The Epidermal Growth Factor Receptor Promotes Glomerular Injury and Renal Failure in Rapidly Progressive Crescentic Glomerulonephritis; the Identification of Possible Therapy

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Contribution made by the co-authors M.F. G.B. and PL.T. conceived the project and experiments. PL.T and N.E. supervised the project. S.S., C.F., M.M., S.V. and E.S. developed methods to culture and analyze primary podocytes and conceived experiments for gene expression analysis. E.R. and M.M. performed electron microscopy studies. S.W.S., S.E.Q., J.B.K., D.W.T., I.C. and C.B. helped generate mice with targeted deficiency of Hbegf, TGF-alpha, Epirerulin, Egfr. A.G. and S.G. performed in situ hybridization studies. D.S. and L.M. provided nephrototoxic serum and discussed data with PL.T. K.E. C.B. and JC.D. also discussed experiments with PL.T. and N.E. P.L.T., G.B. and M.M. conducted all in vivo studies. M.F., A.R and P.C. analyzed human kidney biopsies collected by X.B. G.B. and M.F. contributed equally to the study.

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Abstract

Rapidly progressive glomerulonephritis (RPGN) is a clinical a morphological expression of severe glomerular injury. Glomerular injury manifests as a proliferative histological pattern (“crescents”) with accumulation of T cells and macrophages, and proliferation of intrinsic glomerular cells. We show de novo induction of heparin-binding epidermal growth factor-like growth factor (HB-EGF) in intrinsic glomerular epithelial cells (podocytes) from both mice and humans with RPGN. HB-EGF induction increases phosphorylation of the EGFR/ErbB1 receptor in mice with RPGN. In HB-EGF-deficient mice, EGFR activation in glomeruli is absent and the course of RPGN is improved. Autocrine HB-EGF induces a phenotypic switch in podocytes in vitro. Conditional deletion of the Egfr gene from podocytes of mice alleviates the severity of RPGN. Pharmacological blockade of EGFR also improves the course of RPGN, even when started 4 days after the induction of experimental RPGN. This suggests that targeting the HB-EGF/EGFR pathway could also be beneficial for treatment of human RPGN.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the epidermal growth factor (EGF) family, is expressed during inflammatory and pathological conditions. Transient expression of HB-EGF has been reported in mesangial and epithelial renal cells in an anti-GBM serum-induced rat model of RPGN. However, the evoked pathophysiological effects of HB-EGF and the EGF receptor (EGFR) in this experimental RPGN have been restricted to modulation of vasomotor tone and acute transient regulation...
of glomerular filtration rate, and have not been reported to lead to the major clinical and morphological endpoints. Yet HB-EGF has been shown to be upregulated in vascular endothelial cells by cytokines (IL-1β, TNF-1α) and lysophosphatidylcholine, mediators that may be elicited in RPGN. In addition, HB-EGF has been detected in conditioned medium of macrophages and macrophage-like U-937 cells, and in CD4+ T cells within atherosclerotic plaques. Surprisingly, no role for HB-EGF or its tyrosine kinase receptor (EGFR) has been reported in inflammatory diseases. Studies of crescentic forms of human and experimental GN suggest that T cells and macrophages have important effector roles in promoting the formation of destructive cellular crescents. Therefore, we investigated whether HB-EGF is induced in an anti-GBM-serum-induced mouse model of RPGN and in human renal biopsies with RPGN. We also addressed whether lack of HB-EGF, loss of EGFR or EGFR tyrosine kinase inhibition could influence the course of fatal RPGN in mice.

We found that renal expression of HB-EGF was markedly up-regulated after the onset of crescentic glomerulonephritis in parallel with sustained phosphorylation of the EGFR in podocytes. HB-EGF-deficient mice did not exhibit activation of the EGFR in glomeruli, and were markedly protected from RPGN compared to their wild-type littermates. This phenotype was recapitulated by selective deletion of the Egfr gene in podocytes. Pretreatment of Hbegf (+/+) animals with two different EGFR tyrosine kinase inhibitors suppressed albuminuria and glomerular injury and prevented renal failure. Moreover, delayed EGFR inhibition with a clinically available EGFR inhibitor, even after the onset of acute renal failure, effectively reduced renal damage and renal failure. We also identified de novo production of HB-EGF protein in kidneys from humans with RPGN from various etiologies. These data demonstrate a prominent pathophysiological role for EGFR in crescentic RPGN and suggest that inhibitors of the HBEGF/EGFR cascade may be useful for preventing severe renal damage and renal failure.

RESULTS

Activation of the proHB-EGF gene during crescentic glomerulonephritis

We tested for proHB-EGF mRNA by real-time RT-PCR in kidneys harvested 8 days after injection of nephrotoxic serum (NTS) into mice: proHB-EGF mRNA was three times more abundant in treated than control animals (proHB-EGF cDNA / 18S cDNA ratio 10.8 ± 1.7 vs. 3.5 ± 1.4, respectively, n=6, P<0.05) (not shown). Four days after NTS injection and before the first appearance of crescents, proHB-EGF mRNA was detected in parietal epithelial glomerular cells and podocytes. On day 8, crescents were observed and in situ hybridisation showed diffuse proHB-EGF mRNA labelling (Fig. 1a). In freshly isolated podocytes proHB-EGF mRNA was increased 3.6± 0.4-fold 6 days after NTS injection as compared to untreated controls (n=3 per group, P<0.05) (Fig. 1b).

Activation of EGFR tyrosine kinase during crescentic glomerulonephritis

HB-EGF binds to and activates both EGFR/ErbB1 and ErbB4 receptors. We studied the phosphorylation of EGFR by immunoblot analysis of phospho-EGFR (pEGFR) and total EGFR with lysates extracted from renal cortex 8 days after the beginning of experimental crescentic glomerulonephritis (Fig. 1c). Phosphorylation of EGFR was increased by 2.6-fold...
in wild type (Hbegf (+/+)) tissues after NTS than in non-immunized Hbegf (+/+ or Hbegf (-/-) mice (CT) (n=6, P<0.01 vs. CT). Anatomic visualization of EGFR phosphorylation by immunofluorescence demonstrated strong receptor activation in glomeruli with intense pEGFR-specific staining in podocytes (Fig. 1d), far stronger than signals emanating from tubules. We used western blotting and immunofluorescence to demonstrate the specificity of EGFR phosphorylation in vivo in immunized mice which were injected daily with an EGFR tyrosine kinase inhibitor (AG1478) or vehicle alone. On day 8 post injection of NTS, EGFR phosphorylation remained low in the renal cortex of AG1478-treated Hbegf (+/+)) animals (P<0.01 vs. Hbegf (+/+)) (Fig. 1c,d). Similarly, Hbegf (-/-) mice showed no increase in EGFR phosphorylation in the renal cortex on day 8 post NTS (P<0.01 vs. Hbegf (+/+)) (Fig. 1c,d). Altogether, these data demonstrate a prevailing and specific role for HB-EGF in EGFR phosphorylation in wild-type mice injected with NTS.

**HB-EGF induces a migratory phenotype in podocytes in vitro**

_in vivo_ podocytes are terminally differentiated and stationary cells. During crescent formation in mice, however, podocytes assume a migratory phenotype, attach with their apical membrane onto the parietal basement membrane and start to proliferate\textsuperscript{12-14}. Recent data confirm that podocytes contribute to crescents also in humans\textsuperscript{15,16}. As an assay for podocyte crescent formation, we measured outgrowth of WT-1 positive cells from isolated decapsulated mouse glomeruli. The area of podocyte outgrowth strongly depended on the presence of a functional Hbegf allele (Fig. 2a,b). Of note, there was no gene-dose effect as similar results were obtained with Hbegf (+/-) and Hbegf (+/+ cells (not shown). Treatment with AG1478 suppressed podocyte outgrowth in mice carrying one or two functional Hbegf alleles, and addition of HB-EGF to the medium rescued podocyte outgrowth from Hbegf (-/-) glomeruli (Fig. 2a,b). Similar to the steps that characterize crescent formation, glomerular outgrowth of podocytes from decapsulated glomeruli involves formation of apical protrusions in podocytes, attachment and migration away from glomerular capillaries on the substrate as well as proliferation (Fig. 2c). We assessed podocyte outgrowth _in vitro_ employing a podocyte cell line\textsuperscript{17} with endogenous expression of proHB-EGF mRNA (Supplementary Fig. 1a). Applying specific inhibitors, we examined the involvement of intracellular signalling pathways in podocyte outgrowth: the PI3 kinase pathway that is involved in actin dynamics; the classical MAP kinase pathway that is essential for proliferation; and the p38 MAP kinase pathway that may be relevant for directed migration. HB-EGF caused a complete reorganisation of the F-actin cytoskeleton, and massive formation of RiLiS (ring-like actin structures) and dorsal ruffles (Fig. 2d), indicating apically directed motility. Apical actin protrusions were completely abrogated by PI3 kinase inhibitors wortmannin and LY294002, but insensitive to a highly selective inhibitor of MEK1 (PD 98059) and to a p38 MAPK inhibitor (SB 203280) (Fig. 2e). Proliferation as determined by BrdU incorporation (Supplementary Fig. 1b) was reduced by PD 98059 in the presence of HB-EGF (Fig. 2f). The migration rate of podocytes was doubled by addition of recombinant HB-EGF (Fig. 2g and Supplementary Fig. 1c), which is known to involve a variety of cellular processes such as polarisation, protrusion dynamics, proliferation, cell-matrix and cell-cell adhesion\textsuperscript{18}. Inhibition of PI3 kinase, MEK1 and p38 MAPK all significantly reduced the migration rate. The effects of HB-EGF on podocytes were mediated by the EGFR as they were blocked by AG1478 (Fig. 2d-g). Notably, EGFR
tyrosine kinase inhibition mimicked the effect of HB-EGF deficiency on glomerular outgrowth (Fig. 2a,b) indicating that EGFR/ErbB1 is the prominent, if not only receptor, mediating podocyte proliferation and migration. Thus, HB-EGF triggers a phenotypic switch in podocytes that is required for crescent formation, and that involves multiple signalling pathways.

**Attenuated crescentic RPGN and protection from lethal acute renal failure in Hbegf (−/−) mice**

Twelve Hbegf (−/−) and 14 Hbegf (+/+) male littermates had similar renal histology and functional parameters (microalbuminuria, serum creatinine and blood urea nitrogen (BUN)) at baseline (not shown). Therefore, we used pre-immunized but unchallenged Hbegf (+/+) mice as baseline controls (CT), unless otherwise specified. Histological examination of NTS injected Hbegf (+/+) mice revealed severe glomerulonephritis and reduced survival by day 8 (Fig. 3a), while Hbegf (−/−) littermates were significantly spared from renal damage (Fig. 3b). Nephrotic syndrome is caused by hypoproteinemia due to massive urinary loss of large proteins, particularly albumin, ensuing hypoalbuminemia and is reflected by ascites. HB-EGF deficiency significantly prevented both incidence and severity of ascites (Fig. 3c) as well as renal dysfunction as reflected by albuminuria (Fig. 3d) and also by BUN concentrations, which kept within normal range in Hbegf (−/−) (Fig. 3e). Consistent with renal protection associated with lack of HB-EGF, all Hbegf (−/−) animals survived whereas 30% of Hbegf (+/+) littermates died within 8 days (P<0.01, Fig. 3a) with nearly 100% crescentic glomeruli and terminal renal failure (not shown). Similar protective effects of HB-EGF deficiency were observed in an independent experiment where animals were followed for 21 days (not shown).

**HB-EGF deficiency is associated with reduced podocyte bridge formation and effacement of foot processes**

HB-EGF is induced early in RPGN and promotes podocyte migration and proliferation in vitro and in vivo, leading to the formation of crescentic lesions (see above). Because glomerular induction of proHB-EGF mRNA and loss of glomerular permselectivity with heavy albuminuria preceded the development of the crescents, we evaluated the morphological features of podocytes in Hbegf (+/+) and Hbegf (−/−) animals on day 4 after injection of NTS. Of note, podocyte ultrastructure was identical in Hbegf (+/+) and (−/−) mice under control conditions (Supplementary Fig. 2a–d). In response to NTS, Hbegf (+/+) mice displayed podocytes with mild to severe effacement of foot processes and bridge formation between the parietal and glomerular basement membrane (Supplementary Fig. 2e,f). These ultrastructural alterations were markedly attenuated in Hbegf (−/−) animals (Supplementary Fig. 2g,h). Slender processes (diameter <0.1 μm) protruding into the urinary space were observed in both groups of NTS-treated mice.

**Role of HB-EGF in the immuno-inflammatory response associated with RPGN**

Although T cells and macrophages are central players both in our mouse model of NTS-induced GN and in human crescentic RPGN, antibody deposition may also play a pathophysiological role during the very early steps of the disease, promoting activation of
complement. Therefore, we assessed the humoral response of Hbegf (−/−) and (+/+) littermates to sheep IgG. Serial dilutions of sera from pre-immunized animals showed similar titers of mouse anti-sheep antibodies (Supplementary Fig. 3a) with similar patterns of IgG deposition in glomerular basement membranes in the kidneys in both groups after NTS injection (Supplementary Fig. 3b). Thus, we found no evidence that HB-EGF altered the humoral immune response in this model. By contrast, HB-EGF deficient mice displayed fewer infiltrated CD3-positive T cells in and around their glomeruli than their wild type counterparts following NTS administration (P<0.01) (Supplementary Fig. 3c,d) and fewer F4/80-positive macrophages in the kidney cortex on day 8 after NTS (not shown).

As HB-EGF was previously found in macrophages and T lymphocyte subsets, we sought to evaluate the potential role of non glomerular HB-EGF in RPGN. To this end, we lethally irradiated Hbegf (+/+) control mice and reconstituted them with bone marrow from either Hbegf (+/+) or Hbegf (−/−) congenic mice. Ten weeks after transfer, experimental RPGN was induced and renal histology and function were determined 14 days later. Interestingly, severity of RPGN in Hbegf (+/+) recipient mice was significant and was independent of the source of bone marrow as albumin urinary excretion and crescent formation were similar in both groups (Supplementary Fig. 3e,f), as was BUN (not shown). Thus, the cells responsible for producing pathophysiological HB-EGF are not of hematopoietic origin.

TGF-α– and epiregulin-deficient mice are not resistant to severe crescentic RPGN

Enhanced EGFR phosphorylation in the renal cortex in response to nephro toxic serum correlated with glomerular damage (Supplementary Fig. 4a) and was dependent on HB-EGF, suggesting a non-redundant mechanism for EGFR activation and a direct implication in crescent formation. We nevertheless addressed glomerular expression of alternative EGFR ligands. In the absence of suitable antibody to mouse epiregulin, we took advantage of Ereg promoter driven lacZ expression in Ereg (−/−) mice as a monitoring tool for epiregulin expression in the kidney. As previously described, lacZ staining was observed in punctuate patches throughout the digestive tract and in vascular smooth muscle cells. In contrast, no lacZ staining was found in glomeruli at baseline or after NTS administration (not shown). We also assessed epiregulin antigen expression in kidney biopsies from subjects diagnosed with RPGN and found no epiregulin immunoreactivity in normal nor crescentic glomeruli (Supplementary Fig. 4b,c). Immunoreactive TGF-α was also not found in experimental nor in human RPGN (Supplementary Fig. 4d,e and not shown). We nevertheless addressed the roles of epiregulin and TGF-α in the pathophysiology of experimental RPGN. Ten C57BL/6J, 10 TGF-α-deficient (wa1/wa1) and 10 epiregulin-deficient Ereg (−/−) C57BL/6J males displayed similar renal histology and functional parameters (microalbuminuria, serum creatinine and BUN) at baseline (not shown) and similar similar albuminuria levels and crescentic lesions in response to NTS (Supplementary Fig. 4f,g). Thus, our results support a non-redundant role for HB-EGF in EGFR- driven aspects of RPGN pathogenesis.

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Specific deletion of *Egfr* in podocytes prevents crescentic glomerulonephritis and renal failure

To determine if EGFR expression in podocytes *in vivo* is necessary for crescent formation and renal failure, we selectively deleted *Egfr* from renal podocytes. We used a conditional expression model (Tet-On system) in which the target gene is deleted only in the presence of a tetracycline derivative to achieve temporal podocyte specific deletion of the *Egfr* gene in mice (Supplementary Fig. 5a,b). Mice of all genotypes were born at the expected Mendelian frequency and appeared healthy. Ten weeks after doxycycline administration, kidney histology was normal (Supplementary Fig. 5c), no apoptotic cells were observed in glomeruli (Supplementary Fig. 5d), and albuminuria was within physiological range (not shown). Ten Podocin-rtTA-Tet-O-Cre *Egfr*<sup>loxP/loxP</sup> males and 12 Podocin-rtTA-Tet-O-Cre *Egfr*<sup>wt/wt</sup> gender-matched littermates were subjected to severe, life-threatening RPGN with high dose NTS. Podocyte specific deletion of *Egfr* alleviated albumin urinary excretion (*P*<0.05) (Fig. 4a), the rise of BUN (*P*<0.05) (Fig. 4b), and crescent formation (*P*<0.05) (Fig. 4c) and completely protected mice from death (0% mortality vs. 66% mortality for control mice with unperturbed EGFR expression in podocytes; *P*<0.01; Fig. 4d). Improved kidney function and survival also correlated with less severe changes to podocyte ultrastructure in tetracycline induced Podocin-rtTA-Tet-O-Cre *Egfr*<sup>loxP/loxP</sup> mice relative to tetracycline induced Podocin-rtTA-Tet-O-Cre *Egfr*<sup>wt/wt</sup> littermate controls (Fig. 4e).

Pharmacological blockade of the EGFR tyrosine kinase prevents crescentic glomerulonephritis and renal failure

Together, our data suggested that HB-EGF-dependent activation of the EGFR in podocytes plays an important role in crescent formation, albuminuria and renal failure. We therefore tested whether pharmacological inhibition of the EGFR pathway prevented renal damage.

AG1478 is a synthetic inhibitor of EGFR autophosphorylation<sup>23</sup>. Daily intra-peritoneal administration of AG1478 to wild-type mice not only prevented the EGFR phosphorylation observed in animals receiving vehicle control (Fig. 1c,d); it also substantially reduced the development of crescentic lesions (AG1478 vs. vehicle, n=7 per group, *P*<0.01) (Supplementary Fig. 6a,b) and kept the distribution of CD3+ cells normal (Supplementary Fig. 6c,d). AG1478 also reduced the severity of ascites (AG1478 vs. vehicle, n=7 per group, *P*<0.01) (Supplementary Fig. 6e), albuminuria (n=7 per group, *P*<0.01) (Supplementary Fig. 6f) and preserved renal function, as assessed by BUN (n=6–7 per group, *P*<0.05 for AG1478 vs. vehicle and *P*=NS for AG1478 vs. CT) (Supplementary Fig. 6g). No AG1478-treated *Hbegf* (+/+) mouse died within 8 days of RPGN.

Delayed pharmacological blockade of the EGFR tyrosine kinase stops crescentic glomerulonephritis and prevents renal failure

To determine whether inhibition of the EGFR pathway could represent a therapeutic option for RPGN, we administered a clinically available EGFR inhibitor, erlotinib, on day 4 after infusion of anti-GBM serum. This time point was chosen because it is when albuminuria and acute renal failure peak, and thus a time that is relevant to clinical applications. This regimen was compared to the effect of vehicle alone and to the administration of erlotinib 12 hours before the first infusion of anti-GBM serum. The dose of erlotinib used significantly

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inhibited EGFR phosphorylation after 14 days of experimental RPGN (Fig. 5a). Again, “preventive” EGFR tyrosine kinase inhibition reduced the proportion of crescentic glomeruli (erlotinib-treated vs. vehicle only-treated group; $P<0.01$) (Fig. 5b), and the rise in BUN ($P<0.01$) (Fig. 5c). Erlotinib treatment also attenuated ultrastructural alterations of podocytes and full loss of interdigitating foot process pattern. (Fig. 5d). Overall, early and delayed erlotinib administration had similar marked therapeutic effects on renal damage, inflammation and renal failure in our experimental model (Fig. 5b–d).

**The EGFR pathway is not required for foot processes effacement and proteinuria**

To explore whether EGFR kinase activity has a direct role in regulating podocyte foot process structure and function, we next compared microalbuminuria, glomerular histopathology and foot process morphology of vehicle- or erlotinib-treated wild-type 129Sv mice before and after administration of LPS, a model that has been useful for defining the role of pathways important for the dynamic regulation of the podocyte cytoskeleton.\(^{24}\)

Morphologically, there was no difference between vehicle and erlotinib-treated mice for any of these indexes at baseline (not shown). Moreover, after LPS injection, we found equal albuminuria and podocyte foot process effacement in mice treated with erlotinib or vehicle control (Supplementary Fig. 7a,b). This is in agreement with the lack of induction of EGFR phosphorylation in glomeruli (Supplementary Fig. 7c) and in the renal cortex (Supplementary Fig. 7d) of LPS-injected mice by contrast with what was observed during NTS-induced RPGN.

Phosphorylation levels of EGFR were similar upon LPS administration to those observed at baseline in controls (Supplementary Fig. 7d). These data indicate that intact EGFR tyrosine kinase activity is not mandatory for pathological urinary protein loss or effacement of podocyte foot processes and that different pathways cause RPGN and LPS-induced proteinuria.

**Elevated expression of HB-EGF in human kidneys with RPGN of various etiologies**

To evaluate the relevance of these findings to human disease, we used immunostaining with three unrelated antibodies to human HB-EGF to address upregulation of HB-EGF in glomeruli from subjects with crescentic RPGN (Supplementary Fig. 8a). All three antibodies displayed similar patterns of HB-EGF labelling both in normal human kidney and in renal biopsies from subjects with RPGN (Supplementary Fig. 8a). In normal human kidneys, HB-EGF labelling was weak and restricted to tubules and vascular smooth muscle cells (Supplementary Fig. 8a). Little or no epithelial expression of HB-EGF was found in glomeruli from subjects diagnosed with noncrescentic glomerulopathies (Fig. 6). HB-EGF labelling in glomeruli from subjects with crescentic RPGN was substantially stronger overall, particularly in podocytes, in parietal epithelial cells (Fig. 6 and Supplementary Fig. 8b), and in tubules. Moreover, HB-EGF staining was more intense and diffuse in glomeruli with crescents than in less affected glomeruli within the same tissue samples (Supplementary Fig. 8a). Weaker but consistent HB-EGF expression was also observed in inflammatory infiltrates (Supplementary Fig. 8b). A similar pattern or HBEGF expression was observed in all kidney samples with RPGN whatever the immune cause (Fig. 6 and...
Supplementary Fig. 8b). These studies show that HB-EGF is specifically increased, sometimes in a sustained fashion, in conditions associated with crescent formation and immune destruction of the glomeruli.

DISCUSSION

The results presented herein are consistent with a pathogenic pathway of RPGN in which HBEGF can be induced in the kidney to elicit EGFR activation primarily in podocytes, which in turn promotes glomerular lesions. Although delineated in an experimental mouse model of RPGN, immunolabeling of human kidney biopsies suggests that the same pathway may be active in human disease. Enhanced expression of the HB-EGF gene in the kidneys of streptozotocin-induced diabetic rats had been reported, as well as in models of membranous and minimal change nephropathies. Nevertheless, it was not known whether EGFR phosphorylation is induced in these models and whether HB-EGF has any pathophysiological actions. In the puromycin aminonucleoside nephritis rat model, administration of affinity-purified monoclonal antibody to human HB-EGF induced earlier onset of albuminuria with no subsequent effect on renal function or other major renal endpoints. Based on these reports, we decided to address the role of HB-EGF in a more severe and inflammatory model of renal damage reminiscent of human proliferative glomerulonephritis. Crescentic RPGN are the most severe class of glomerulopathies in humans and require costly and side-effect-prone immunosuppressive therapies. The inflammatory infiltrate in glomeruli and interstitium is composed of T lymphocytes, mononuclear phagocytes and neutrophils, much as has been described in other animal models of crescentic glomerulonephritis and human RPGN. HB-EGF was shown to be rapidly induced in mesangial cells and glomerular epithelial cells in the anti-Thy-1.1 model of mesangial injury. It was reported that proHB-EGF mRNA expression was induced glomeruli of rats 30 minutes after anti-GBM antibodies administration and contributed to acute glomerular hemodynamic alterations. Although elegant, this was a short-term study with no assessment of clinically relevant end-points, such as renal damage, proteinuria and death. Indeed, upregulation of HB-EGF in this model decreased spontaneously to baseline by 24 hours. Nevertheless, a clinical report of high mesangial expression of HB-EGF in a variety of human glomerulonephritis disorders, suggests that HB-EGF may be chronically induced in renal disease. However, in the NTS model of severe glomerular injury, HB-EGF deficiency and EGFR blockade both prevented the development of ascites and renal leukocytic infiltrates prior to the appearance of patent crescentic proliferative lesions and interstitial fibrosis; therefore, we suggest that the HBEGF/EGFR pathway acts very early to promote renal damage. Several other cell types may participate in early HB-EGF release. HB-EGF has been reported in wound fluid, macrophages, and T cell subsets in tumors and atherosclerotic plaques, although its functions in these settings are unknown. In a lupus model of accelerated crescentic glomerulonephritis, a marked and sustained increase in renal proHB-EGF mRNA has been reported to take place, in part in macrophages. Nevertheless, HB-EGF expression within inflammatory infiltrates was much weaker than within crescentic glomeruli in mouse and human kidney cortex. Accordingly, immunolocalization of phosphorylated EGFR revealed a prominent distribution in glomeruli. Furthermore, deficiency of HB-EGF
restricted to bone marrow-derived cells did not reduce the severity of experimental RPGN, contrasting with results obtained with systemic or podocyte specific alteration of the HB-EGF/EGFR pathway. Although these observations do not exclude the involvement of the HB-EGF/EGFR pathway in the early inflammatory phase of the disease, they suggest a prominent pathophysiological role for HB-EGF released by intrinsic renal cells, probably upon stimulation by immune mediators. Of note, residual podocyte alterations and albuminuria could still be measured in Hbegf (−/−) or after EGFR inhibition. This may occur because NTS can cause albuminuria due to a direct non-inflammatory effect on podocytes. For example, it was reported that NTS revealed several podocyte antigens such as aminopeptidase A (APA). These findings, in combination with previous studies showing that monoclonal anti-APA antibodies induce proteinuria in mice, suggest that some anti-APA antibodies may be responsible for complement-independent proteinuria in this model\textsuperscript{40}. Although we acknowledge there must be other pathways contributing to crescent formation, this is the first report to our knowledge to demonstrate a pathophysiological role for HB-EGF in a model of human disease and a role for EGFR in a model of renal immune-mediated destruction. The use of a mouse genetic model of HB-EGF deficiency allowed us to overcome the issues of specificity, affinity and bioavailability of neutralizing antibodies. Moreover, although HB-EGF is a growth factor, its observed actions appear to extend beyond its known mitogenic properties: HB-EGF-deficient mice were protected from inflammatory renal infiltrate and albuminuria prior to the development of marked renal cell proliferation. Several hallmarks engaging the HB-EGF/EGFR axis were observed in podocytes such as RiLiS formation (an assay for apical protrusions and apical migration) and migration. Of note, wound closure involves protrusion dynamics, cell polarity and cell-cell adhesion, but appears to be largely independent of proliferation\textsuperscript{18}. To confirm that these effects were not due to a developmental effect of chronic HB-EGF deficiency on the maturation of the immune system or of the kidney, we verified that podocyte-specific deletion in adult animals of the EGFR, the main receptor for HB-EGF, mimicked the phenotype induced by genetic targeting of the Hbegf gene. Although downregulation of EGFR expression has been associated with increased apoptosis in other cell types\textsuperscript{41}, specific deletion of the EGFR in adult podocytes per se did not increase podocyte apoptosis or proteinuria. Moreover, inducible and specific deletion of the Egfr gene in adult podocytes significantly alleviated the course of fatal RPGN, indicating a pathophysiological role of the HB-EGF/EGFR pathway in these cells \textit{in vivo}. Induction of EGFR phosphorylation during disease progression and phenocopy of HB-EGF deficiency with EGFR deficiency suggests that activation of ErbB4, another receptor for HB-EGF that may promote distinct cellular effects\textsuperscript{42}, plays a marginal, if any, role in our model. Our results also demonstrated that EGFR was not highly phosphorylated in the renal cortex of HB-EGF-deficient mice after injection of NTS, implicating HB-EGF in EGFR activation in RPGN. Although some participation of other ligands cannot be excluded in other kidney compartments, lack of protection from glomerulonephritis in NTS challenged TGF-α- or epiregulin-deficient mice supported a non-redundant role for HB-EGF. Epiregulin was a potentially important target to investigate because its gene (Ereg) was shown to be induced by serum in quiescent human mesangial cells\textsuperscript{43}. Furthermore, comprehensive expression profiling using an inducible WT1 expression model identified Ereg, as well as Hbegf, as a direct WT1 target gene\textsuperscript{44}. Hbegf was the only gene product that was unpressed in crescentic RPGN whereas WT1
expression vanished from glomerular epithelial cells, suggesting that Hbegf is also controlled by WT1 independent pathways.

Use of an EGFR kinase inhibitor was previously shown to attenuate increased glomerular volume and tubular volume in diabetic rats\(^\text{45}\) and in a diabetic rat (TGR(mRen-2)27) model with overactivation of the renin angiotensin system\(^\text{46}\). However, no specific activation of the EGFR and of EGFR ligands was described then. Although no beneficial effect or EGFR inhibition on glomerulosclerosis or kidney failure could be demonstrated then, the results suggest that the EGFR pathway may be involved in the control of the glomerular hemodynamics\(^\text{46}\). On the other hand, the findings do not appear to be relevant to the potential therapy we have identified in aggressive proliferative glomerulonephritis.

Finally, the efficacy of two distinct EGFR inhibitors administered either shortly before anti-GBM serum infusion or 4 days after the beginning of RPGN, (i.e. after the period of pre-immunization) suggests that recruitment of the HB-EGF-EGFR pathway is involved during the effector phase of the disease and does not play a significant role in the pre-immunization process. Accordingly, the humoral response was similar in HB-EGF-deficient and normal animals that exhibited similar levels of anti-sheep IgG.

In line with our measurement of induction of proHB-EGF mRNA expression in freshly sorted podocytes from nephritic mice, we observed consistently more HB-EGF protein in glomeruli of human kidneys with crescentic RPGN than in normal tissues or in a variety of non crescentic glomerular diseases. Expression of HB-EGF was mainly observed in podocytes, in parietal epithelial cells and in tubules, a pattern similar to that reported in rat models of focal adhesive glomerular sclerosis or passive Heymann nephritis although no strong chronic up-regulation could be observed in these models\(^\text{47}\). Interestingly, in the same renal biopsies, the most severely affected glomeruli displayed the most intense staining to HB-EGF in podocytes, further implicating HB-EGF in the pathophysiology of human RPGN. Furthermore, some kidney biopsies from individuals with RPGN relapse despite chronic immunosuppressive therapy displayed sustained glomerular elevation of HB-EGF expression, again consistent with a specific role for this mediator.

In conclusion, the data presented herein provide evidence that immune-mediated glomerular injury leads to active and sustained pathophysiological recruitment of glomerular EGFR by HB-EGF. In contrast to reports suggesting a protective or a negligible role for HB-EGF in models of mild glomerular injury\(^\text{2,27}\), we demonstrate here that activation of EGFR in podocytes is involved in a severe form of glomerular injury that leads to death from renal failure. These findings raise the possibility that specific EGFR inhibitors may be of therapeutic value for treating crescentic and other types of inflammatory glomerulonephritis.

**METHODS**

**Animals**

We used mice that have been described previously: HBEGF-deficient (Hbegf \((-/-)\)\(^\text{48}\), TGF-\(\alpha\)-deficient \(wal/wal\)\(^\text{49,50}\), epiregulin-deficient mice\(^\text{22}\) and Podocin-Cre\(^\text{51}\) × Z/EGFP\(^\text{52}\) mice. To specifically generate a time-specific and cell-specific knockout of \(Egfr\), we bred mice

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carrying the reverse tetracycline transactivator protein under control of the podocin promoter (Pod-rtTa)\textsuperscript{53} with mice carrying the Tet-O-Cre transgene\textsuperscript{54} and with mice carrying a floxed \textit{Egfr} allele\textsuperscript{55}. Doxycycline was administered for 2 weeks prior to NTS administration. Experiments were conducted according to the French veterinary guidelines and those formulated by the European Community for experimental animal use (L358-86/609EEC), and were approved by the Institut National de la Santé et de la Recherche Médicale.

**Bone Marrow Transplantation**

Hbegf (+/+) mice were lethally irradiated and then transplanted with bone marrow cells extracted from Hbegf (−/−) or Hbegf (+/+) mice. We documented robust and equivalent long-term hematopoietic reconstitution in all recipients.

**Induction of crescentic glomerulonephritis and EGFR kinase inhibition**

We used the accelerated anti-GBM RPGN model, as previously described\textsuperscript{11,30}. After nephrotoxic serum (NTS) injection, mice were given repeated intraperitoneal injections of either AG1478 (LC Laboratories) (30 mg.kg\textsuperscript{−1}.day\textsuperscript{−1}), or vehicle (n=7 per group). On day 8, we collected urine and euthanized mice. In another set of experiments, three groups of mice (n=9 per group) received erlotinib (10 mg.kg\textsuperscript{−1}.day\textsuperscript{−1} per gavage), started erlotinib either on day 0 or day 4, or vehicle, until sacrifice on day 14. We repeated the experiment for electron microscopy study, mice receiving erlotinib or vehicle (n=3 per group), from day 0 to sacrifice on day 5.

**Lipopolysaccharide-induced albuminuria and EGFR inhibition**

We induced albuminuria in male 129S2 mice (n= 7 per group) by intraperitoneal injection of LPS on days 1 and 2. Mice were given erlotinib (10 mg.kg\textsuperscript{−1}.day\textsuperscript{−1}) or vehicle alone before the first LPS injection, and then every 24 hours. We compared these two groups to erlotinib or vehicle-treated animals that were not injected with LPS (n=3 per group). On day 3, we collected urine and euthanized mice.

**Isolation of podocytes**

We isolated podocytes from five Pod-Cre × Z/EGFP mice 6 days after treatment with NTS and from six untreated PodCre × Z/EGFP mice. Glomeruli were isolated glomeruli by sieving and dissociated. Then podocytes were stained with an APC-conjugated antibody to mouse podocalyxin antibody and sorted by FACS. We sorted the cell suspension by FACS isolating podocytes as GFP and APC double-positive cells.

**Real-time RT-PCR and \textit{in situ} hybridization analysis of proHB-EGF mRNA**

We did quantitative RT-PCR for HB-EGF expression using RNA recovered from cultured and extracted freshly isolated podocytes. We generated a mouse \textit{proHB-EGF} probe and performed \textit{in situ} hybridization as previously described\textsuperscript{56}.

**Western blot analysis**

We performed western blotting on lysates prepared in a lysis buffer using rabbit antibodies to phospho-EGFR or EGFR.
Outgrowth from isolated glomeruli

We isolated decapsulated glomeruli as reported earlier\textsuperscript{17}. We verified podocyte phenotype by positive staining for synaptopodin and WT-1 and quantified podocyte outgrowth. Outgrowth of >24 glomeruli was analysed per condition.

Podocyte cell line and \textit{in vitro} assays

Cells of a well-established podocyte cell line were used and cultured as described previously\textsuperscript{17}. We examined the effects of HB-EGF (30 nM HB-EGF for 7 min) on induction of apical actin-based protrusions in differentiated podocytes. We applied inhibitors 1 h prior to stimulation with HB-EGF. We determined proliferation of differentiated podocytes by BrdU incorporation. Cells were incubated with 10 μM BrdU and 30 nM HB-EGF in the absence or presence of inhibitors for 48 h. We used a wound assay to assess migration of podocytes incubated with 30 nM HB-EGF in the absence or presence of inhibitors.

To study the signalling pathways mediating HB-EGF effects, we employed the following inhibitors: AG1478, the PI3 kinase inhibitors wortmannin and LY294002 the MEK1 inhibitor PD98059 and the p38 MAP kinase inhibitor SB203580.

Assessment of ascites, renal function and albuminuria

Ascites was quantified with a 5 point scale (0–4) the day of sacrifice by examining the peritoneum and collecting peritoneal fluid with a syringe. Urinary creatinine and blood urea nitrogen (BUN) were quantified spectrophotometrically using colorimetric methods. Urinary albumin excretion was measured using a specific ELISA assay (CellTrend GmbH).

Histopathological study in mouse tissues

We evaluated histopathological changes in kidneys using Masson’s trichrome coloration of formalin fixed paraffin embedded sections. The proportion of crescentic glomeruli was evaluated by examination of at least 80 glomeruli per section, by an examiner blind to the experimental conditions. We stained formalin fixed paraffin-embedded sections for F4/80, CD3ε, and TGF-α. Staining for apoptotic cells was also performed, using in situ Cell Death Detection Kit, POD (Roche). We did immunofluorescence staining in mouse kidneys for mouse IgG, phospho-EGFR, EGFR and Synaptopodin,

Electron microscopy

Small pieces of renal cortex were fixed in 4% glutaraldehyde and post-fixed in 1% osmium tetroxide and embedded in Araldite M (Sigma). Ultrathin sections were counter-stained with uranyl acetate and lead citrate and examined in a ZEISS 910 transmission electron microscope.

Immunohistochemistry in human tissues

We obtained formalin-fixed paraffin-embedded sections of renal biopsies from thirty subjects with RPGN. Informed consent was obtained in all cases before renal biopsy. We did immunohistochemical staining for HB-EGF in normal human kidneys and biopsies from
subjects with crescentic RPGN. We also studied kidney biopsies from other subjects with various glomerular diseases but no crescentic lesions.

**Statistical analysis**

Data are expressed as means ± SEM. The 2-tailed Mann-Whitney test and Student's t-test were used as appropriate. For experiments with more than two subgroups, the nonparametric Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test were used. Values of $P<0.05$ were considered significant. Survival curves were calculated according to the Mantel-Haenszel method. All analyses were performed using Prism version 5.03 for Windows, GraphPad Software.

**Additional methods**

Detailed methodology is described in the Supplementary Methods.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Induction of renal HB-EGF synthesis leads to glomerular activation of EGFR during RPGN

(a) Representative image of in situ hybridization study in NTS-injected wild-type (Hbegf (+/+)) animals, showing proHB-EGF expression in glomeruli (G), especially in parietal glomerular epithelial cells (Pec), in podocytes (day 4) and in crescents (Cr) (day 8). White arrow indicates abundant proHB-EGF mRNA expression in areas where tuft-capsular podocyte bridges were present. Scale bar (orange), 50 μm. (b) Quantification by real-time RT-PCR of proHB-EGF mRNA in freshly isolated podocytes on day 6 after nephrotoxic serum injection (NTS) and in podocytes from non-injected control mice (CT) (n=3 per group). * P<0.05 versus controls. (c) Western blot analysis of phosphorylated EGFR and total EGFR in the renal cortex from non-challenged controls (CT), wild-type (Hbegf (+/+)) mice infused with NTS ((+/+)NTS), HB-EGFR deficient mice infused with NTS ((−/−)NTS), and from Hbegf (+/+) animals that were given intraperitoneal injections of the EGFR tyrosine kinase inhibitor AG1478 ((+/+)NTS+AG1478). Values reported are means ± sem. (n=6–8 per group). (d) Immunofluorescence staining for phosphoEGFR in renal cortex from controls (CT), NTS-injected Hbegf (+/+) mice ((+/+)NTS), HB-EGFR deficient mice infused with NTS ((−/−)NTS), and from NTS-injected Hbegf (+/+) animals treated by AG1478 ((+/+)NTS+AG1478), on day 8 after NTS administration. * P<0.05 versus controls at baseline, ** P<0.01 versus controls at baseline. ## P<0.01 versus mice treated with vehicle only. Pec: parietal glomerular epithelial cells; G: glomerulus; Cr: crescent.
Figure 2. HB-EGF induces a migratory phenotype in podocytes \textit{in vitro}

(a) Podocyte outgrowth over 6 days from decapsulated glomeruli of Hbegf (+/+) or Hbegf (−/−) mice (arrow). Cells are stained for WT-1 expression. (b) Outgrowth area from glomeruli of Hbegf (−/−) mice in the absence (light blue bar) or presence of the EGFR inhibitor AG1478 (500 nM) (AG, dark blue bar) and from glomeruli. Sparse outgrowth from glomeruli of Hbegf (−/−) mice in the absence (light grey bar) was rescued by addition of 30 nM HB-EGF (black bar). (c) Schematic drawing of podocyte outgrowth from isolated glomeruli, which was used as a combined migration/proliferation assay to assess the ability of crescent formation \textit{in vitro}. Podocytes are in a stationary state (blue color) on the surface of capillary loops (grey circle), when glomeruli are plated. Subsequently, podocytes assume a migratory phenotype (orange color), characterized by apical protrusions, by attachment and by migration on the substratum. Later stages of outgrowth also involve proliferation. (d) Representative image of F-actin reorganisation and formation of ring-like actin structures (RiLiS) induced by HB-EGF (30 nM for 7 min) in differentiated podocytes, in the absence or presence of AG1478 (500 nM). The effect of HB-EGF to induce apical protrusions is abrogated in the presence of AG1478 (500 nM). (e) Quantitative analysis of RiLiS formation in differentiated podocytes. HB-EGF (30 nM) was added in the absence (Ctl) or presence of inhibitors: AG - AG1478 (500 nM), Wo - Wortmannin (100 nM), LY - LY294002 (30 μM), PD - PD98059 (25 μM), SB - SB203580 (25 μM). (f) BrdU incorporation in differentiated podocytes over 48 h. (g) Distance of migration of differentiated podocytes within 8 h in the wound assay. Data are means ± SEM (n=3–4 experiments). * \( P<0.05 \) vs. untreated Hbegf (+/+) glomeruli in (b) and vs. HB-EGF alone in (e–g). Scale bar : 300 μm in (a) and 30 μm in (d).
Figure 3. Deletion of Hbegf gene prevents fatal renal destruction
(a) Survival curve for challenged Hbegf (+/+ ) and Hbegf (−/− ) mice. In all cases, death was associated with severe renal damage with macroscopic hematuria (blood leakage into the urine) and animals died from renal failure (with 100% crescentic and necrotizing lesions at autopsy). (b) Masson Trichrome staining of kidneys and proportion of crescentic glomeruli in control mice and in NTS-injected Hbegf (+/+ ) and Hbegf (−/− ) mice (day 8 post NTS) (Cr: crescents, G: glomeruli, Tc: tubules with proteinaceous casts) Scale bar, 50μm. (c) Ascites score as an index of albumin plasma loss and water and sodium retention, (d) albuminuria and (e) blood urea nitrogen concentrations in NTS-challenged Hbegf (+/+ ) and Hbegf (−/− ) animals on day 8 post NTS, and in unchallenged controls (CT). Values reported are means ± sem. (n=9–12 per group). * \( P<0.05 \) versus controls at baseline, ** \( P<0.01 \) versus baseline, *** \( P<0.001 \) versus baseline. # \( P<0.05 \) versus NTS-treated (+/+ ), ## \( P<0.01 \) versus NTS-treated (+/+ ).
Figure 4. Selective deletion of Egfr from podocytes protects from RPGN
(a) Albuminuria (b) blood urea nitrogen concentration and (c) proportion of crescentic glomeruli in Pod-Tet on-Cre Egfr<sup>wt/wt</sup> and Pod-Tet on-Cre Egfr<sup>loxP/loxP</sup> mice, 8 days after NTS-induced RPGN (*P<0.05 for all comparisons). (d) Survival curve of challenged Pod-Tet on-Cre Egfr<sup>loxP/loxP</sup> and littermate control mice in a severe model of RPGN. (*P<0.01). (e) Ultrastructural analysis of podocytes by transmission electron microscopy in NTS-treated Pod-Tet on-Cre Egfr<sup>wt/wt</sup> and Pod-Tet on-Cre Egfr<sup>loxP/loxP</sup> mice. More severe foot process effacement and irregular thickening of the GBM is visible in Pod-Tet on-Cre Egfr<sup>wt/wt</sup> animals. Scale bars: upper panels 2μm, lower panels 1μm.
Figure 5. Delayed EGFR tyrosine kinase inhibition stops the development of crescentic RPGN

(a) Quantification by western blot analysis of phosphorylated EGFR and total EGFR in the renal cortex from non-challenged controls (CT), NTS-injected mice treated with vehicle alone, and NTS-injected mice treated with erlotinib either started twelve hours before administration of NTS (days 0–14) or in a curative protocol, started four days later (d4–14). Mice were euthanised after 14 days of RPGN. (b) Blood urea nitrogen concentration and (c) proportion of crescentic glomeruli in CT in the different groups of mice as in (a). Data are means ± sem, (n=9 mice per group). ** P<0.01 versus controls at baseline (CT), *** P<0.001 versus CT, ## P<0.01 versus vehicle, ## P<0.001 versus vehicle.

(d) Ultrastructural analysis of podocytes by transmission electron microscopy in erlotinib-treated and vehicle-treated mice, five days after injection of NTS. Scale bar 2 μm.

(e) Masson trichrome staining of renal cortex from a mouse treated with erlotinib (day 4–14) (left panel) and a vehicle-treated mouse (right panel) on day 14. Ne: necrotic glomerular lesions, Cr: cellular crescents, Tc: tubular proteinaceous casts, Infilt: diffuse CD3-positive cell infiltrates (Infilt) seen in vehicle-treated mice. Scale bar 100 μm.
Figure 6. HB-EGF expression is induced in human crescentic glomerulonephritis
Representative images of immunostaining for HB-EGF using monoclonal sc-74526 antibody in sections of kidney biopsies from 8 random subjects diagnosed with non crescentic glomerulopathies (upper panels), including diabetic nephropathy, amyloidosis, minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), IgA nephropathy (IgAN), and membranous nephropathy (MN). Similarly, lower panels show immunostaining for HB-EGF in renal biopsies from 8 random subjects RPGN of various etiologies, including lupus nephritis, microscopic polyangiitis (MP), endocarditis (End), Goodpasture disease (Gp), and Wegener disease (Wg). Scale bar 50 μm.