EGFR ligands in renal dedifferentiation and proliferation.

EGFR may be also activated by transactivation, a mechanism that is not involved in the direct interaction of EGFR with its ligands at the initial step. During this process, some other stimuli such as reactive oxygen species and non-EGFR ligands can induce activation of EGFR though activation of some intracellular signaling molecules such as Src (5, 9). In agreement with this concept, we have recently demonstrated that Src is activated in renal tubular cells following plating, leading to EGFR phosphorylation and renal tubular cell dedifferentiation and proliferation.

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The signaling pathway that leads from EGFR activation to renal tubular dedifferentiation remains undefined in vivo. Our
in vitro studies showed that the PI3K/Akt pathway acts downstream of the EGFR and regulates renal tubular cell dedifferentiation and proliferation in primary cultures of renal tubular cells (31, 32). Here, we showed that in response to FA, Akt was phosphorylated in the kidney and that EGFR inhibition suppressed its phosphorylation, suggesting that this pathway may play a role in signaling EGFR activation to renal regenerative responses. Although the ERK1/2 pathway is also activated in response to ischemia and other insults such as cisplatin and oxidative stress, their activation has been shown to be involved in renal tubular cell death but not cell proliferation (16, 33, 35). Therefore, the PI3K/Akt pathway may be the major signaling pathway that mediates renal epithelial cell dedifferentiation and proliferation.

Nevertheless, the PI3K/Akt pathway may also mediate the survival of renal tubular cells. Cellular stresses such as oxidative stress have been reported to stimulate the PI3K/Akt pathways in renal tubular cells (34), and Akt inhibits apoptosis after the release of cytochrome c by phosphorylating and inhibiting caspase-9 (30). By using a murine model of cisplatin-induced AKI, Kuwana et al. (18) showed that the PI3K-Akt pathway is activated after cisplatin administration and blockage of the PI3K/Akt pathway accelerates renal tubular cell death and leads to poor prognoses, indicating the importance of the PI3K/Akt pathway in mediating renal tubular cell survival after toxic injury to the kidney. In support of this concept, we demonstrated that inhibition of EGFR activity results in AKT dephosphorylation and an increase in apoptotic renal tubular cells, implying that EGFR-mediated activation of the PI3K pathway is necessary for renal tubular cells to confer against the toxic effect of FA.

To our knowledge, this is the first time it has been demonstrated that the EGFR-mediated signaling pathway is responsible for

Fig. 8. Effects of FA administration on Akt activation in WT, gefitinib-treated, and waved-2. Whole renal cell extracts were immunoblotted with the specific antibodies against p-AKT, AKT, or GAPDH in WT and gefitinib-treated mice (A) or waved-2 (B) on day 2 after FA administration. Expression levels of p-AKT and AKT were quantified by densitometry analysis and normalized with AKT (C and E) and GAPDH (D and F), respectively. Data are represented as the means ± SE (n = 5). Bars with different superscript letters are significantly different from one another (P < 0.05).
renal tubular cell dedifferentiation in an in vivo model of AKI. However, we cannot rule out the possibility that other growth factor receptors are also involved in these processes. In this respect, it has been reported that hepatocyte growth factor c-Met and platelet-derived growth factor receptors are expressed in renal tubular cells and mediate renal tubular cell proliferation in vitro and vivo (21). Moreover, hepatocyte growth factor administration protects renal epithelial against apoptosis in vitro by activating the PI3K/Akt pathway, resulting in phosphorylation and inactivation of proapoptotic protein Bad and promoting expression of Bel-xL, an antiapoptotic factor (29). Thus it is likely that EGFR may function alone or cooperatively with other receptors to regulate renal regenerative responses. On the other hand, some receptors may regulate renal regenerative responses through signaling to EGFR, since EGFR is a unique cellular membrane receptor, which can be activated by signals initiated from other growth factor receptors through a transactivation-dependent mechanism as described above.

In summary, our study supports the importance of EGFR as a major regulator of dedifferentiation and proliferation in the early phase of renal injury. In addition, EGFR activation is also required for renal tubular survival. Thus EGFR activation following AKI may minimize acute tubular damage and promote effective renal regeneration. Given that proliferation of dedifferentiated renal epithelial cells has been recognized as the predominant mechanism of renal repair, EGFR-mediated dedifferentiation of renal tubular cells may be the key step to restore renal structure and function after acute tubular injury.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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REFERENCES

