

Inhibition of EGF Receptor Blocks the Development and Progression of Peritoneal Fibrosis

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ABSTRACT

Inhibitors of EGF receptor (EGFR) have antifibrotic effects in several organs, but the effect of these inhibitors on the development of peritoneal fibrosis is unknown. Here, we explored the therapeutic effect of gefitinib, a specific inhibitor of EGFR, on the development and progression of peritoneal fibrosis in a rat model. Daily intraperitoneal injections of chlorhexidine gluconate induced peritoneal fibrosis, indicated by thickening of the submesothelial area with an accumulation of collagen fibrils and activation of myofibroblasts, accompanied by time-dependent phosphorylation of EGFR. Administration of gefitinib immediately after injury prevented the onset of peritoneal fibrosis and delayed administration after the onset of peritoneal fibrosis halted fibrosis progression. Gefitinib treatment abrogated the increased phosphorylation of EGFR, Smad3, signal transducer and activator of transcription 3, and NF- κ B during peritoneal fibrosis; it also inhibited the accompanying overproduction of TGF- β 1 and proinflammatory cytokines and the infiltration of macrophages to the injured peritoneum. Moreover, gefitinib significantly reduced the peritoneal increase of CD31-positive blood vessels and vascular EGF-positive cells after injury. Finally, gefitinib also attenuated high glucose-induced peritoneal fibrosis in rats and abrogated TGF- β 1-induced phosphorylation of Smad3 and the epithelial-to-mesenchymal transition of cultured human peritoneal mesothelial cells. These results demonstrate that EGFR contributes to peritoneal fibrosis, inflammation, and angiogenesis, suggesting that EGFR inhibitors may have therapeutic potential in attenuating peritoneal fibrosis.

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Peritoneal dialysis (PD) has been an alternative therapy to hemodialysis for patients with ESRD. Its efficacy depends on the structural and functional integrity of the peritoneum. However, long-term PD leads to peritoneal injury with structural changes and functional decline. At its worst, peritoneal injury leads to peritoneal fibrosis and sclerosis, a serious complication of PD. Characteristic features of fibrotic sclerosing peritoneum include loss of mesothelial cells, abnormal proliferation of α -smooth muscle actin (α -SMA)-positive myofibroblasts and thickening of the submesothelial area with an accumulation of collagen.^{1,2} The thickened submesothelial fibrotic layer reduces efficacy of exchanges, leading to functional failure of PD. Much attention has been paid to

investigating the factors and mechanisms that contribute to peritoneal fibrogenesis and to developing

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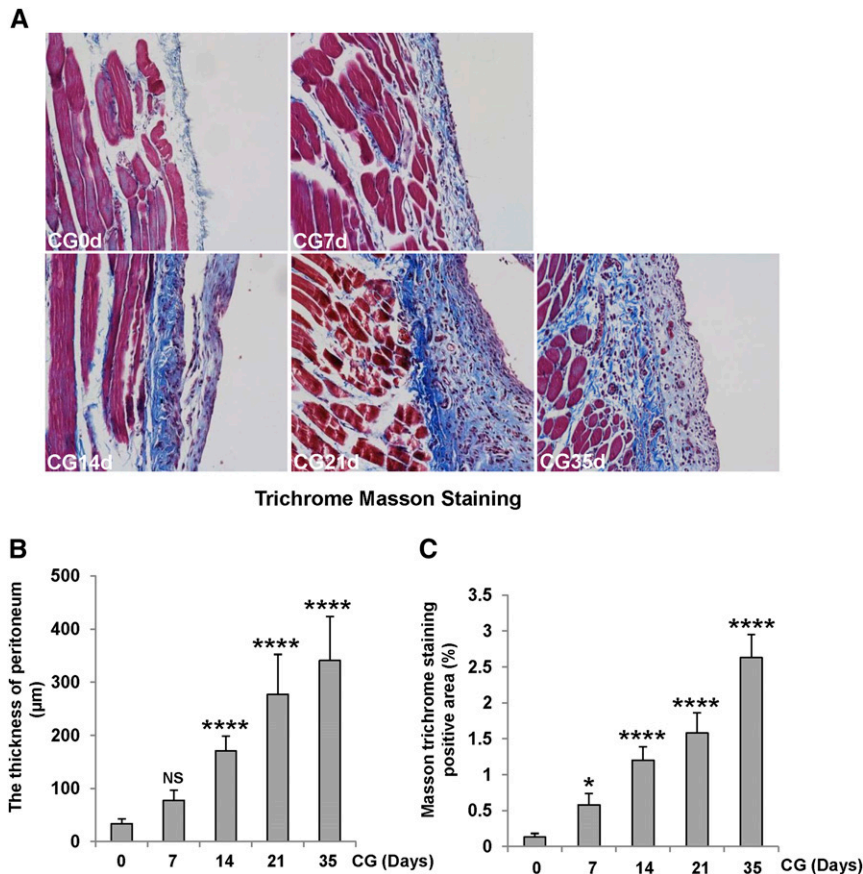


Figure 1. CG injection induces peritoneal fibrosis in a time-dependent manner. (A) Photomicrographs illustrating Masson trichrome staining of peritoneal membrane collected after CG injury at different times. (B) The graph shows the thickness of the compact zone measured from ten random fields (original magnification, $\times 200$) of five rat peritoneal samples. (C) The graph shows the fibrosis score of Masson-positive sub-mesothelial area (blue) from ten random fields (original magnification, $\times 200$) of five rat samples. Data are the mean \pm SEM ($n=5$). * $P<0.05$; **** $P<0.001$ versus sham controls.

therapies to preserve the structure and function of the peritoneal membrane.

Many factors have been proven fundamental to the development of peritoneal fibrosis in patients undergoing PD. One of the most important factors is the conventional bioincompatible PD solution, which contains a high concentration of glucose, as well as glucose degradation products and an acidic pH.^{3,4} Factors other than those related to the PD fluid, such as uremic toxins and peritonitis, also contribute to this process.⁵ All of these factors can damage the peritoneal membrane and subsequently lead to inflammation, fibrosis, and angiogenesis.

Chronic inflammation is an important mechanism in the initiation and maintenance of peritoneal fibrosis. This process is associated with activation of the NF- κ B pathway,⁶ overproduction of multiple proinflammatory factors (including TNF- α , IL- β 1, IL-6, and monocyte chemoattractant protein-1 [MCP-1]), and infiltration of various inflammatory cells (including macrophages).^{4,7} Activated macrophages in the peritoneum release several cytokines and chemokines and produce growth

factors that enhance extracellular matrix (ECM) production and exacerbate the fibrotic process.⁷

Peritoneal fibrosis and angiogenesis are typical morphologic changes leading to loss of peritoneal functions in patients undergoing PD. Many factors have been described as inducing these pathologic changes. On a molecular level, TGF- β , and vascular endothelial growth factor (VEGF) are two important cytokines and growth factors related to fibrosis and angiogenesis, respectively.⁷ TGF- β 1 induces peritoneal fibrosis through activation of the Smad signaling pathway.⁸ VEGF contributes to increasing endothelial permeability and induces angiogenesis.⁹ In addition, heparin-binding EGF (HB-EGF) was detected in peritoneal mesothelial cells and peritoneal macrophages in patients undergoing PD¹⁰; peritoneal mesothelial cells also express EGF receptor (EGFR) and human epidermal growth factor receptor 4, two receptors for HB-EGF.¹¹ Furthermore, exposure of cultured human peritoneal mesothelial cells (HPMCs) to HB-EGF and its homologous factor, EGF, was reported to induce a morphologic change of each factor toward a fibroblastic phenotype.^{10,11} Because phenotypic transition of peritoneal mesothelial cells to mesenchymal cells (fibroblast-like cells) is an important mechanism of peritoneal fibrosis,¹² EGF signaling may also be involved in the development of peritoneal fibrosis.

EGFR is a tyrosine kinase receptor that mediates multiple biologic effects on stimulation by its ligands, such as EGF and HB-

EGF. Ligand binding to EGFR induces its dimerization and phosphorylation and subsequently leads to activation of several intracellular signaling pathways, namely phosphatidylinositol 3-kinase/Akt, extracellular signal-regulated kinases, and signal transducers and activators of transcription 3 (STAT3), NF- κ B.^{13,14} STAT3 and NF- κ B are two transcriptional factors that can drive the expression of multiple cytokine/chemokines.¹⁵ STAT3 can also regulate expression of ECM proteins, such as type 1 collagen, and plays an important role in mediating the differentiation of renal interstitial fibroblasts to myofibroblasts and the development of renal fibrosis.¹⁶

EGFR has been linked to tissue fibrosis in a variety of organs, including the liver,¹⁷ lung,¹⁸ and kidney.^{13,19,20} However, it is unknown whether EGFR is activated in the peritoneum or whether it is involved in peritoneal fibrosis after injury. In this study, we investigated EGFR activation in response to peritoneal injury and explored the therapeutic potential of gefitinib, a specific inhibitor of EGFR, in rat models of peritoneal fibrosis induced by chlorhexidine gluconate (CG) and high glucose.

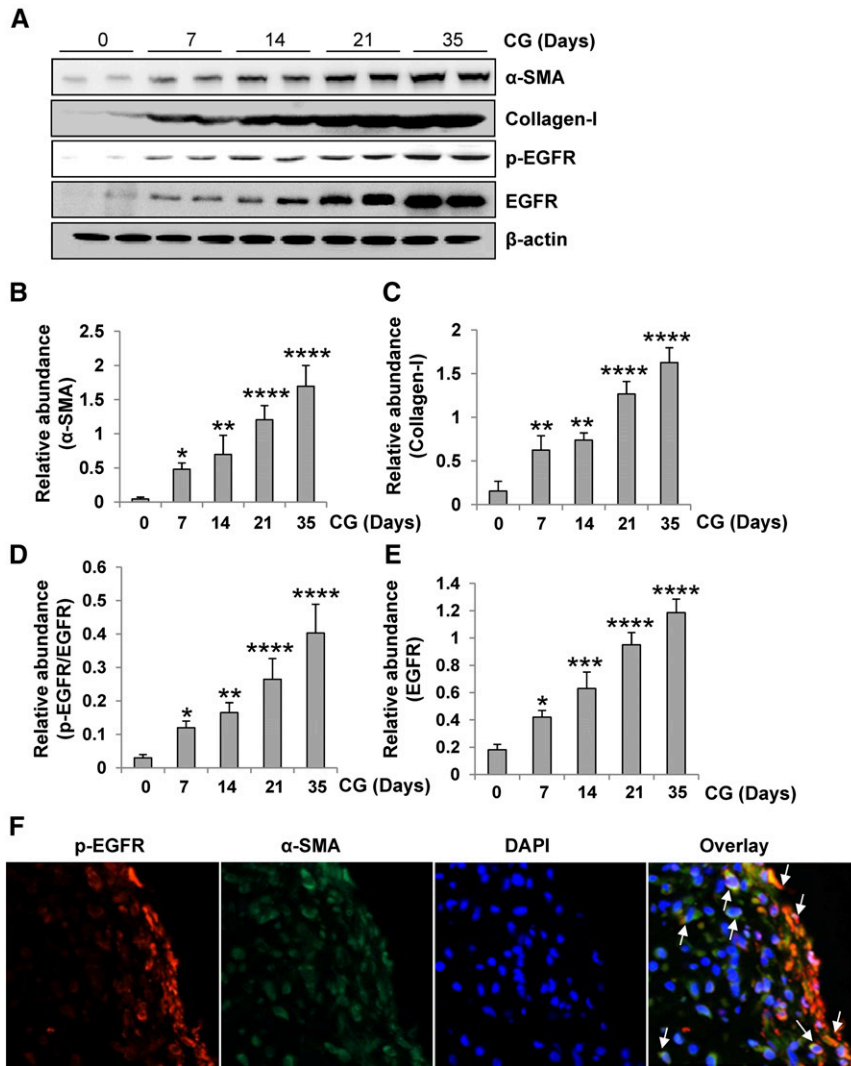


Figure 2. CG injection induces expression of Collagen-I, α -SMA, p-EGFR phosphorylation in peritoneum in a time-dependent manner. (A) Peritoneal membrane lysates were subjected to immunoblot analysis with specific antibodies against α -SMA, collagen-I, p-EGFR, EGFR, or β -actin; the representative results with two samples are shown. (B and C) Expression levels of α -SMA and collagen-I were separately quantified by densitometry and normalized with β -actin. (D) Expression levels of p-EGFR were quantified by densitometry and normalized with EGFR. (E) Expression levels of EGFR were quantified by densitometry and normalized with β -actin. Data are represented as the mean \pm SEM ($n=5$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.001$ versus sham controls. (F) Photomicrographs illustrate costaining of α -SMA and EGFR (white arrows) in the peritoneum collected 35 days after CG injection. DAPI, 4',6-diamidino-2-phenylindole.

RESULTS

CG Injection Induces Progression of Peritoneal Fibrosis in a Time-Dependent Manner in Rats

It is well documented that long-term PD can lead to peritoneal fibrosis, which is characterized by activation of renal interstitial fibroblasts and thickening of the submesothelial area with an accumulation of collagens.²¹ To elucidate the role of EGFR in the development of peritoneal fibrosis, we first established a

model of peritoneal fibrosis in rats by daily injection of CG. As shown in Figure 1, repeated injection of CG resulted in the thickening of the submesothelial compact zone, as well as peritoneal interstitial expansion with collagen accumulation and increased cellularity as indicated by Masson trichrome staining (Figure 1, A–C). These changes were observed 7 days after CG injection and gradually increased over time. By day 14 of CG injury, the animals exhibited advanced fibrosis, with a fibrotic score of 1.2. After 21 days of CG injury, animals had dramatic fibrosis, with a fibrotic score of 1.5 (Figure 1B). Measurement of the thickness of the submesothelial compact zone indicated a time-dependent increase in peritoneal thickness after CG injury. After 35 weeks of CG administration, the thickness of peritoneum was three times greater than on day 7.

CG Administration Induces Phosphorylation/Activation of EGFR and Upregulation of Peritoneal Fibrosis Markers in a Time-Dependent Manner

We also explored CG-induced peritoneal fibrosis by examining expression of collagen-I and α -SMA by immunoblot analysis. Type 1 collagen is the major component of ECM, and α -SMA is the hallmark of activated fibroblasts (myofibroblasts).²² Figure 2, A and C shows that collagen-I was barely detected in the peritoneum of sham-operated rats, but its expression was increased 7 and 14 days after CG injection, was further elevated at 21 days, and then was sustained for at least 35 days. α -SMA expression was also clearly observed in the peritoneum of CG-treated rats at 7 days, gradually increasing between days 14 and 21 and peaking at 35 days. Quantitative analysis (Figure 2B) showed that compared with the controls injected with saline, α -SMA expression levels increased up to 15-fold at day 14 after injection with CG and approximately 25-fold and 35-fold at days 21 and 35, respectively.

To examine whether EGFR would be activated in peritoneal fibrosis, we examined expression of phosphorylated-EGFR (p-EGFR) and total EGFR in the peritoneum. Along with the fibrotic changes, expression levels of p-EGFR increased in a time-dependent manner. After CG injection, EGFR phosphorylation occurred as early as 7 days, gradually increased, and peaked on 35 days, as indicated in Figure 2, A and D. Expression of total EGFR was increased at 14 days and then

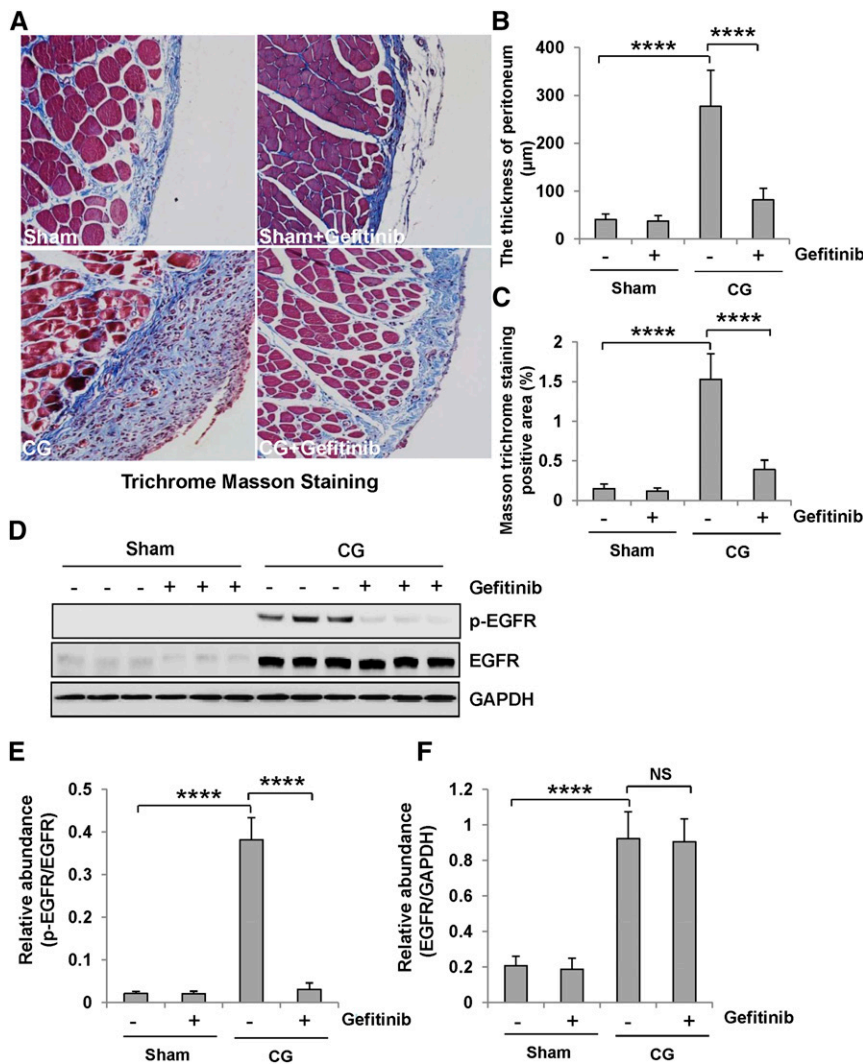


Figure 3. Gefitinib attenuates development of CG-induced peritoneal fibrosis. Peritoneal membrane was collected at 21 days after CG injury with or without administration of gefitinib (100 mg/kg daily). (A) Photomicrographs illustrate Masson trichrome staining of the peritoneum. (B) The graph shows the thickness of the compact zone measured from ten random fields (original magnification, $\times 200$) of six rat peritoneal samples. (C) The graph shows the score of the Masson-positive submesothelial area (blue) from ten random fields (original magnification, $\times 200$) of six rat peritoneal samples. (D) The peritoneal tissue lysates were subjected to immunoblot analysis with specific antibodies against p-EGFR, EGFR, or glyceraldehyde 3-phosphate dehydrogenase. (E) Expression levels of p-EGFR were quantified by densitometry and normalized with EGFR. (F) Expression levels of EGFR were quantified by densitometry and normalized with glyceraldehyde 3-phosphate dehydrogenase. Data are mean \pm SEM ($n=6$). **** $P<0.001$.

gradually increased until the maximum level at 35 days (Figure 2, A and E). Immunostaining showed that p-EGFR is most expressed in the α -SMA-positive cells (Figure 2F). These results illustrated that CG-induced peritoneal fibrosis was accompanied by persistent activation of EGFR and that p-EGFR is preferentially colocalized with cells expressing α -SMA in the peritoneum.

EGFR Inhibition Attenuates Development of Peritoneal Fibrosis in the Peritoneum after CG Injury

Previous studies have shown that sustained EGFR activation is involved in renal vascular and glomerular pathologic changes and interstitial fibrosis.^{23,24} Additionally, EGFR and one of its ligands, HB-EGF, were detected in PD effluent and peritoneal membrane biopsy samples.¹¹ To assess whether EGFR activation contributes to the onset of peritoneal fibrosis, 100 mg/kg gefitinib, a specific EGFR inhibitor, was injected intraperitoneally immediately after CG injection at day 1. Gefitinib was then administered daily for 21 days. As shown in Figure 3, A and B the thickness of the submesothelial zone in CG-injured rats with gefitinib administration was significantly less than that in rats subjected to CG alone. Gefitinib treatment also inhibited the thickening of the submesothelial compact zone and reduced the area of collagen fibrils. Peritoneal tissues of control rats were almost normal, with no thickening of the submesothelial zone. Repeated injections of gefitinib alone did not affect the thickening of the peritoneum compared with the control. Application of gefitinib blocked CG-induced EGFR phosphorylation in the peritoneum, but expression levels of total EGFR were not affected by this agent. No p-EGFR signal was detected in the peritoneum of sham-operated animals treated with DMSO or gefitinib alone (Figure 3D). Densitometry analysis indicated a 90% reduction of p-EGFR in rats treated with gefitinib and CG compared with rats with CG alone (Figure 3E). These data indicated that gefitinib is a potent agent for blocking EGFR activation and preventing peritoneal fibrosis.

EGFR Inhibition Reduces Deposition of ECM and Activation of Fibroblasts in the Peritoneum after CG Injury

To confirm the preceding observations, we further assessed the ability of gefitinib to suppress the deposition of ECM and activation of fibroblasts in the peritoneum by immunoblot analysis. As shown in Figure 4, A–C treatment with gefitinib inhibited CG-induced expression of collagen-I on the basal level and also largely suppressed α -SMA expression. Immunohistochemistry staining showed that gefitinib treatment dramatically reduced collagen 1 expression in the submesothelial compact zone (Figure 4D).

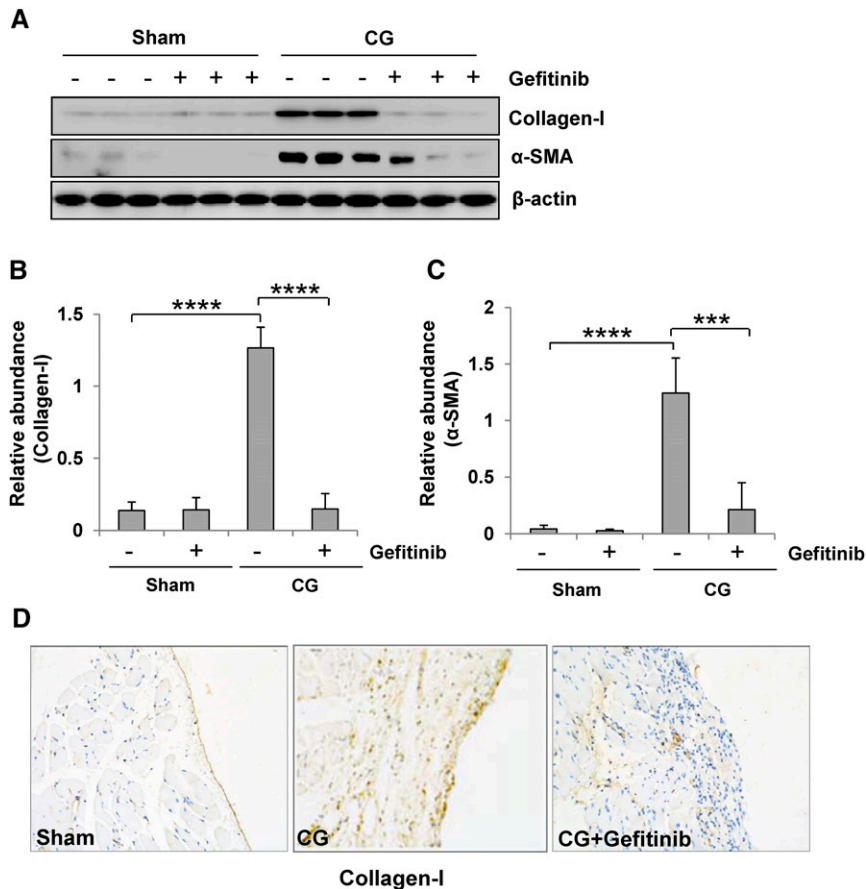


Figure 4. Gefitinib inhibits deposition of ECM and activation of fibroblasts in the injured peritoneum. Peritoneal membrane was collected at 21 days after CG injury with or without administration of gefitinib (100 mg/kg daily). (A) The peritoneal tissue lysates were subjected to immunoblot analysis with specific antibodies against collagen-I, α -SMA, or β -actin. (B) Expression level of collagen-I was quantified by densitometry and normalized with β -actin. (C) Expression level of α -SMA was quantified by densitometry and normalized with β -actin. Data are mean \pm SEM ($n=6$). *** $P<0.001$; **** $P<0.001$. (D) Photomicrographs illustrate immunohistochemical staining of type 1 collagen in the submesothelial compact zone. Original magnification, $\times 200$.

These data, together with the results from Figure 3, further indicate that EGFR activation is critically involved in the development of peritoneal fibrosis induced by CG.

EGFR Inhibition Attenuates Development of Peritoneal Fibrosis, Deposition of ECM, and Activation of Fibroblasts in the Peritoneum after Administration of Dialysis Fluid with High Glucose

To understand whether EGFR activation may also mediate peritoneal fibrogenesis in a more clinically relevant model, we examined the effect of gefitinib on peritoneal fibrosis induced by high glucose in rats. As shown in Figure 5, daily injection of 4.25% PD fluid with 4.25% glucose for 4 weeks induced thickening of the peritoneum and deposition of collagen fibrils as evidenced by Masson trichrome staining. Moreover, simultaneous administration of 100 mg/kg gefitinib inhibited these changes (Figure 5, A–C). Immunoblot analysis also indicated

that gefitinib was effective in suppressing high glucose-induced expression of collagen 1 and α -SMA in the peritoneum (Figure 5, D–H). These results compiled from a different model provide additional evidence for the critical involvement of EGFR in the development of peritoneal fibrosis.

EGFR Inhibition Blocks TGF- β 1 Signaling Pathway in the Peritoneum after CG Injury

Activation of TGF- β 1 signaling is implicated in the development and progression of peritoneal fibrosis.² To explore the mechanism by which EGFR activation contributes to peritoneal fibrosis, we examined the effect of EGFR inhibition on the expression of TGF- β 1 and the activation of Smad3, a key signaling molecule of the TGF- β signaling pathway, in the peritoneum after CG injury. As shown in Figure 6A, the expression level of TGF- β 1 markedly increased after CG injection, while inhibition of EGFR by gefitinib reduced its expression by 50% in the peritoneum. CG treatment also increased the expression of phospho-Smad3 (p-Smad), while gefitinib treatment suppressed its expression (Figure 6, B and C). Costaining of p-Smad3 and α -SMA indicates that p-Smad3 is most expressed in α -SMA-positive cells (Figure 6D). Thus, these results suggest that the antifibrotic effect of EGFR inhibitors is associated with the suppression of the TGF- β /Smad signaling pathway in CG-induced peritoneal fibrosis. Moreover, Smad3 can be activated in peritoneal myofibroblasts.

EGFR Inhibition Abrogates STAT3 and NF- κ B(p65) Phosphorylation in the Peritoneum after CG Injury

Both STAT3 and NF- κ B are transcriptional factors that drive gene expression of proinflammatory cytokines/chemokines,^{15,25} and STAT3 is also involved in the regulation of collagen 1 expression.²⁶ Phosphorylation of STAT3 at Tyr 705 or NF- κ B(p65) at S536 will lead to their translocation from the cytosol to the nucleus, thereby exerting transcriptional activities. Thus, we examined the effect of EGFR inhibition on the phosphorylation and expression of these two molecules. As shown in Figure 7, phosphorylated STAT3 and NF- κ B(p65) were barely detectable in the sham-operated and gefitinib-treated rat peritoneum without CG injection; injection of CG, however, increased their expression. Administration of gefitinib almost completely blocked STAT3 phosphorylation and also significantly inhibited NF- κ B phosphorylation in CG-injured peritoneum (Figure 7, A–C). Although expression

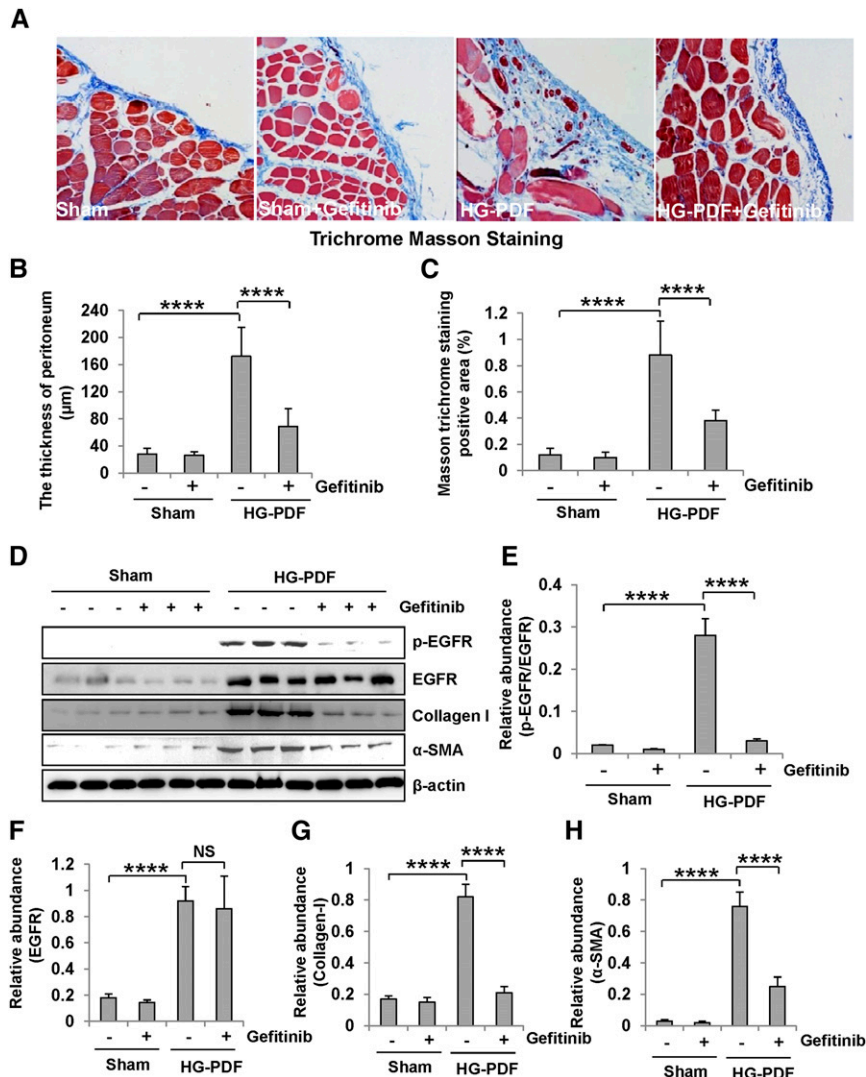


Figure 5. Gefitinib attenuates development of high glucose-induced peritoneal fibrosis. Peritoneal membrane was collected at 28 days after injection of PD fluid containing 4.25% glucose (HG-PDF) with or without daily administration of gefitinib (100 mg/kg). (A) Photomicrographs illustrate Masson trichrome staining of the peritoneum. (B) The graph shows the thickness of the compact zone measured from ten random fields (original magnification, $\times 200$) of five rat peritoneal samples. (C) The graph shows the score of the Masson-positive submesothelial area (blue) from ten random fields (original magnification, $\times 200$) of five rat peritoneal samples. (D) The peritoneal tissue lysates were subjected to immunoblot analysis with specific antibodies against p-EGFR, EGFR, collagen-I, α -SMA, or β -actin. (E) Expression levels of p-EGFR were quantified by densitometry and normalized with EGFR. Expression levels of (F) EGFR, (G) collagen-I, and (H) α -SMA were quantified by densitometry and normalized with β -actin, respectively. Data are mean \pm SEM ($n=6$). **** $P<0.01$.

of total STAT3 and NF- κ B(p65) also increased in the fibrotic peritoneum, gefitinib treatment did not affect their expression (Figure 7, A–C). Immunostaining showed that both phosphorylated STAT3 (p-STAT3) and NF- κ B(p65) were most colocalized with the α -SMA(+) cells in the submesothelial area, and their expression was also seen in the few α -SMA (+) mesothelial cells (Supplemental Figure 7). These data

indicate that EGFR mediates activation of STAT3 and NF- κ B signaling pathways in peritoneal fibroblasts and activated mesothelial cells during peritoneal fibrosis, suggesting that these two signaling pathways may act downstream of EGFR to mediate peritoneal fibrogenesis.

EGFR Inhibition Suppresses Production of Multiple Inflammatory Cytokines and Infiltration of Macrophages in the Peritoneum after CG Injury

Increased expression of proinflammatory cytokines/chemokines and infiltration of macrophages in the thickened submesothelial compact zone were regarded as typical pathologic changes in the fibrotic peritoneum.²⁷ To determine whether EGFR mediates these responses after CG injury, we examined the expression of TNF- α , IL-6, IL1- β , and MCP-1 by ELISA and examined protein level of CD68, a marker of macrophage infiltration, by immunoblot and immunohistochemistry. Figure 8 shows that expression of all these cytokines/chemokines in the peritoneum was significantly elevated after CG injury, while treatment with gefitinib completely suppressed expression of TNF- α and significantly inhibited that of IL-6, IL1- β , and MCP-1.

Moreover, macrophage infiltration detected by immunohistochemistry dramatically increased in the submesothelial layer of rat peritoneum after CG injury but was significantly reduced by gefitinib treatment (Figure 9, A and B). Similar results were also demonstrated by immunoblot analysis of CD68 (Figure 9, C and D). Collectively, these data indicate that EGFR activation is required for the production of multiple cytokines and the accumulation of macrophages in the fibrotic peritoneum.

EGFR Inhibition Attenuates Angiogenesis and Expression of VEGF in the Peritoneum after CG Injury

Long-term PD is frequently associated with angiogenesis in the fibrotic submesothelial zone, and local production of VEGF during PD plays a central role in the processes leading to peritoneal neoangiogenesis and functional decline.²⁸ To understand the role of EGFR in angiogenesis after injury to the peritoneal membrane, we first examined the expression of endothelial cell marker CD31 in the peritoneum. The number of

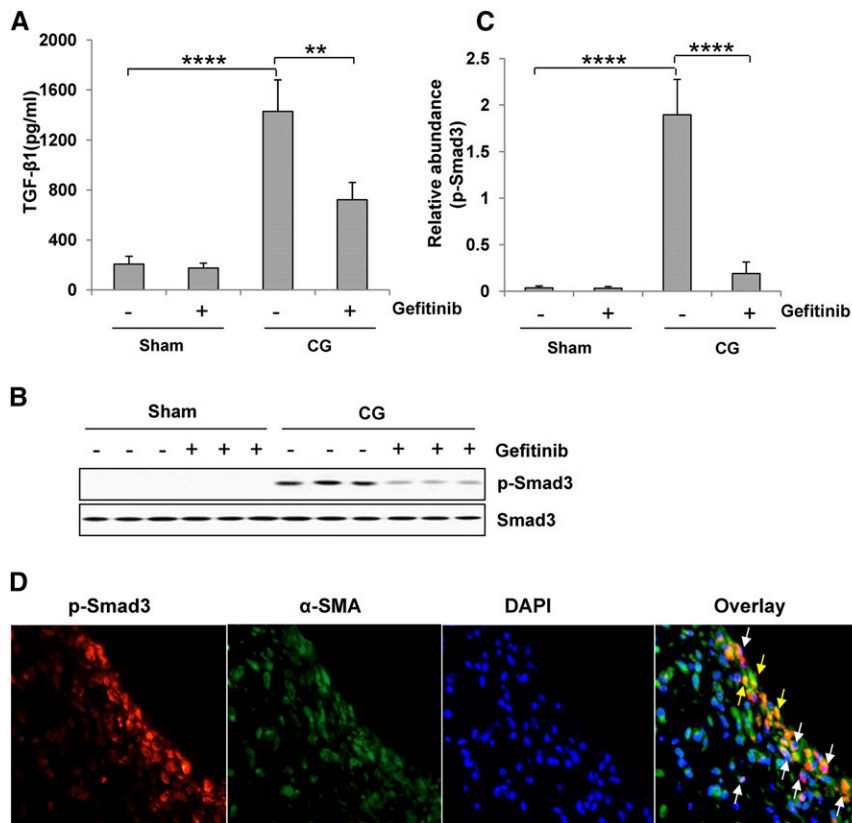


Figure 6. Gefitinib inhibits production of TGF- β 1 and activation of Smad3 in the injured peritoneum. Peritoneal membrane was collected at 21 days after CG injury with or without administration of gefitinib (100 mg/kg daily). (A) Graph shows the expression level of TGF- β 1 by ELISA. Data are mean \pm SEM ($n=6$). $**P<0.01$; $****P<0.001$. (B) Peritoneum tissue lysates were subjected to immunoblot analysis with specific antibodies against p-Smad3 and Smad3. (C) Expression level of p-Smad3 was quantified by densitometry and normalized with Smad3. Data are mean \pm SEM ($n=6$). $**P<0.01$; $****P<0.001$. (D) Double immunofluorescence staining shows the costaining of α -SMA and Smad3 in the injured peritoneum. Yellow arrows and white arrows indicate location of Smad3 in the cytosol and nucleus of myofibroblasts, respectively. DAPI, 4',6-diamidino-2-phenylindole; M, muscle layer.

CD31(+) vessels in the peritoneum markedly increased after CG injection compared with the sham group. Administration of gefitinib significantly suppressed the increase of CD31(+) vessels in the peritoneum induced by CG (Figure 10, A and B).

Next, we examined the effect of EGFR inhibition on the expression of VEGF by immunohistochemical staining. VEGF-positive cells increased markedly in the peritoneal membrane of rats injected with CG, while gefitinib treatment largely reduced this population of cells (Figure 10, A and C). Notably, VEGF-positive cells were located not only in the mesothelial layer but also in the submesothelial zone. This is consistent with previous observations that VEGF is expressed in multiple cell types, including peritoneal mesothelial cells, vascular endothelial cells, and fibroblasts.²⁹ Furthermore, our experiments showed that VEGF is colocalized with α -SMA and CD68, respectively, in mesothelial and submesothelial

(Supplemental Figure 8), confirming expression of VEGF in activated mesothelial cells and macrophages.

Collectively, these data indicate that EGFR blockage by gefitinib can inhibit neoangiogenesis, perhaps through a mechanism involved in the suppression of VEGF expression.

Delayed Administration of Gefitinib Attenuates Progression of Peritoneal Fibrosis after CG Injury

To explore the therapeutic effect of gefitinib on the progression of peritoneal fibrosis, we treated animals with gefitinib beginning 21 days after CG injury (Figure 11A), when peritoneal fibrosis had already developed to an advanced stage, as indicated in Figures 1 and 2. After an additional 14 days of treatment with gefitinib, rats were euthanized, and peritoneum was removed to assess the deposition of collagen fibrils by Masson trichrome staining and expression of type 1 collagen by immunoblot analysis (Figures 11, A–D, and 12, A–C). The thickness of the submesothelial compact zone and Masson trichrome–positive areas increased within 21 days and was further elevated at 35 days after CG injury. In contrast, these changes of peritoneum in animals treated with gefitinib starting at 21 days were not further increased at 35 days after CG injury (Figure 11, B–D). Consistently, immunoblot and immunohistochemical analysis showed that administration of gefitinib at 21 days after CG injury prevented further expression of type 1 collagen (Figure 12, A–C). Gefitinib treatment also significantly reduced α -SMA expression (Figure 12, B

and D). Treatment with gefitinib beginning at 21 days after CG injection prevented further increase of EGFR phosphorylation at 35 days, but the expression of total EGFR was not affected by gefitinib. Thus, these data suggest that delayed administration of gefitinib can block the progression of peritoneal fibrosis.

Delayed Administration of Gefitinib Inhibits Activation of the TGF- β /Smad, STAT3, and NF- κ B Signaling Pathways in the Peritoneum after CG Injury

To explore the mechanisms of delayed gefitinib treatment in blocking peritoneal fibrosis progression, we further examined the role of EGFR inhibition in regulation of TGF- β /Smad, STAT3, and NF- κ B signaling pathways. As shown in Supplemental Figures 1 and 2, expression of TGF- β and phosphorylation of Smad, STAT3, and NF- κ B increased in the

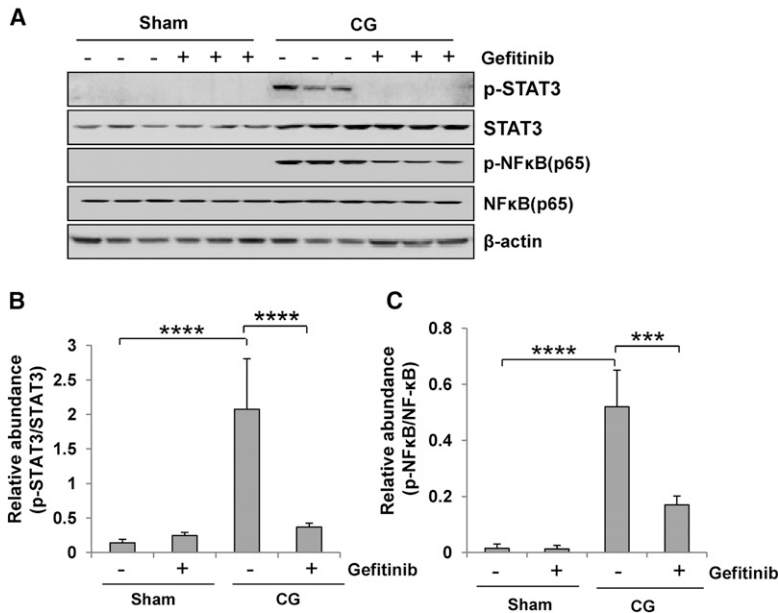


Figure 7. Gefitinib inhibits STAT3 and NF-κB(p65) phosphorylation in the injured peritoneum. Peritoneal membrane was collected at 21 days after CG injury with or without administration of gefitinib (100 mg/kg daily). (A) Peritoneum tissue lysates were subjected to immunoblot analysis with specific antibodies against p-STAT3, STAT3, phosphorylated NFκB(p65) (p-NF-κB(p65)), NF-κB(p65), or β-actin. (B) Expression levels of p-STAT3 were quantified by densitometry and normalized with STAT3. (C) Expression level of p-NFκB(p65) was quantified by densitometry and normalized with NFκB(p65). Data are mean±SEM (n=6). ***P<0.001; ****P<0.001. DAPI, 4',6-diamidino-2-phenylindole; S, muscle layer.

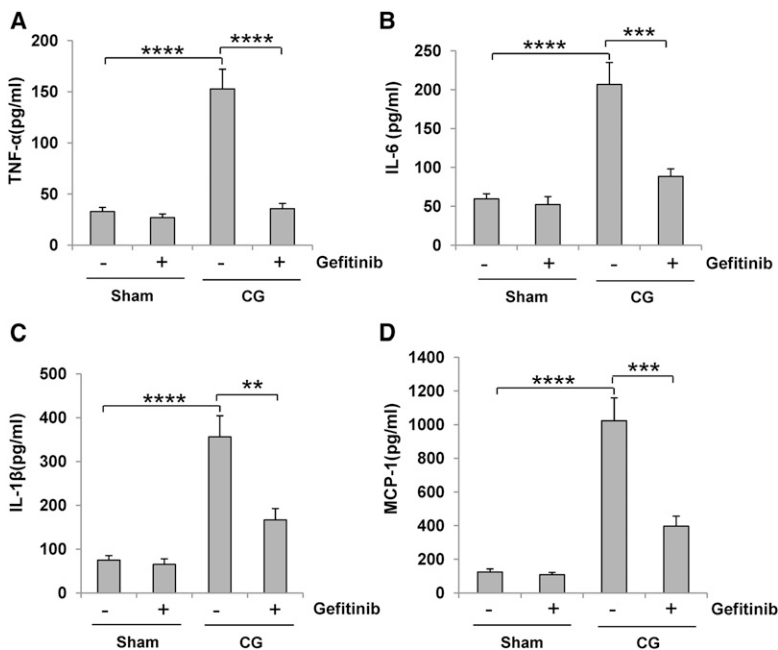


Figure 8. Gefitinib suppresses production of multiple proinflammatory cytokines and chemokines in the injured peritoneum. Peritoneal membrane was collected at 21 days after CG injury with or without administration of gefitinib (100 mg/kg daily). Graphs show the expression level of (A) TNF-α, (B) IL-6, (C) IL-1β, and (D) MCP-1 by ELISA. Data are mean±SEM (n=6). **P<0.01; ***P<0.001; ****P<0.001.

peritoneum 21 days after CG injection and were further elevated at 35 days after CG injury. Treatment with gefitinib starting at 21 days after CG injection blocked further expression of TGF-β and phosphorylation of all three signaling molecules. These data suggest that EGFR activation also mediates CG-induced expression of TGF-β and activation of the Smad, STAT3, and NF-κB signaling pathways in the late phase of CG-induced peritoneal injury.

Delayed Administration of Gefitinib Suppresses CG-Induced Production of Proinflammatory Cytokines, Infiltration of Macrophages, and Expression of VEGF in the Peritoneum

We also analyzed the effect of delayed administration of gefitinib on the expression of TNF-α, IL-6, IL-1β, MCP-1, CD68, CD31, and VEGF. As shown in Supplemental Figures 3–5, when gefitinib was administered at 21 days after CG injection, CG-induced expression of these molecules was not further increased at 35 days. Thus, these data indicate that gefitinib is also able to prevent continuous expression of these cytokine/growth factors, accumulation of macrophages, and induction of angiogenesis in the peritoneum during the late phase of CG-induced peritoneal injury and fibrosis.

EGFR Mediates TGF-β-Induced Epithelial-to-Mesenchymal Transition In Vitro

It has been reported that the partial epithelial-to-mesenchymal transition (EMT) of mesothelial cells is involved in peritoneal fibrosis and that TGF-β1 is a potent cytokine that stimulates EMT and induces peritoneal fibrosis.² Thus, we further examined whether EGFR would play an important role in TGF-β1-stimulated EMT in HPMCs *in vitro*. Exposure of HPMCs to TGF-β1 induced expression of α-SMA and fibronectin, two hallmarks of EMT, as well as phosphorylation of EGFR and Smad3. Treatment with gefitinib blocked all these responses (Supplemental Figure 6). These data support our *in vivo* observations that EGFR is a critical mediator in the regulation of fibrotic responses and activation of the TGF-β signaling pathway.

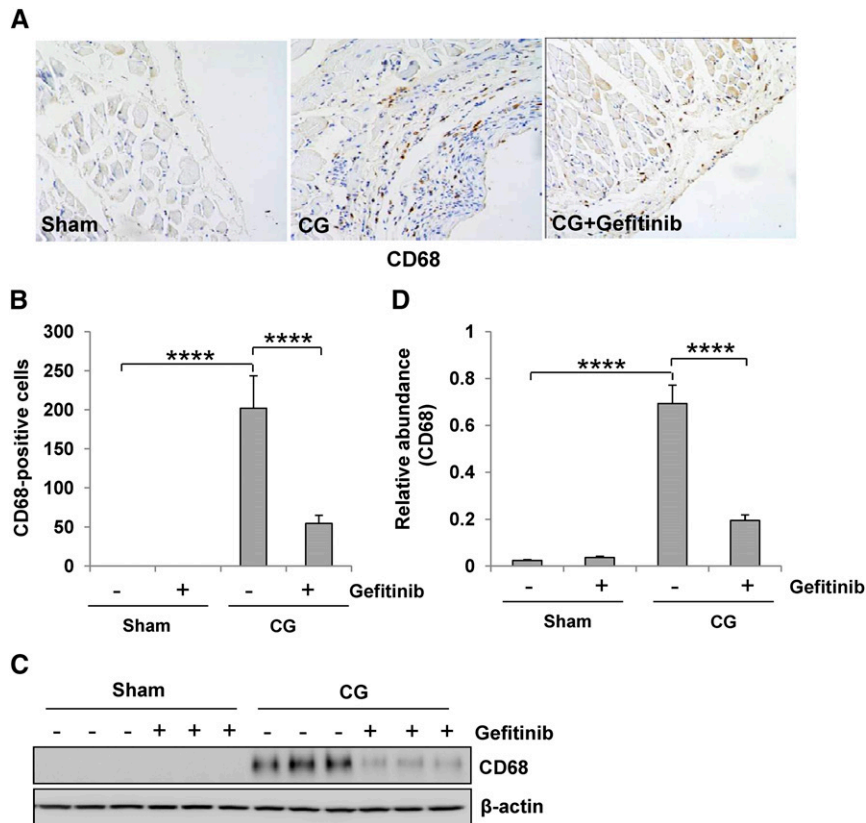


Figure 9. Gefitinib inhibits peritoneal macrophage infiltration in the injured peritoneum. Peritoneal membrane was collected at 21 days after CG injury with or without administration of gefitinib (100 mg/kg daily). (A) Photomicrographs illustrate immunohistochemical staining of CD68 in the submesothelial compact zone. (B) The number of CD68-positive cells was calculated from ten random fields of six rat peritoneal samples. (C) Peritoneum tissue lysates were subjected to immunoblot analysis with specific antibodies against CD68 or β -actin (original magnification, $\times 200$). (D) Expression levels of CD68 were quantified by densitometry and normalized with β -actin. Data are mean \pm SEM ($n=6$). **** $P<0.001$.

DISCUSSION

Exposing the peritoneum to PD fluids induces structural and functional deterioration of the peritoneal membrane, leading to fibrosis. Thus, one of the most important challenges in PD is to preserve the integrity of the peritoneal membrane and prevent the development and progression of peritoneal fibrosis. The antifibrotic effects of EGFR inhibition have been reported in several experimental models of chronic diseases, including renal fibrosis.^{13,17–20} However, the potential of EGFR inhibitors to act as therapeutic agents to ameliorate peritoneal membrane damage has not been studied yet. In this study, we showed that early or late administration of gefitinib, an EGFR inhibitor, reduced deposition of ECM with inactivation of myofibroblasts, suppression of inflammation responses, and attenuation of angiogenesis in the injured peritoneum. To our knowledge, this is the first study to demonstrate the beneficial effect of EGFR inhibition on peritoneal fibrosis and suggest EGFR as a potential target for the treatment of patients undergoing PD who have developed this complication.

CG-induced peritoneal fibrosis is a common animal model used for studying chronic peritoneal changes. In this model, persistent chemical irritation by CG induces tissue damage with inflammation, followed by excessive proliferation of myofibroblasts,³⁰ accumulation of interstitial collagens, and increased vascular density in the peritoneum. All of these changes are key pathologic features of human peritoneal sclerosis. Therefore, we agree with a statement made by Yoshio *et al.* that the CG model is an ideal model for examining the efficacy of potential therapeutic reagents for treating peritoneal fibrosis. In this model, we also observed that CG injury induces a time-dependent increase in the expression and activation of EGFR in the peritoneum, which is consistent with the time course of ECM accumulation and fibroblast activation. Immunostaining showed that active (phospho)-EGFR was most localized in α -SMA(+) cells in the injured peritoneum, suggesting that EGFR is activated in peritoneal myofibroblasts. In addition, we found that p-EGFR is elevated in the peritoneum of high glucose-induced peritoneal fibrosis in rats.

Currently, the underlying mechanism by which EGFR is activated during peritoneal fibrosis is unclear, but it may be associated with persistent stimulation by EGFR ligands. It is evident that HB-EGF, an EGFR ligand, is produced in the peritoneal cavity by peritoneal macrophages and mesothelial cells in PD recipients.¹¹ EGF also stimulates the conversion of peritoneal mesothelial cells to a fibroblastic phenotype.¹⁰ Thus, these and other EGFR ligands may accumulate in the peritoneum to stimulate EGFR activation. Peritoneal EGFR may also be activated by stimuli that do not directly act on the EGFR through a mechanism called transactivation. EGFR transactivation can be induced by many non-EGFR ligands, such as G protein-coupled receptor agonists, cytokines, and other stimuli (*i.e.*, high glucose, reactive oxygen species).^{31–33} During this process, the signal initiated by diverse stimuli can induce activation of some intracellular kinases, such as protein kinase C and Src, subsequently activating proteases, and disintegrin and metalloprotease family members. These proteases then cleave EGFR ligands, releasing their soluble forms, which bind to and activate EGFR.^{34,35} Because all the aforementioned stimuli for EGFR transactivation are also able to induce peritoneal damage and fibrosis, it is possible that EGFR plays an essential role in transmitting these stimuli to the deterioration of plasma membrane. Further studies are needed to address cellular and molecular basis for EGFR activation in the injured peritoneum.

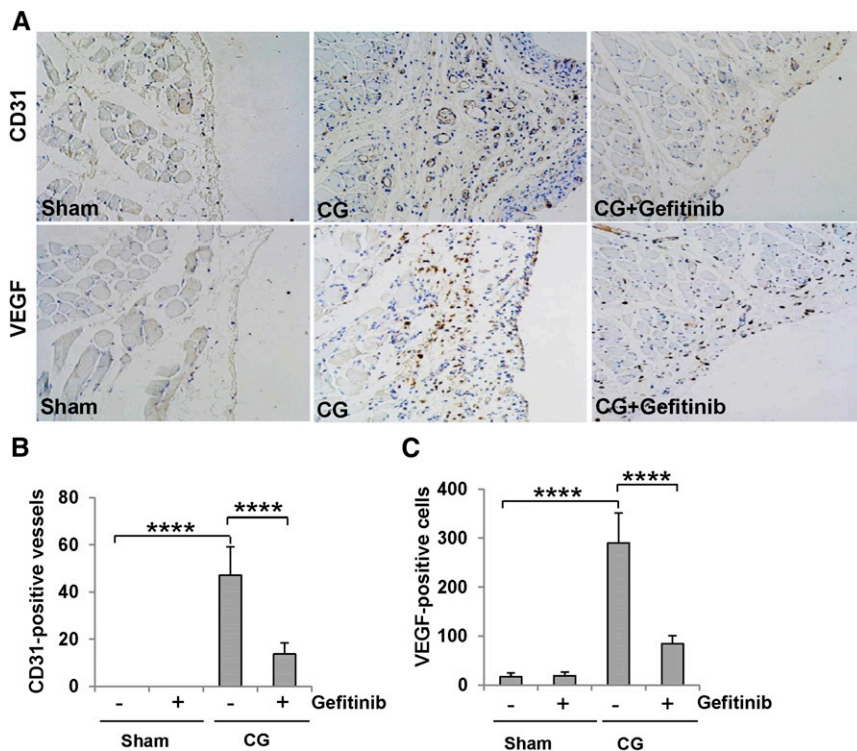


Figure 10. Gefitinib inhibits CD31 and VEGF expression in the injured peritoneum. Peritoneal membrane was collected at 21 days after CG injury with or without administration of gefitinib (100 mg/kg daily). (A) Photomicrographs illustrate immunohistochemical staining of CD31 or VEGF in the submesothelial compact zone. The number of (B) CD31-positive vessels or (C) VEGF-positive cells was calculated from ten random fields (original magnification, $\times 200$) of six rat peritoneal samples. Data are mean \pm SEM ($n=6$). **** $P<0.001$.

The mechanisms behind antifibrotic actions of EGFR inhibition are multifaceted in the peritoneum. Because TGF- β is widely implicated in the pathogenesis of peritoneal fibrosis,^{36,37} we first examined the effect of gefitinib on the activation of this signaling pathway and found that CG-injured fibrotic peritoneum is accompanied by overproduction of TGF- β 1 and phosphorylation of Smad3, a key mediator of TGF- β signaling. Administration of gefitinib inhibited CG-induced TGF- β 1 production and Smad3 phosphorylation, and exposure of HPMCs to gefitinib also suppressed TGF- β 1-induced Smad 3 phosphorylation and EMT. Given that TGF- β 1 contributes to peritoneal injury through a Smad-dependent signaling pathway,^{38–40} blockade of TGF- β 1 signaling may be a prominent mechanism for EGFR inhibition-mediated peritoneal protection. In addition, we also found that treatment with gefitinib inhibited phosphorylation of STAT3, an important transcriptional regulator associated with driving the expression of ECM and/or cytokines or chemokines. Previously, we demonstrated that STAT3 is a critical mediator of renal fibroblast activation and renal fibrogenesis.¹⁶ A recent study showed that blocking JAK2, the direct upstream activator of STAT3, attenuated peritoneal membrane inflammation, mesothelial cell injury, fibrosis, and hypervascularity induced by bioincompatible PD fluids. Thus,

JAK2/STAT3 signaling could be another important signaling pathway in linking EGFR activation to peritoneal fibrosis.

EGFR inhibition may also antagonize peritoneal fibrogenesis through suppression of inflammation. Inflammation is considered one of the main pathologic processes contributing to peritoneal fibrosis during long-term PD.^{4,7} In this study, the expression of multiple proinflammatory factors (TNF- α , MCP-1, IL- β 1, and IL-6) increased in the peritoneum of CG-injured rats. Macrophages also accumulated in the submesothelial compact zone after injury. EGFR blocking inhibited expression of all those cytokines or chemokines and infiltration of macrophages with attenuation of peritoneal fibrosis. Because inflammation is an early response of the peritoneum to injury, reduction of inflammation by gefitinib may result in the attenuation of peritoneal fibrosis. EGFR inhibition-elicited anti-inflammatory response may be through the suppression of NF- κ B and STAT3 signaling. This is supported by our observation that inhibition of EGFR with gefitinib inhibited the phosphorylation of NF- κ B(p65) and STAT3 during CG-induced peritoneal fibrosis. It is well known that both NF- κ B and STAT3 are critical transcriptional factors that mediate expression or production of multiple inflammatory cytokines and chemokines under various pathologic conditions.⁴¹

Angiogenesis is an important promoter of peritoneal fibrosis and ultrafiltration failure.^{9,42} A correlation between peritoneal vascular density and peritoneal fibrosis was observed in patients undergoing long-term PD.⁴³ Animal studies have also indicated that inhibition of VEGF, a key angiogenic growth factor, by the use of neutralizing anti-VEGF monoclonal antibody, TNP-470, or thalidomide, can significantly ameliorate fibrotic changes with the reduction of VEGF expression.^{29,44,45} These investigations offer compelling evidence to support the substantial connection between peritoneal fibrosis and angiogenesis. In support of this notion, we demonstrated that CD31-positive cells increased in the thickened submesothelial area of CG-treated mice and that gefitinib treatment significantly reduced this population of cells. In addition, application of gefitinib also decreased the expression of VEGF in the injured peritoneum. Costaining of VEGF with α -SMA(+) or CD68 in injured peritoneum suggests that this growth factor is generated by multiple cell types, including myofibroblasts and macrophages. Therefore, antiangiogenesis may also be an important mechanism for EGFR inhibitors to exhibit their antifibrotic effects in the peritoneum.

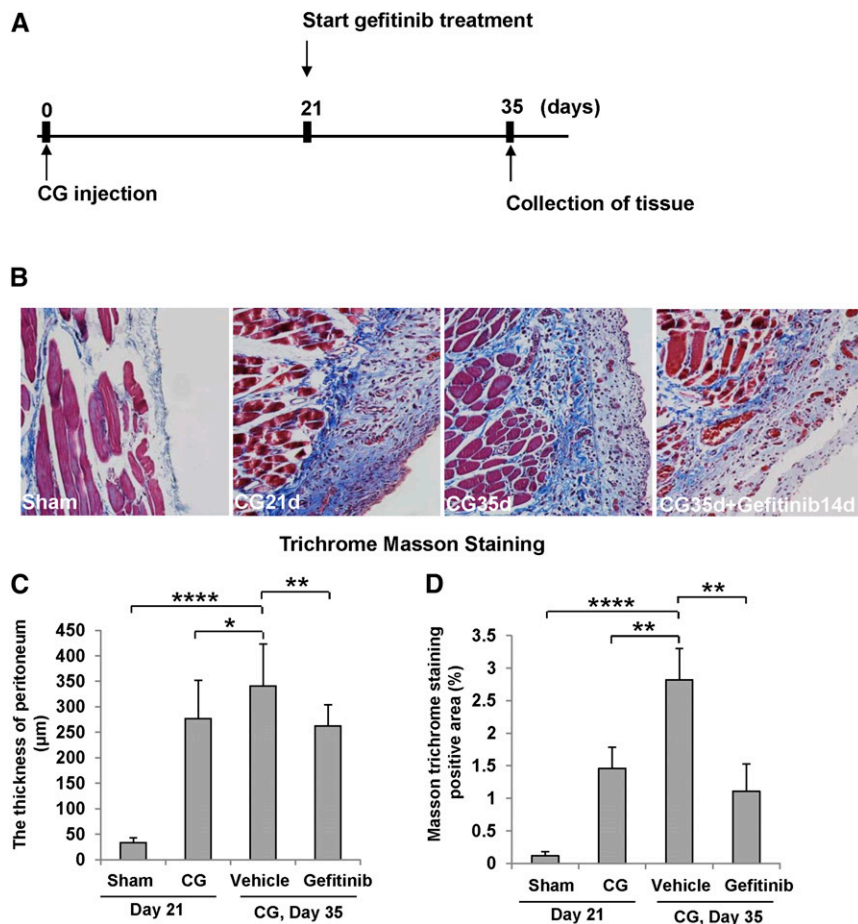


Figure 11. Delayed administration of gefitinib halts progression of peritoneal fibrosis. (A) Schematic of the experimental design for late treatment with gefitinib. Peritoneal membrane was collected at 35 days after CG injury with or without administration of gefitinib (100 mg/kg daily). (B) Photomicrographs illustrate Masson trichrome staining of peritoneum tissue after various treatments (original magnification, $\times 200$). (C) Thickness of the compact zone measured from ten random fields (original magnification, $\times 200$) of six rat peritoneal samples. (D) The graph shows the score of Masson-positive submesothelial area (blue) from ten random fields (original magnification, $\times 200$) of six rat peritoneal samples. Data are mean \pm SEM ($n=6$). * $P<0.05$; ** $P<0.01$; **** $P<0.001$.

To date, there is no established treatment for peritoneal fibrosis. Many therapeutic interventions have been investigated with the aim of preventing some of the major pathologic processes, such as the fibroblast activation, inflammation, and angiogenesis, involved in peritoneal fibrosis.^{46,47} Our studies indicate that inhibition of EGFR with gefitinib can suppress all those processes and attenuate both the generation and progression of peritoneal fibrosis. These results were consistent with the results of previous studies designed to examine the renoprotective effects of EGFR inhibition in renal fibrosis, and they suggest that the inhibition of EGFR may help protect against peritoneal fibrosis. Given that the EGFR inhibitor erlotinib has been approved by the US Food and Drug Administration to treat tumors, our results here may have important clinical translational implications for peritoneal fibrosis.

In summary, we demonstrated that the blockage of EGFR can prevent both the generation and progression of peritoneal fibrosis. These beneficial effects may be through inhibition of multiple profibrotic signaling pathways, inflammatory responses, and angiogenesis. Thus, targeting EGFR may be an effective approach to preserve peritoneal membrane ultrafiltration capacity. Further studies are needed to assess the efficacy of EGFR inhibitors in patients with peritoneal fibrosis, through monotherapy or combination therapy with more biocompatible fluids that better preserve the mesothelial cell monolayer.

CONCISE METHODS

Reagents and Antibodies

Antibodies to phosphorylated NF- κ B(p65), NF- κ B (p65), p-STAT3, STAT3, p-Smad3, Smad3, and p-EGFR were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to fibronectin, collagen 1(A2), glyceraldehyde 3-phosphate dehydrogenase, EGFR, CD31, and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to CD68 was purchased from Boster (Wuhan, China). Antibody to β -actin was purchased from TransGen Biotech (Beijing, China). TNF- α , IL-1 β , MCP-1, IL-6, and TGF- β 1 ELISA kits were from R&D Systems (Minneapolis, MN). Gefitinib was purchased from LC Laboratories (Woburn, MA). α -SMA, CG, and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Establishment of Rat Peritoneal Fibrosis Models and Gefitinib Administration

The peritoneal fibrosis model was established in male Sprague-Dawley rats that weighed 200 ± 10 g (Shanghai Super-B&K Laboratory Animal Corp. Ltd.), as described in our previous study.⁴⁸ Briefly, peritoneal fibrosis in rats was generated by daily intraperitoneal injection of 0.1% CG. Control rats were injected with an equal volume of 0.9% saline. To explore the preventive effect of EGFR inhibition on peritoneal fibrosis, gefitinib was administered intraperitoneally at a dose of 100 mg/kg in 50 μ l DMSO at the first day of CG injection and then given daily. Animals were divided into four groups ($n=5-6$): the sham group with DMSO or gefitinib and the peritoneal fibrosis group induced by 0.1% CG with DMSO or gefitinib. At 21 days, rats were euthanized and the parietal peritoneum apart from the injection points was harvested for further analysis. To investigate the therapeutic effect of gefitinib on established peritoneal fibrosis, gefitinib was administered starting at 21 days after injection with CG and then

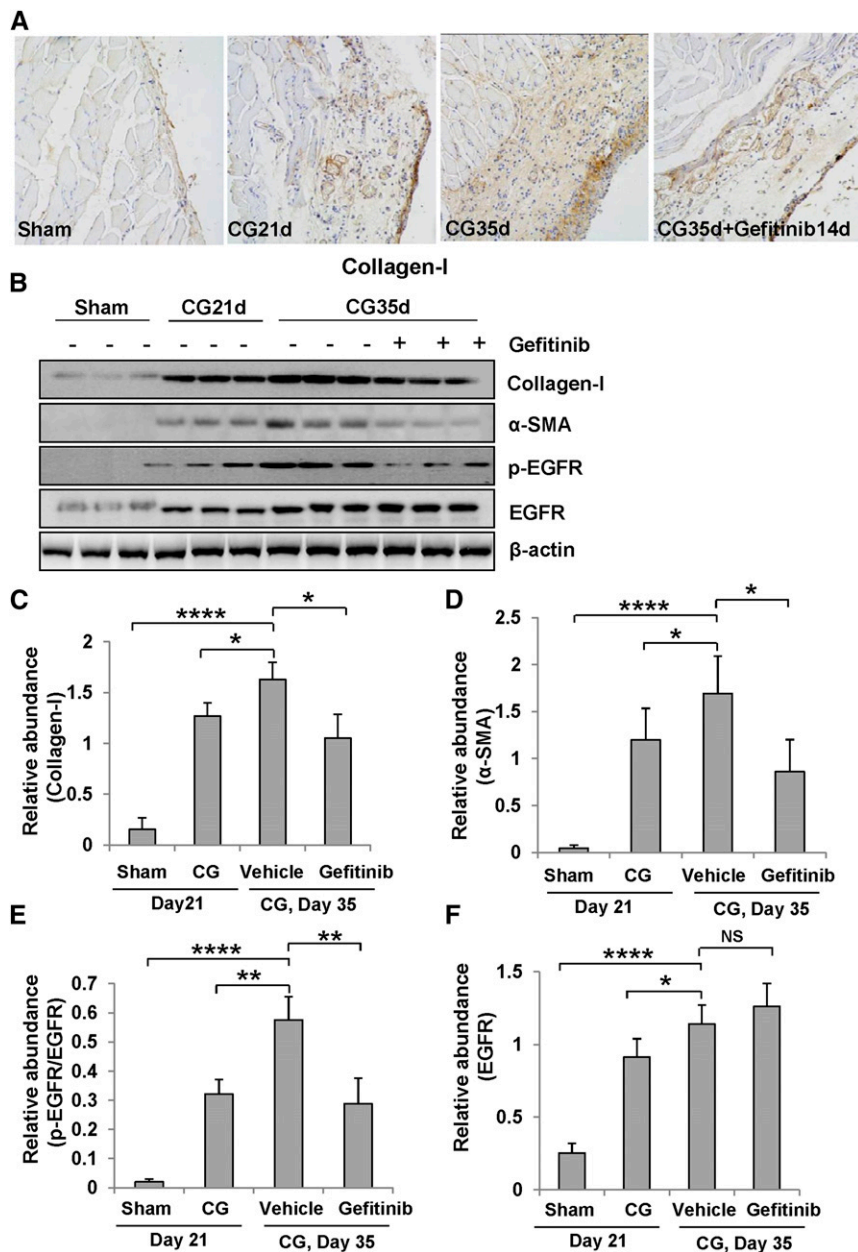


Figure 12. Delayed administration of gefitinib inhibits ECM protein deposition and fibroblast activation. Experimental design was the same as indicated in Figure 11A. (A) Photomicrographs illustrate the immunohistochemical staining of collagen-I in the submesothelial compact zone after various treatments (original magnification, $\times 200$). (B) Peritoneum lysates were prepared and subject to immunoblot analysis with antibodies to type 1 collagen, α -SMA, p-EGFR, EGFR, or β -actin. (C and D) Expression levels of α -SMA and collagen-I were separately quantified by densitometry and normalized with β -actin. (E) Expression levels of p-EGFR were quantified by densitometry and normalized with EGFR. (F) Expression level of EGFR was quantified by densitometry and normalized with β -actin. Data are mean \pm SEM ($n=6$). * $P<0.05$; ** $P<0.01$; **** $P<0.001$.

given daily for 14 days. At 35 days, animals were euthanized for collection of peritoneum (see Figure 11A). To establish the rat model of high glucose, rats were intraperitoneally injected daily with 100 ml/kg PD fluid containing 4.25% glucose (Baxter). Control rats were injected with an equal volume of 0.9% saline. Animals were divided into four groups

($n=6$) as described above and the same dose of gefitinib was used. At 28 days after various treatments, rats were euthanized for collection of peritoneum. All the experiments were conducted in accordance with the animal experimentation guideline of Tongji University School of Medicine.

Cell Culture and Treatments

HPMCs were cultured in DMEM (Sigma-Aldrich) containing 10% FBS, 1% penicillin, and streptomycin in an atmosphere of 5% CO₂ and 95% air at 37°C. To determine the effect of EGFR inhibition on HPMCs in response to TGF- β 1, cells were starved with 0.5% FBS for 24 hours and then exposed to TGF- β 1 (10 ng/ml) for 36 hours in the absence or presence of gefitinib (20 nM) before being harvested for protein analysis.

Immunoblot Analysis

Immunoblot analysis of peritoneum tissue samples was conducted as described previously.⁴⁸ The densitometry analysis of immunoblot results was conducted using ImageJ software (National Institutes of Health, Bethesda, MD).

Morphologic Studies of Peritoneum

Formalin-fixed peritoneum were embedded in paraffin and prepared in 3- μ m-thick sections. For evaluation of peritoneal fibrosis, Masson trichrome staining was performed according to the protocol provided by the manufacturer (Sigma-Aldrich). The collagen tissue area (blue) was quantitatively measured using Image Pro-Plus software (Media-Cybernetics, Silver Spring, MD) by drawing a line around the perimeter of the positive staining area. The average ratio to each microscopic field (original magnification, $\times 200$) was calculated and graphed. The thickness of the submesothelial tissue was evaluated (in micrometers), and the average of ten independent measurements was calculated for each section (original magnification, $\times 200$).

Immunohistochemical Staining

Immunohistochemical staining was conducted on the basis of the procedure described in our previous studies.⁴⁸ For quantitative assessment, the positive staining area was measured by Image Pro-Plus software, and the average ratio to each microscopic field (original magnification, $\times 200$) was calculated and graphed. Immunofluorescent staining was carried out according to the procedure described in our previous studies.¹⁹ Images were taken using a Zeiss 710 Duo microscope.

ELISA Detection

ELISA detection of TGF- β 1, MCP-1, IL-1 β , TNF- α , and IL-6 protein was performed in accordance with the manufacturer's instructions.

Statistical Analyses

Samples from five to six animals were used for the experiments in each group as indicated in figure legends. Immunoblots and tissue histologic images are representative of at least three experiments from different animals. Data depicted in graphs represent the mean \pm SEM for each group. For all the experiments, the differences between two groups were made using one-way ANOVA followed by the Tukey test. Statistically significant difference between mean values was marked in each graph. $P > 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.001$ were considered NS, significant, very significant, highly significant, and extremel significant, respectively. The statistical analyses were conducted by using IBM SPSS Statistics 20.0 (Beijing, China).

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An ethics statement was not required for this work.

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DISCLOSURES

None.

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