Epidermal growth factor: a new therapeutic target in glomerular disease

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Abstract
Glomerular kidney diseases are of major public health importance because of their strong impact on the quality of life of patients and of their costly management. A relatively neglected area of study is the local factors that influence development of glomerular disease. The involvement of a glomerular factor has now been demonstrated in glomerulonephritis with cell proliferation such as crescentic rapidly progressive glomerulonephritis (RPGN). Various unrelated immune disorders promote RPGN, such as antibodies directed against the glomerular basement membrane, deposition of immune complexes or antibodies directed against neutrophils. Despite the heterogeneity of these causing diseases, RPGNs share similar histopathological features, which suggest involvement of common final pathways. De novo expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF) in glomerular epithelial cells is found specifically in human glomerulonephritis with proliferation of these cells and dedifferentiation of podocytes. A receptor for HB-EGF, the EGF receptor (EGFR), is expressed by parietal epithelial cells and podocytes. Furthermore, in a mouse model of RPGN, HB-EGF deficiency or conditional targeting of the Egrf alleles in podocytes markedly alleviated RPGN, renal failure and death. This indicates that the HB-EGF/EGFR pathway plays a pivotal role in RPGN and opens therapeutic perspectives as EGFR inhibitors are clinically available.

Introduction
Glomerular kidney diseases are of major public health importance not only because of their high prevalence and costly management but also because they are a major independent risk factor for cardiovascular morbidity and mortality. Despite the crucial importance of the kidney, both as target and as determinant of the prognosis of patients with chronic vascular diseases, knowledge of the mechanisms underlying glomerular damage is limited. A relatively neglected area of study is the local factors that promote or prevent development of glomerular damage. The recent demonstrations of the important role of the vascular endothelial growth factor [1, 2], the nitric oxide [3, 4] and the angiopoietin-1 (Aplt1)/tyrosine kinase with immunoglobulin and EGF homology domains pathways (Tie2) [5, 6] in protecting the glomerular filtration barrier have unravelled the crucial homeostatic importance of local factors. The involvement of a glomerular factor has now also been demonstrated in glomerulonephritis with proliferative podocytopathy such as crescentic glomerulonephritis or rapidly progressive glomerulonephritis (RPGN). In this field, most of the research efforts have focused on the immunopathological aspects [7–14]. For example, very distinct immune disorders promote RPGN such as antibodies directed against the glomerular basement membrane (GBM) (Type I RPGN), the deposition of immune complexes in the glomerulus (Type II RPGN) or antibodies directed against neutrophils (anti-neutrophil cytoplasmic antibodies) (Type III RPGN or pauci-immune RPGN) [15, 16]. Strikingly, despite the heterogeneity of these causing diseases, RPGN shares remarkably similar histopathological features with change in phenotype of resident cells that suggest involvement of the final common pathways [17–31]. De novo expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF family of proteins in parietal epithelial cells (PECs) lining the Bowman capsule and in podocytes, provides the first example of a molecular interaction between these two cell types [32]. The main receptor for HB-EGF, the EGF receptor (EGFR), is ubiquitously expressed, including in PECs and podocytes. In contrast, HB-EGF is not present in normal glomerular epithelial cells but is found to be specifically expressed in human glomerulonephritis with proliferation of these cells and dedifferentiation of podocytes. This feature is not observed in other types of glomerular diseases with the notable exception of collapsing glomerulopathies (unpublished data) and, to a milder extent, in glomerular capsular adhesions in some severe cases of focal segmental glomerulosclerosis (FSGS). Furthermore, in a mouse model...
of RPGN, genetic deletion of the **Hbegf** alleles or tetracycline-inducible conditional gene targeting of the **Egrf** gene in podocytes markedly alleviated RPGN and prevented renal failure and death. These recent findings complement previous studies that suggest that the **EGFR** pathway may contribute to glomerular diseases.

**The **EGFR** and **ErbB** receptor family**

The **EGFR** belongs to the family of receptor tyrosine kinases **ErbB**, which plays a fundamental role in the development of many cellular functions, such as proliferation, migration and differentiation [33]. The **ErbB** protein family consists of four members: **EGFR** (ErbB1), **ErbB2**, **ErbB3** and **ErbB4**. These receptors are brought into play through complex interactions among themselves and with their respective ligands: the **EGF**, **HB-EGF**, transforming growth factor-α (TGF-α), amphiregulin, betacellulin, epiregulin and the neuregulins 1 to 4 (NRGs). With the exception of neuregulins, all are able to bind to **EGFR** [34].

While **ErbB** receptors display a monomeric conformation in the basal state, ligand binding leads to the formation of homo- or hetero-dimers, activation of the tyrosine kinase domain and phosphorylation of specific tyrosine residues in the cytoplasmic domain, allowing the recruitment and activation of adapter proteins and the initiation of signalling cascades [35]. **EGFR** is capable of forming homodimers or heterodimers with the other three **ErbB** receptors, and signalling properties differ depending on the ligand and the receptor with which it is coupled into homo- or hetero-dimer [36], which results in a wide variety of responses [34]. The major signalling cascades downstream of **EGFR** activation pathways are Ras-Raf-mitogen-activated protein kinase, phosphoinositide 3-kinase (PI3K)-AKT and signal transducers and activators of transcription factors 3 and 5 [37]. The expression of **EGFR** in many cell types and multiple signalling pathways put into play by this receptor explain its importance in many cellular processes such as survival, proliferation, apoptosis and migration in the development of many organs [38] and in some cancers.

**The heparin-binding EGF-like growth factor**

Since the discovery of **HB-EGF** in culture media from human macrophages and monocytes [39, 40], the expression of this factor has been reported in many tissues and cells, including various types of epithelial cells [41]. Like other ligands of **EGFR**, **HB-EGF** is synthesized as a transmembrane precursor (pro-HB-EGF), whose cleavage by a metalloproteinase releases the soluble mature form of **HB-EGF** [42], responsible for the majority of its biological effects. Cleavage of pro-HB-EGF under the action of several metalloproteinases can be induced by various factors, including agonists of G protein-coupled receptors, thrombin, endothelin and angiotensin II [43–45], leading to the release of a soluble fragment and transactivation of **EGFR** [46]. A peculiar property of pro-HB-EGF is the natural receptor for the diphtheria toxin in humans and monkeys [47, 48].

The **HB-EGF** is a potent inducer of proliferation and cell migration and has been involved in a wide variety of physiological and pathological processes, including development, the phenomena of cell renewal and tissue repair, implantation of blastocyst, the smooth muscle cell hyperplasia, atherosclerosis and tumour proliferation [49, 50].

**Involvement of **EGFR** in renal pathophysiology**

The **EGFR** is expressed in virtually all cells of the body. Specificity of biological processes is mainly ensured by spatial restriction of the activating ligands (EGF, TGF-alpha, amphiregulin, AR, **HB-EGF**, epiregulin and betacellulin), and of upstream activator systems. In addition, **EGFR** can signal through heterodimerization with the related receptors. In the kidney, **EGFR** is expressed by tubular cells throughout the nephron [51] but also in the glomerulus including the podocytes [32, 52]. The **HB-EGF** is expressed in tubular cells but not in normal glomeruli [53]. The involvement of **HB-EGF** and **EGFR** has been reported in various situations related to renal pathophysiology. **EGFR** plays a role in the regulation of ion transport and particularly in magnesium re-absorption by activating the transient receptor potential melastatin carrier 6 [54], which explains why **EGFR** inhibitors used in cancer often result in hypomagnesaemia [55]. Various *in vitro* and *in vivo* studies have demonstrated the important role of **EGFR** and its ligands, including **HB-EGF**, in the proliferation and migration of tubular epithelial cells, promoting the epithelial regeneration response to acute tubular damage [56–61]. Meanwhile, if the activation of **EGFR** can exert beneficial effects by promoting tubular regeneration, excess of **EGFR** activity in tubular cells may be involved in the development of kidney diseases as detailed below.

A few studies conducted in animals have shown that **EGFR** promotes the development of glomerulosclerosis and renal interstitial fibrosis in hypertensive models [62, 63]. This could be partly explained by the fact that significant effects of angiotensin II and endothelin-1 (ET-1), which play a major role in the development of renal fibrosis in these models [64–66], could be mediated by the transactivation of **EGFR**, particularly through the cleavage and extra-cellular release of **HB-EGF** [67–70]. Thus far, the precise mechanisms of **EGFR** implication on the development of hypertensive glomerulosclerosis are not known. Beyond promotion of extracellular matrix deposition by mesangial cells, increased glomerular capillary pressure leading to podocyte stretch and albuminuria may be favoured by **EGFR** activity. Similar pathophysiology may be partially at play in diabetes-associated glomerulosclerosis. Indeed, administration of a pharmacological inhibitor of **EGFR** to diabetic Sprague-Dawley rats was associated with reduced glomerular volume and albuminuria, without any reduction of glomerulosclerosis [71]. Similar findings were recently reported in another model of diabetic glomerulosclerosis with overactivation of the renin–angiotensin system, the heterozygous streptozotocin-diabetic TGR(mRen-2)27 rats [72]. Interestingly, such **EGFR**-dependent change in glomerular volume may have reflected changes in the local haemodynamics such as reduction of the glomerular capillary ultrafiltration coefficient, which, in combination with **EGF**-induced
constriction of both pre-glomerular and post-glomerular arterioles, results in acute major reductions in the rates of glomerular filtration and perfusion upon renal infusion of exogenous EGF [73]. Thus, in the context of diabetic nephropathy, and likely, in hypertensive glomerulosclerosis, regulation of the glomerular haemodynamics by the EGFR system is suspected. Further ultrastructural and immunohistochemical studies are needed to evaluate potential podocyte preservation through direct effect or through attenuation of the glomerular capillary pressure and glomerular volume. Additionally, some puzzling data indicate that unknown mechanisms may be at play. In particular, mice overexpressing a dominant negative isoform of EGFR in the proximal tubule were protected from glomerular lesions during chronic Ang II infusion [63].

HB-EGF glomerular expression data in experimental nephropathies

Until recently, the involvement of HB-EGF and EGFR was not demonstrated in glomerular diseases, although expression of the ligand was reported in various animal models. Thus, increased expression of HB-EGF in podocytes and lesions of FSGS has been reported in a model of glomerulopathy induced by puromycin aminonucleoside (PAN) in rats [74, 75]. Induction of mesangial expression of HB-EGF was shown in the model of glomerulonephritis induced by anti-Thy-1.1 and biopsies of human patients with various types of glomerulonephritis involving mesangial proliferation [76, 77]. Sustained expression of HB-EGF in myofibroblasts during remodelling of the peri-infarct region of the remnant kidney model has also been reported [78], suggesting a potential role in the progression of chronic kidney disease. While it has previously been reported that HB-EGF is induced during the development of poly (I:C)-induced lupus nephritis, macrophages and type I interferon were found to be the predominant contributors to the development of crescentic disease in this model [79]. Another study looked at the HB-EGF in the model of RPGN induced by anti-GBM serum and showed induction of expression of HB-EGF in the glomerulus [80]. However, this study did not examine the development of RPGN and showed an increased expression of HB-EGF in the hours following the injection of anti-GBM serum, that correlated with transient alterations of the glomerular capillary ultrafiltration coefficient and increasing arteriolar pre and post-glomerular resistance, resulting in a decrease in glomerular filtration rate [80]. In addition, the transactivation of EGFR by G protein-coupled receptors (GPCRs) is important for the vasoconstriction induced by angiotensin II [81] and endothelin-1 [69, 82], potential deterioration co-factors of the filtration barrier (Figure 1).

Fig. 1. Summary of the pathophysiological hypothesis in crescentic RPGN: upon immune-mediated injury of the endothelium and the filtration barrier, detrimental gene program is induced leading to de novo expression of HB-EGF in glomerular epithelial cells (podocytes and parietal cells). Activation of the EGFR on podocytes induces sustained intracellular signals leading to cytoskeleton rearrangement, loss of polarization, shedding of nephrin, effacement of foot processes, loss of glomerular permselectivity and subsequent proliferation and migration. Additionally, the EGFR cascade may synergize with ligands associated with vasculitis such as thrombin, endothelin and sphingosine-1-phosphate (S1P). Their respective GPCRs, protease-activated receptors (PARs), endothelin receptors (ETRs) and S1P receptors (S1PRs) may further amplify EGFR signalling through transactivation. The sequence or hierarchy of GPCRs and EGFR, their crosstalk and downstream mediators in this process are yet to be deciphered. Crosstalk is also envisioned at the level of cells as reciprocal influence between podocytes and surrounding cells may occur in RPGN.
Recent developments in glomerular pathophysiology: the HB-EGF/EGFR pathway promotes glomerular demolition in experimental RPGN

We have utilized both pharmacological and genetic models to demonstrate that HB-EGF activation of the EGFR plays a critical role in the development of RPGN in the murine anti-GBM nephrotoxic serum (NTS)-induced model.

HB-EGF expression was increased in mice with crescentic glomerulonephritis induced by the anti-GBM NTS model. This increased expression was largely limited to PECs and podocytes. Furthermore, de novo HB-EGF expression was associated with increased activation of the EGFR in glomeruli from mice after NTS injection as compared to non-immunized mice or HB-EGF-deficient mice [32]. In this NTS model of severe glomerular injury, HB-EGF deficiency prevented the development of ascites and renal leukocytic infiltrates prior to the appearance of patent crescentic proliferative lesions and interstitial fibrosis; therefore, this suggests that the HB-EGF/EGFR pathway acts very early to promote renal damage.

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 dynamic ring-like F-actin structures (RiLiS) formation (an assay for apical protrusions and apical migration), activation of the phosphatidylinositol 3-kinase (PI3K) pathway and migration [32] (Figure 1). While podocytes in vivo are terminally differentiated, the process of crescent formation in mice causes podocytes to lose polarity and to become migratory [24]. Interestingly, wound closure involves protrusion dynamics, cell polarity and cell–cell adhesion, but appears to be largely independent of proliferation [83]. Furthermore, thanks to inducible and specific deletion of the Egfr gene in adult podocytes, we observed significant alleviation of the course of fatal RPGN, indicating a pathophysiological role of the HB-EGF/EGFR pathway in these cells in vivo.

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.

Several other cell types may participate in early HB-EGF release. For example, HB-EGF has been reported in wound fluid [84] with high concentrations in conditioned medium of macrophages and macrophage-like U-937 cells [39]. HB-EGF has also been observed in T-cell subsets in vitro and in tumours and atherosclerotic plaques [85, 86], although its functions in these settings are unknown. Thus, because HB-EGF synthesis by leukocytes might provide additional ligand for EGFR in the kidney, we performed bone marrow transfer of Hbegf (−/−) or Hbegf (+/+) cells. Deficiency of HB-EGF restricted to bone marrow-derived cells neither limited albuminuria nor mitigated glomerular damage and renal failure in NTS-induced 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. Although there must be other pathways contributing to crescent formation, this is the first demonstration to our knowledge of a pathophysiological role for HB-EGF in a model of human disease and of a role for EGFR in a model of renal immune-mediated destruction.

Clinical-pathological correlation in human kidney biopsies

In line with measurement of induction of proHB-EGF messenger RNA expression in freshly sorted podocytes from nephritic mice, more HB-EGF protein was found in glomeruli of human kidneys with crescentic RPGN than in normal tissues or in a variety of non-crescentic glomerular diseases [32]. Expression of HB-EGF was mainly observed in glomerular epithelial cells, in parietal cells of Bowman’s capsule (Figures 2 and 3) and in tubules, a pattern similar to that observed by in situ hybridization in mouse kidneys after NTS administration and to that reported in a rat model of focal adhesive glomerular sclerosis induced by PAN or passive Heymann nephritis although no strong chronic up-regulation could be observed in these two latter models [75]. 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.

Fig. 2. Focal and selective immunoreactive HB-EGF expression is seen with a pattern typical of podocytes (Po) and glomerular PECs (representative pattern in pauci-immune vasculitis). Magnification ×1000.
relapse despite chronic immunosuppressive therapy displayed sustained glomerular elevation of HB-EGF expression, again consistent with a specific role for this mediator. These findings suggest a pathogenic pathway of RPGN in which HB-EGF can be induced in the glomerular epithelial cells to elicit EGFR tyrosine kinase activity primarily in podocytes, which in turn promotes glomerular inflammation, damage and renal failure. Although delineated in an experimental mouse model of RPGN, immunolabelling of human kidney biopsies suggests that the same pathway may be active in human disease.

**Potential proof of principle for the therapeutic use of HB-EGF or EGFR antagonists to tackle crescentic and immune-mediated glomerular disease**

Finally, the efficacy of two distinct EGFR inhibitors administered either shortly before anti-GBM serum infusion or 4 days after the beginning of RPGN suggests that recruitment of the HB-EGF-EGFR pathway is involved during the effector phase of the disease. The proof of principle for a potent therapeutic approach was provided by the efficacy of EGFR tyrosine kinase inhibition with a clinically available drug introduced after the onset of massive proteinuria and inflammation of the kidneys.

**Conclusion**

In conclusion, pre-clinical and human clinicopathological data unravelled a novel mechanism whereby autoimmune diseases may promote life-threatening rapid destruction of the kidneys. Immune-mediated glomerular injury leads to active and sustained pathophysiological recruitment of glomerular EGFR by HB-EGF (Figures 1, 2 and 3). From these studies, a major role of auto/paracrine HB-EGF/EGFR signalling in podocytes leading to a switch in podocyte phenotype can be deduced because either Hbegf gene deficiency or podocyte-specific deletion of Egfr significantly alleviated features of RPGN and, remarkably, the death rate [32]. These findings raise the possibility that specific EGFR inhibitors may be of therapeutic value for treating crescentic and other types of inflammatory glomerulonephritis, on top of current immunosuppressive regiments. Alternative strategies may be to block the activation of EGFR by interfering with HB-EGF, either by preventing its release with disintegrin and metalloprotease inhibitors or neutralizing its binding with an antibody. In addition, as HB-EGF is the diphtheria toxin receptor [47, 48], blocking its actions with non-toxic diphtheria toxin derivatives could represent an attractive therapeutic approach [87]. It may be thus envisioned to use an anti-HB-EGF or anti-EGFR strategy for initiation of therapy, in order to block crescent formation while immunosuppression is not fully active. Such a strategy.

Fig. 3. 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 four random subjects diagnosed with minimal-change disease (MCD), or with RPGN of various aetiologies, including microscopic polyangiitis (MP) and endocarditis (Endo). Magnification ×400.
could also be planned for patients who relapse despite immunosuppression or for those who need to have their immunosuppressive regimen decreased.

Future directions

Beyond the therapeutic implications of these findings, the results demonstrate a pivotal molecular pathway involved in several features of phenotypic switch in podocytes, namely: motility, proliferation and cytoskeleton reorganization. This may add a novel pathophysiological paradigm in nephrology, supporting a key role of podocyte phenotype switch in renal vasculitis caused by various immunological disorders. Furthermore, expression studies in mice and in human kidneys with various degrees of crescentic GN suggest that de novo glomerular expression of HB-EGF occurs primarily in PECs, then in podocytes ([32] and Figures 2 and 3). Thus, this could be the first demonstration of a molecular pathway mediating pathophysiological cross-talk between PECs and podocytes. Further studies will be required to determine whether the HB-EGF pathway in PECs is involved in RPGNs and other glomerulopathies. Several other questions remain. The triggers for Hbegf gene activation are still to be deciphered and further work will also be required to determine whether urinary HB-EGF might serve as a biomarker of glomerular disease activity.

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