Genetic or Pharmacologic Blockade of EGFR Inhibits Renal Fibrosis

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ABSTRACT

Although enhanced activation of the EGF receptor (EGFR) associates with the development and progression of renal fibrosis, the mechanisms linking these observations are not completely understood. Here, after unilateral ureteral obstruction (UUO), wild-type mice exhibited sustained EGFR phosphorylation in the kidney and developed renal fibrosis that was more severe than the renal fibrosis observed in waved-2 mice, which have reduced EGFR tyrosine kinase activity. Waved-2 mice also showed fewer renal tubular cells arrested at G2/M, reduced expression of α-smooth muscle actin (α-SMA), downregulation of multiple genes encoding profibrogenic cytokines, including TGF-β1, and dephosphorylation of Smad3, STAT3, and ERK1/2. Administration of the specific EGFR inhibitor gefitinib recapitulated this phenotype in wild-type mice after UUO. Furthermore, inactivation of either EGFR or STAT3 reduced UUO-induced expression of lipocalin–2, a molecule associated with the pathogenesis of CKD. In cultured renal interstitial fibroblasts, inhibition of EGFR also abrogated TGF-β1– or serum-induced phosphorylation of EGFR, STAT3, ERK1/2, and Smad3 as well as expression of α-SMA and extracellular matrix proteins. Taken together, these data suggest that EGFR may mediate renal fibrogenesis by promoting transition of renal epithelial cells to a profibrotic phenotype, increased production of inflammatory factors, and activation of renal interstitial fibroblasts. Inhibition of EGFR may have therapeutic potential for fibrotic kidney disease.


CKD is a leading cause of death in the United States, affecting approximately 10% of the population in the developed world.1 Tubulointerstitial fibrosis is considered the final common pathway leading to ESRD.2,3 The pathogenesis of renal fibrosis is characterized by proliferation of activated fibroblasts (myofibroblasts) and overproduction and deposition of extracellular matrix (ECM), ultimately leading to fibrotic lesions and tissue scarring.2–4 Although the cellular mechanisms that facilitate tubulointerstitial fibrosis after injury remain poorly defined, genetic tracing studies show that the resident fibroblasts are the major source of myofibroblasts and play an important role in this process.5 As such, understanding the mechanism of myofibroblast activation and proliferation is critical for the development of novel treatments to slow or halt the progression of CKD.

Numerous cytokines/growth factors seem to modulate activation of renal interstitial fibroblasts and progression of glomerular and tubulointerstitial scarring.6,7 Of these cytokines/growth factors, TGF-β is the single most important profibrogenic mediator in renal fibrosis.8 The fibrogenic effects of TGF-β are thought to occur through its interaction with TGF-β receptors4,9 and subsequent activation of Smad3. Activated Smad3, together with Smad4, is translocated to the nucleous, where it drives expression of...
TGF-β1-responsive genes. TGF-β1 can also signal independently of Smads through transactivation of EGF receptor (EGFR). Receptor transactivation is defined as a stimulus other than ligand-induced activation of a cellular membrane receptor. In addition to TGF-β1, many other profibrotic factors, such as angiotensin II and endothelin 1, can also induce EGFR transactivation. Thus, it is possible that the signaling stimulated by diverse stimuli would converge on EGFR, which in turn, induces activation of renal fibroblasts and renal fibrogenesis.

EGFR is a transmembrane protein with intrinsic tyrosine kinase activity. Several ligands, including heparin-binding EGF and TGF-α, are expressed in renal epithelial cells and released after injury. Ligand binding to EGFR induces dimerization and phosphorylation of tyrosine residues in its cytosolic domains. The phosphorylated tyrosine residues become docking sites for signaling molecules that activate cellular signaling pathways such as extracellular-regulated kinase (ERK) and signal transducer and activator of transcription 3 (STAT3). Activation of these pathways triggers a number of cellular responses, including cell proliferation and survival as well as protein expression. Our recent studies show that activation of STAT3 after chronic kidney injury is required for renal fibroblast to myofibroblast transformation and expression of ECM proteins such as type I collagen and fibronectin. STAT3 also mediates up-regulation of some proinflammatory mediators, including TNF-α, intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1).

EGFR is expressed in both renal interstitial fibroblasts and renal epithelial cells. Recent studies have shown that overexpression of the dominant negative isoform of EGFR was quantified by densitometry and normalized with GAPDH or α-tubulin. Kidney tissue collected at day 7 was used for staining with antibodies to p-EGFR in sham and UUO groups. (G) Both epithelial cells (white arrows) and interstitial cells (yellow arrows) were stained with p-EGFR. Data are represented as the mean ± SEM (n=6). Means with different superscript letters are significantly different from one another (P<0.05).

Figure 1. EGFR activation in the kidney after UUO injury in wild-type and waved-2 mice. The left ureter was ligated in both wild-type (WT) and waved-2 (Wa-2) mice. (A–C) At days 1, 3, 7, and 14 or (D–G) 7 days, the kidneys were taken for immunoblot analysis of p-EGFR, EGFR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or α-tubulin as indicated (A–F). (B, C, E, and F) Expression levels of p-EGFR and EGFR.
EGFR in renal tubular cells attenuates the renal fibrotic lesions induced by prolonged renal ischemia and chronic infusion of angiotension II, suggesting that activation of epithelial EGFR is also involved in renal fibrogenesis. However, how epithelial cells orchestrate interstitial fibrosis development is not fully understood and is being studied. Recently, the work by Yang et al. showed that, after diverse AKIs, a large number of renal tubular cells are arrested in the G2/M stage of the cell cycle, which acts to generate profibrotic cytokines TGF-β1 and connective tissue growth factor, a key downstream effector of profibrotic TGF-β1 activity. This finding suggests that renal epithelial cells may contribute to the development of renal fibrosis by transition to a phenotype that produces TGF-β1, which indirectly regulates renal interstitial fibroblast activation. However, the fibrotic action of EGFR has not been linked to cell cycle dysregulation and production of cytokines in the injured kidneys.

The purpose of this study was to investigate the molecular events of EGFR-mediated renal fibrosis in a mouse model of obstructive nephropathy using waved-2 mice, which contain a point mutation in EGFR that reduces receptor tyrosine kinase activity by >90% and a specific EGFR inhibitor, gefitinib. We show that obstructive injury induces a sustained increase in EGFR phosphorylation, which is essential for driving of renal epithelial cells to arrest at G2/M phase of cell cycle, production of profibrotic cytokines, and activation of renal fibroblasts.

**RESULTS**

**EGFR Phosphorylation and Expression in the Kidney after Unilateral Ureteral Obstruction**

EGFR has been shown to be involved in renal regeneration after AKI; however, its role in CKD is less investigated. As an initial step to understanding the role of EGFR in the progression of renal fibrosis, we examined activation and expression of EGFR in a mouse model of renal fibrosis induced by unilateral ureteral obstruction (UUO), which is characterized by activation and accumulation of myofibroblasts. As shown in Figure 1, A–C, UUO injury induced EGFR tyrosine phosphorylation in the kidney of wild-type mice, which was observed at day 1, peaked at day 7, and remained at that level for at least 14 days. An increase in total EGFR was also clearly observed on day 3 and remained elevated at 14 days after UUO injury. However, increased levels of phosphorylated EGFR (p-EGFR) were not only caused by upregulation of total EGFR, because the ratio of p-EGFR to EGFR increased over time during the time course of UUO injury (Figure 1B). In contrast to wild-type animals, waved-2 mice displayed a dramatic decrease in p-EGFR in the kidney after UUO injury, although renal EGFR expression was comparable in UUO-injured waved-2 and wild-type mice. Densitometry analysis indicates a 92% reduction of p-EGFR in waved-2 mice relative to wild-type mice.
To the p-EGFR expressed in wild-type mice subjected to UUO injury (Figure 1E), which is consistent with approximately 90% decrease of kinase activity in wave-2 mice as reported previously.\textsuperscript{23} No p-EGFR was detected in the kidneys of wild-type and wave-2 mice without UUO injury (Figure 1, D and F). Immunofluorescent staining indicated the distribution of p-EGFR and total EGFR in both tubular cells and interstitial fibroblasts in the fibrotic kidney (Figure 1G and Supplemental Figure 1).

To identify the tubular cell types that express EGFR in obstructive kidney, we first costained the injured kidney tissues for EGFR with an FITC-labeled lectin, peanut agglutinin (PNA), which binds and stains proximal and distal tubules and collecting ducts.\textsuperscript{25–27} As shown in Supplemental Figure 2, many tubules were labeled by FITC-PNA from cortex through medulla, but only tubules in the cortex were costained with EGFR. Because the majority of renal tubules in the cortex are proximal tubules, this finding suggests that EGFR is predominantly expressed in proximal tubular cells.

To localize the active EGFR (p-EGFR) in renal tubules, we also costained the kidney tissues for p-EGFR with three tubular segment-specific markers: megalin (proximal tubules), aquaporin 2 (collecting ducts), and tamm-horsfall protein (thick ascending limb of the loop of henle), respectively. p-EGFR expression was observed in proximal tubular cells and thick ascending limbs in the kidney of wild-type mice after UUO injury, which was decreased in the injured kidney of wave-2 mice. In contrast, p-EGFR-positive cells did not colocalize with aquaporin 2-positive cells (Supplemental Figure 3).

Collectively, these data illustrate that UUO injury induces a persistent activation of EGFR in the kidney of wild-type mice, which is markedly reduced in wave-2 mice. p-EGFR is primarily localized in the proximal tubules and also expressed in thick ascending limbs but not collecting ducts.

**UUO-Induced Renal Fibrosis Is Attenuated in Waved-2 Mice**

To assess the role of EGFR in the pathogenesis of renal fibrosis, we examined the expression of interstitial collagen fibrils by Masson trichrome staining in the kidney of both wild-type and wave-2 mice after UUO injury. As shown in Figure 2A, kidneys with ureteral obstruction for 7 days displayed tubular dilation and interstitial expansion with collagen deposition, which was evidenced by an increase in Masson trichrome-positive areas within the tubulointerstitium in wild-type mice. By contrast, kidneys in wave-2 mice exhibited decreased fibrotic lesions in the interstitium. Quantitative analysis of Masson trichrome-positive areas revealed an approximately 10-fold increase of ECM in the obstructive kidney compared with sham-operated kidneys in wild-type mice. In wave-2 mice, the expression of ECM components was reduced by ~80% (Figure 2B).

Collagen type 1 and fibronectin are two key components of the interstitial matrix. Immunoblot analysis of kidney tissue showed that their expression was increased after UUO injury in wild-type mice compared with sham-operated animals. In contrast, fibronectin expression was abolished, and expression of collagen type 1 was reduced to the basal level in injured wave-2 mice (Figure 2, C–E). These data, together with results from Masson trichrome staining, indicate that EGFR activation contributes to the development of renal interstitial fibrosis and production of ECM proteins after UUO injury.

**UUO-Induced Renal Fibroblasts Activation Is Suppressed in Waved-2 Mice**

Because myofibroblasts are the principle cell type responsible for production of ECM proteins and α-smooth muscle actin (α-SMA) is the hallmark of myofibroblasts,\textsuperscript{4} we also determined the level and distribution of α-SMA-positive cells in this model. As shown in Figure 3, A and B, wild-type mice displayed increased α-SMA–positive cells in the obstructive kidneys, whereas this population of cells was significantly reduced in wave-2 mice. Immunoblot analysis of renal tissue
showed that α-SMA was barely detectable in the renal tissue of sham-operated wild-type and waved-2 mice and that UUO injury resulted in a marked increase in α-SMA expression. In contrast, α-SMA expression level was largely suppressed in waved-2 mice (Figure 3, C and D). We also examined the expression of fibroblast-specific protein-1, another hallmark of myofibroblasts, by immunostaining. As shown in Supplemental Figure 3, the obstructive kidney in wild-type mice displayed an increased number of fibroblast-specific protein-1–positive cells, whereas this cell type was not observed in sham-operated animals. These data indicate that EGFR plays a critical role in the activation of renal fibroblasts after obstructive injury.

**Inhibition of EGFR by Gefitinib Attenuates Development of Renal Fibrosis and Renal Fibroblast Activation in Wild-Type Mice**

To examine whether EGFR can be used as a therapeutic target to attenuate renal fibrosis, we also examined the effect of gefitinib, a specific EGFR inhibitor, on the development of renal fibrosis and activation of renal fibroblasts. As shown in Figure 4, A and B, daily intraperitoneal injection of gefitinib at 100 mg/kg significantly reduced the deposition of ECM components as shown by Masson trichrome staining. Of note, this dose of gefitinib largely inhibited UUO-induced EGFR phosphorylation in the obstructive kidney, whereas expression levels of total EGFR were not affected (Figure 4, C–E). We also examined the effect of gefitinib on expression of type 1 collagen, fibronectin, and α-SMA in the obstructive kidney. Consistent with the results obtained in waved-2 mice, gefitinib administration inhibited UUO injury-induced expression of all these proteins (Figure 5). These data provide additional evidence for the importance of EGFR in mediating renal fibrogenesis after injury, and they suggest that pharmacological inhibition of EGFR activity may be an approach to attenuate development of renal fibrosis.

**EGFR Activity Is Required for Production of TGF-β1 and Activation of TGF-β Signaling in the Obstructive Kidney**

Activation of TGF-β1 signaling is central to the development of renal fibrosis in various models of renal fibrosis.8,28,29 To explore the mechanism by which EGFR activation contributes to renal fibrosis, we examined the role of EGFR in regulation of this signaling pathway. First, we determined expression levels of TGF-β1 in the obstructed kidneys by quantitative real-time PCR. As shown in Figure 6, A and B, UUO injury markedly increased expression of TGF-β1 in wild-type but not waved-2 mice. The basal level of renal TGF-β1 was also slightly lower in waved-2 than wild-type mice. Administration of gefitinib also significantly reduced the expression of TGF-β1 in the obstructive kidney (Figure 6B). These data indicate that EGFR is required for production of TGF-β1 in the injured kidney.

Smad3 is the key downstream mediator of TGF-β signaling, and it controls the transcription of TGF-β1–responsive
genes. To verify the regulatory role of EGFR in activation of this signaling pathway after UUO injury, we also compared the level of phosphorylated Smad3 (p-Smad3) in wild-type and waved-2 mice as well as in wild-type mice after gefitinib treatment. p-Smad3 was not detectable in the kidney of wild-type and waved-2 mice without injury. After UUO injury, a high level of p-Smad3 was observed in the renal tissue of wild-type mice. In contrast, Smad3 was not detected in the injured kidney of waved-2 mice (Figure 6C). Similar to these observations, gefitinib treatment also reduced expression of p-Smad3 (Figure 6D). These data, therefore, indicate that EGFR activity is critically involved in the regulation of TGF-β signaling in the kidney after UUO injury.

**EGFR Activity Contributes to Renal Epithelial Cell Arrest in the G2/M Phase of Cell Cycle after UUO**

Recent studies showed that renal epithelial cells arrested at the G2/M boundary resulted in a prominent profibrotic phenotype that produces profibrotic growth factors/cytokines. This phenotype is characterized by renal tubular cells arrested at the G2/M stage. Because phosphorylation of histone H3 at serine 10 (p-H3) is a hallmark of cells arrested at the G2/M stage, we investigated whether EGFR plays a role in this process by examining p-H3 expression in the obstructive kidney by immunostaining and immunoblot analysis. As shown in Figure 7, A–C, a large number of renal tubular cells labeled with p-H3 was observed in wild-type animals subjected to obstructive injury. In contrast, this population of cells was dramatically reduced in the injured kidney of either waved-2 or wild-type mice receiving gefitinib. Immunoblot analysis showed that p-H3 was barely detectable in sham-operated animals, but its expression was increased in the kidney of wild-type UUO injury (Figure 7, D–G). In contrast, p-H3 expression was barely seen in the kidney of waved-2 mice after UUO injury and the kidney of wild-type mice subjected to obstructive injury and treatment with gefitinib (Figure 7, D–G).

Taken together, our data suggest that EGFR activation is essential for driving tubular cells to arrest at the G2/M phase of cell cycle after obstructive injury.

**EGFR Activation Is Required for Lipocalin-2 Expression in Renal Epithelial Cells after UUO Injury**

It has been reported that expression of lipocalin-2 is associated with the development of renal fibrosis. To determine whether EGFR is also required for expression of lipocalin-2 in the obstructive kidney, we examined its expression in the kidney over time after UUO injury and the effect of EGFR inhibition. Lipocalin-2 was not detectable in the kidney of wild-type animals. After UUO injury, lipocalin expression was detected at day 3, reached the maximum level on day 7, and then elevated for at least 14 days (Figure 8A). Compared with wild-type animals, there was much less expression of lipocalin-2 in the injured kidney of waved-2 or wild-type mice treated with gefitinib (Figure 8, B–E). Immunofluorescent analysis indicates that lipocalin-2 is not expressed in the sham-operated kidney but highly expressed in the kidney of UUO-injured animals (Figure 8F). Consistent with
previous studies, lipocalin-2 was expressed in renal tubules but not interstitial cells. Thus, lipocalin-2 may mediate the profibrotic effect of EGFR in renal epithelial cells.

EGFR Activation Is Required for UUO-Induced STAT3 and ERK1/2 Phosphorylation

STAT3 and ERK1/2 signaling pathways are reported to be activated in the obstructive kidney and involved in the progression of renal fibrosis in the murine model of UUO.\(^3\),\(^3\) We, thus, sought to determine whether EGFR mediates the phosphorylation of these two signaling molecules. As shown in Figure 9, A–C, STAT3 and ERK1/2 phosphorylation was barely detected in the sham-treated kidney but was dramatically increased in the obstructive kidney of wild-type mice. In contrast, the injured kidney of waved-2 mice displayed reduced UUO-induced STAT3 and ERK1/2 phosphorylation. Similarly, inhibition of EGFR with gefitinib in the injured kidney also reduced expression of phosphorylated STAT3 and ERK1/2 (Figure 9, D–F). Although expression of total STAT3 and ERK1/2 was also remarkably increased in either wild-type or waved-2 mice after UUO injury compared with normal kidneys, inactivation of EGFR did not alter its expression. These data indicate that EGFR plays a critical role in mediating UUO injury-induced activation of STAT3 and ERK1/2 signaling pathways.

STAT3 Is a Critical Intermediate between EGFR and Lipocalin-2

STAT3 is a transcriptional factor that drives gene expression of numerous molecules associated with renal fibrogenesis.\(^1\),\(^8\) To determine whether STAT3 mediates lipocalin-2 expression in the obstructed kidney, we treated UUO-injured animals with S3I-201, a novel and specific inhibitor of STAT3, and analyzed lipocalin-2 expression by immunoblot analysis. Similar to what we observed by inactivation of EGFR, inhibition of STAT3 with S3I-201 also significantly reduced lipocalin-2 expression (Figure 10), suggesting that STAT3 may act as an intermediate between EGFR and lipocalin-2.

EGFR Activation Is Required for Production of Multiple Cytokines in the Fibrotic Kidney

Given that upregulation of proinflammatory cytokines contributes to the development of renal fibrosis and that tubular cell arrest at the G2/M phase is associated with production of profibrotic cytokines such as TGF-\(\beta\),\(^2\) we also examined the role of EGFR in the expression in the kidney of three major proinflammatory cytokines (TNF-\(\alpha\), MCP-1, and ICAM-1) by quantitative real-time PCR. Our data reveal that gene expression of all these three cytokines was significantly increased after UUO injury. Expression of TNF-\(\alpha\) and MCP-1
but not ICAM-1 was inhibited in the injured kidney of waved-2 mice and the injured kidney in wild-type mice subjected to gefitinib treatment (Figure 11). These data, together with role of EGFR in TGF-β1 production after UUO injury, suggest that EGFR is critically involved in production of multiple cytokines in the fibrotic kidney.

**DISCUSSION**

There is growing evidence for the role of the EGFR pathway in various types of renal lesions. Increased EGFR activation and expression was correlated with interstitial fibrosis and tubular atrophy in human renal allograft biopsies. EGFR activation is involved in endothelin-induced renal vascular and glomerular fibrosis. Furthermore, interstitial fibrosis and tubular atrophy from prolonged ischemia and chronic infusion of angiotensin II were associated with increased EGFR activity. However, the underlying mechanism by which EGFR activation contributes to renal fibrosis is not well understood.

**EGFR Mediates TGF-β1–Induced Activation of Renal Interstitial Fibroblasts in Vitro**

A lower expression level of α-SMA in the kidney of waved-2 mice relative to the kidney in wild-type mice after UUO injury suggests the possibility that EGFR is involved in regulating renal fibroblast activation. To test this hypothesis, we examined the role of EGFR in TGF-β1–induced renal fibroblast activation in vitro using gefitinib, a specific inhibitor of EGFR. Exposure of cultured renal interstitial fibroblasts (NRK-49F) to TGF-β1 induced expression of α-SMA and fibronectin as well as phosphorylation of EGFR, Smad3, STAT3, and ERK1/2. Treatment with gefitinib inhibited TGF-β1–induced expression of α-SMA and fibronectin and phosphorylation of EGFR, Smad3, STAT3, and ERK1/2 in a dose-dependent manner with a nearly complete inhibition at 10 nM (Figure 12, A–D). Gefitinib also dose-dependently suppressed serum-induced expression of α-SMA, fibronectin, and type 1 collagen and phosphorylation of EGFR, Smad3, STAT3, and ERK1/2 in NRK-49F (Supplemental Figure 5). Of note, 10 nM gefitinib did not induce cleavage of poly (ADP-ribose) polymerase and caspase-3, two hallmarks of apoptosis, suggesting that it does not cause apoptosis at this concentration. As a positive control, we observed that exposure of NRK-49F to 250 μM hydrogen peroxide resulted in cleavage of these two proteins (Supplemental Figure 6). These data support our in vivo observations that EGFR is a critical mediator in the activation of renal interstitial fibroblasts.
fibrosis remains unknown. By using genetic and pharmacological approaches, we endorse the importance of EGFR in the development of renal fibrosis in a model of kidney fibrosis induced by UUO injury and also show that EGFR activity is required for activation of renal interstitial fibroblasts, activation of TGF-β1 signaling, production of proinflammatory cytokines, and conversion of renal epithelial cells to a fibrotic phenotype. We also show that EGFR mediates TGF-β1–induced activation of renal interstitial fibroblasts in vitro. These data suggest that EGFR is a key molecule that integrates, directly or indirectly, the effects of various fibrogenic factors to initiate and promote renal fibrosis in CKD.

In contrast to transient activation/expression of EGFR in AKI, chronic injury to the kidney induces persistent EGFR expression and phosphorylation. This finding is evidenced by our observations that EGFR phosphorylation/expression was induced at day 3 after injury, peaked at day 7, and was sustained for at least 14 days. Increased EGFR phosphorylation is not merely caused by increased EGFR expression, because the p-EGFR/EGFR ratio was increased over time after injury. A persistent activation of EGFR may provide a continuous signal to stimulate EGFR activation and subsequently, induce renal fibrosis by activation of intracellular signaling pathways that trigger profibrotic machinery (see below). Currently, the mechanism leading to the sustained EGFR activation after injury remains elusive but may be associated with increased production of EGFR ligands. In this regards, it has been reported that one EGFR ligand, TGF-α, is the key mediator of genetic predisposition to CKD progression in FVB/N mice. TGF-α is expressed in renal tubular cells, and its increase preceded the development of renal lesions in the lesion-prone FVB/N strain, suggesting that TGF-α may be one of the EGFR ligands involved in the persistent activation of EGFR. In addition, other EGFR ligands may be overproduced in the chronic pathogenetic states of kidneys. Additional investigation is required to identify the EGFR ligands that are increased and participate in the activation of EGFR and renal fibrosis after chronic injury.

Activation of EGFR after chronic injury may also be induced by non-EGFR ligand stimulation. Our study shows that TGF-β1 expression is increased in the fibrotic kidney and that EGFR inhibitor blocked TGF-β1–stimulated renal fibroblast activation in cultured rat renal interstitial fibroblasts. Interestingly, inhibition of EGFR also inhibits TGF-β1–induced activation of Smad3, a key mediator of TGF-β signaling. Furthermore, either genetic or pharmacological blockade of EGFR inhibited Smad3 phosphorylation in the kidney after UUO injury. Other non-EGFR ligands are recognized as profibrotic stimuli in line with our observations. For example, angiotensin II and endothelin induce EGFR activation and renal fibrogenesis in the kidney, and mice overexpressing a dominant negative isoform of EGFR or subjected to EGFR inhibitors have reduced fibrotic lesions. These data suggest that EGFR not only transduces profibrotic signals from its ligands but also from some non-EGFR ligands. With this property, many metabolic, hormonal, and hemodynamic factors may induce renal fibrosis through activation of EGFR. The activation of a cellular membrane receptor by a stimulus other than its ligand is termed receptor transactivation. The mechanisms of EGFR transactivation are not fully understood but may involve inhibition of protein tyrosine phosphatases and induction of metalloproteases or a disintegrin and metalloprotease-dependent Heparin-binding/TGF-α cleavage. The demonstration of TGF-α as the key mediator of the genetic predisposition to CKD progression suggests its importance in mediating EGFR transactivation induced by TGF-β1 or other stimuli. In support of this statement, the work by Lautrette et al. has reported that angiotensin II treatment induces TGF-α production, and angiotensin II–induced fibrotic lesions were substantially reduced in mice lacking TGF-α.

Our data indicate that EGFR is expressed in both renal epithelial cells and renal interstitial fibroblasts. It has long been
thought that injury to renal tubular cells leads to renal fibrosis, but underlying mechanisms are unclear. Yang et al.\textsuperscript{22} have recently observed that, after ischemic, toxic, and obstructive injury, the number of cells expressing phosphohistone (serine 10), a hallmark of cells in G2/M phase, increased in the late stage of injury and that reversal of G2/M arrest reverses the fibrogenic effect,\textsuperscript{22} suggesting that tubular cells arrested in G2/M play a primary role in determining the fibrotic response. The renal epithelial cell with G2/M arrest may contribute to renal fibroblast activation through overproduction of profibrotic factors. It is evident that G2/M-arrested proximal tubular cells promote the release of profibrotic cytokines such as TGF-\(\beta\) and connective tissue growth factor.\textsuperscript{22} However, the mechanism by which renal injury leads to G2/M arrest in tubular cells is not known. In our study, we observed that a large number of epithelial cells are arrested in the G2/M phase in the kidney of wild-type mice after obstructive injury and that this population of cells was dramatically decreased in the injured kidney of waved-2 mice and the kidney of wild-type mice subjected to treatment with the EGFR inhibitor. Furthermore, expression of TGF-\(\beta\) was increased in the fibrotic kidney, and both genetic and pharmacological reduction of EGFR activity inhibited TGF-\(\beta\) expression in the obstructive kidney. These data indicate the importance of EGFR in regulating production of this profibrogenic factor and suggest that EGFR activation may contribute to renal fibrosis by inducing transition of renal epithelial cells to a profibrotic phenotype. Renal tubular cells that express EGFR are localized in the cortex, and the majority of tubular cells in this region are proximal tubular cells. Thus, the proximal tubular cell may be the major cell type that is regulated by EGFR to undergo the transition from epithelial to profibrotic phenotype.

EGFR may also mediate renal fibrotic response through directly acting on renal interstitial fibroblasts. This statement is supported by several observations. First, phosphorylated EGFR was not only localized in the renal tubular cells but also interstitial cells in the fibrotic kidney. Second, a high level of EGFR was detected...
in cultured renal interstitial fibroblasts. Third, serum and TGF-β1 increased EGFR phosphorylation in cultured renal interstitial fibroblasts. Fourth, treatment of cells with gefitinib blocked phosphorylation of EGFR, expression of α-SMA and ECM proteins, and activation of Smad3 in renal interstitial fibroblasts in response to both serum and TGF-β1. Because TGF-β1 is the most important profibrogenic mediator in renal fibrosis and p-Smad3 is primarily localized in myofibroblasts in the injured kidney, which was shown by our immunologic staining, EGFR may be coupled to TGF-β signaling and may positively regulate this pathway at multiple levels in renal interstitial fibroblasts. These data, together with the role of EGFR in mediating G2/M phase arrest, reveal a dual mechanism by which EGFR contributes to renal fibrogenesis. EGFR activation in renal epithelial cells leads to conversion of renal epithelial cells to a fibrotic phenotype that produces a large amount of profibrotic cytokines and/or growth factors; in renal interstitial fibroblasts, EGFR mediates fibroblast activation and proliferation and ECM production in response to numerous growth factors, cytokines, and hormones, including the ones produced from renal tubular cells under various pathologic conditions.

Inflammation is an important mechanism in initiation and maintenance of renal damage, and decreased inflammatory response results in attenuation of renal fibrosis. In addition to TGFβ1, we also observed increased expression of TNF-α and MCP-1 in the obstructed kidney of wild-type mice and decreased expression in wounded mice and wild-type mice receiving EGFR inhibitor treatment. Currently, the mechanism by which EGFR regulates these two cytokines remains undefined. Like TGF-β1, EGFR may regulate production of cytokines through cell cycle arrest in the G2/M phase. EGFR may be coupled to the production of these cytokines through effects on other cell types, such as macrophages and other inflammatory cells, because numerous inflammatory cells are accumulated in the kidney after injury.

Although the pathogenesis of obstructive nephropathy is a complex process involving multiple factors and mechanisms, only limited intracellular signaling pathways are activated during this process. Recently, we showed that UUO injury induces activation of STAT3, which is required for activation of renal interstitial fibroblasts, through induction of inflammatory responses and development of renal fibrosis. In this study, we also examined the role of EGFR in the activation of STAT3 in the obstructed kidney. Our data indicate that both genetic and pharmacological blockade of EGFR inhibited STAT3 phosphorylation, suggesting that EGFR-mediated intracellular events may be transduced through activation of the STAT3 signaling pathway. In support of this hypothesis, we found that inhibition of STAT3 by S3I-201, a novel STAT3 inhibitor, reduces expression of Lcn2, a recently identified target of EGFR that mediates renal fibrosis. Nevertheless, STAT3 may not be the sole intracellular signaling molecule that is initiated by EGFR activation and involved in the pathogenesis of renal fibrosis. In this study, we showed that blockade of EGFR also inhibited UUO-induced phosphorylation of ERK1/2. The functional role of ERK1/2 in renal fibrogenesis has been shown to be associated with formation of renal fibrotic lesions after chronic injury.

In summary, our findings show a critical role for EGFR activation in mediating development of renal fibrosis after obstructive injury. Because EGFR can be activated by its ligands and multiple nonligand stimuli associated with renal fibrogenesis, EGFR may act as a key mediator of renal fibrosis. As such, pharmacological inhibition of the EGFR activation may represent a novel treatment for chronic fibrotic kidney diseases.

CONCISE METHODS

Chemicals and Antibodies
Antibodies to p-EGFR, p-Smad3, Smad3, p-ERK1/2, ERK1/2, p-STAT3, STAT3, cleaved poly (ADP-ribose) polymerase, and cleaved caspase-3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to fibronectin, collagen 1 (A2), EGFR, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to p-histone H3 was purchased from Millipore Corporation (Millipore, MA). Antibodies to lipocalin-2 and TGF-β1 were purchased from R&D Systems (Minneapolis, MN). Antibodies to α-SMA and α-tubulin and all other chemicals were from Sigma (St. Louis, MO). Gefitinib was purchased from AstraZeneca (Macclesfield, England).

Cell Culture and Treatments
NRK-49F cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) containing 5% FBS, 0.5% penicillin, and streptomycin in an
atmosphere of 5% CO₂ and 95% air at 37°C. To determine the effects of gefitinib on fibroblast activation, gefitinib was directly added to subconfluent NRK-49F cells for 36 hours. For TGF-β1 treatment, cells were starved for 24 hours by incubation with 0.5% FBS containing DMEM and then exposed to TGF-β1 (2 ng/ml) in the presence or absence of gefitinib for 36 hours.

UUO Model and Waved-2 Mice
The UUO model was established in male waved-2 mice (maintained on a C57BL/6jeixC3H/HeSnJ background) and their litters that weighed 20–25 g (Jackson Laboratory, Bar Harbor, ME) as described previously.32 Briefly, the abdominal cavity was exposed by a midline incision, and the left ureter was isolated and ligated. The contralateral kidney was used as a control. To examine the efficacy of gefitinib in renal fibrosis after UUO injury, gefitinib at 100 mg/kg in 50 μl DMSO was given intraperitoneally immediately after ureteral ligation and then administered daily. DMSO only-treated animals were used as controls. At days 1, 3, 7, and 14 after surgery, the animals were killed, and the kidneys were collected for protein analysis and histologic examination. Six mice were used in each group.

Immunoblot Analysis
Immunoblot analysis for NRK-49F cells and tissue samples was carried out according to our previous protocols.32

Immunohistochemical and Immunofluorescent Staining
Immunofluorescent staining was carried out according to the procedure described in our previous studies.32 Masson trichrome staining was performed according to the protocol provided by the manufacturer (Sigma, St. Louis, MO). For assessment of renal fibrosis quantitatively, the collagen tissue area was measured using Image Pro-Plus Software (Media-Cybernetics, Silver Spring, MD) by drawing a line around the perimeter of the positive staining area, and the average ratio to each microscopic field (400×) was calculated and graphed.

Figure 11. Expression of TNF-α, MCP-1, and ICAM-1 in the kidney after UUO injury. mRNA was extracted from sham-operated or obstructed kidneys of WT, Wa-2, and with/without gefitinib administration mice and subjected to quantitative real-time reverse-transcription PCR as described in Concise Methods. mRNA expression levels of (A and B) TNF-α, (C and D) MCP-1, and (E and F) ICAM-1 were indicated as fold induction over control (sham-operated mice treated with vehicle) and expressed as means ± SEM. Means with different superscript letters are significantly different from one another (P<0.05).

Quantitative Real-Time PCR
The procedure for quantitative real-time PCR and the primers used for all measurements have been described previously.32

Statistical Analyses
All the experiments were conducted at least three times. Data depicted in graphs represent the means ± SEM for each group. Intergroup comparisons were made using one-way ANOVA. Multiple means were compared using Tukey’s test. The differences between two groups were determined by t test. Statistically significant differences between mean values were marked in each graph.
Figure 12. Effect of gefitinib on TGF-β1-induced activation of cultured renal interstitial fibroblasts. Cultured NRK-49F cells were starved for 24 h and then exposed to 2 ng/ml TGF-β1 for 36 h in the absence or presence of gefitinib (0–10 nM). Cell lysates were subjected to immunoblot analysis using antibodies to (A and B) p-EGFR and EGFR, (C and D) p-Smad3 and Smad3, (E and F) fibronectin, α-SMA, or GAPDH, and (G and H) p-STAT3, STAT3, p-ERK1/2, and ERK1/2. Representative immunoblots from three experiments are shown. Expression levels of all these proteins were quantified by densitometry and expressed as means ± SEM. The ratios of (B) p-EGFR/EGFR, (D) p-Smad3/Smad3, and (H) p-STAT3/STAT3 or p-ERK/ERK are shown. (F) α-SMA and fibronectin were normalized with GAPDH. Means with different superscript letters are significantly different from one another (P<0.05).

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DISCLOSURES

None.

REFERENCES


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LEGENDS FOR SUPPLEMENTAL FIGURES

Supplemental Figure 1. Expression of EGFR in renal epithelial cells and renal interstitial fibroblasts. Confocal photomicrographs (1X400) illustrating EGFR and α-SMA staining of cortex kidney tissue collected at day 7 after UUO injury. A, α-SMA; B, EGFR; C, DAPI; D, merge.

Supplemental Figure 2. Location of EGFR in renal tubular cells. The kidney tissue collected at day 7 after UUO injury was processed for immunofluorescence of EGFR, followed by staining with FITC-labeled PNA and DAPI. Photographs were taken by confocal microscopy (1X400) from the cortex to the medulla.

Supplemental Figure 3. Location of p-EGFR in renal tubular cells. The kidney tissue collected at day 7 after UUO injury was processed for immunofluorescence of p-EGFR (Tyr-1073), followed by staining with antibodies against megalin, Aquaporin2 (AQP2) and Tamm-Horsfall Protein (THP), respectively. Photographs (1X400) were taken by a confocal microscopy. Arrows: collecting ducts.

Supplemental Figure 4. The kidney tissue collected at day 7 after sham surgery or UUO injury was processed for immunofluorescence of FSP-1. Photographs (1X400) were taken by an immunofluorescent microscopy.

Supplemental Figure 5. Effect of gefitinib on serum-induced interstitial fibroblast activation, as well as activation of EGFR, STAT3 and ERK1/2. NRK-49F cells were cultured with 5% FBS for 24 h and then and exposed to the indicated concentrations of gefitinib (0-10 nM) for 36 h (A, B, E, G, I). Cell lysates were subjected to immunoblot analysis using antibodies to fibronectin (A), α-SMA (C), collagen 1(E), α-tubulin, p-EGFR, EGFR (G), p-STAT3, STAT3, p-ERK1/2, ERK1/2 (I).
Representative immunoblots from three experiments are shown (A-J). Expression levels of all these proteins were quantified by densitometry and expressed as means ± SEM. The ratios of p-EGFR/EGFR (H), p-STAT3/STAT3 or p-ERK/ERK (J) were shown. Fibronectin, collagen 1, or α-SMA were normalized with α-tubulin (B, D, F). Means with different superscript letters are significantly different from one another (P < 0.05).

Supplemental Figure 6. Gefitinib treatment does not induce cell death in interstitial renal fibroblasts. NRK-49F cells were cultured with 5% FBS in the absence or presence of gefitinib 10 nM for 36 h or H2O2 250 μM for 3 h. Cells were harvested for immunoblot analysis of cleaved PARP and cleaved caspase-3.
Supplemental Figure 1

$\alpha$-SMA+EGFR+DAPI
Supplemental Figure 4

Sham

UUO

FSP-1
Supplemental Figure 6

GAPDH

50 kDa
75 kDa
100 kDa
20 kDa
15 kDa

control
Gefitinib (10nM)
H2O2 (250μM)

Cleaved PARP

↓ 100 kDa
↓ 75 kDa

Cleaved Caspase3

↓ 50 kDa
↓ 20 kDa
↓ 15 kDa

GAPDH
↓ 37kDa