Inhibition of the VEGF signalling pathway and glomerular disorders

Mario Ollero1,2 and Djillali Sahali1,2,3

1INSERM, U955, Equipe 21, Créteil, France, 2Université Paris-Est Créteil Val-de-Marne, Créteil, France and 3AP-HP, Groupe Hospitalier Henri Mondor–Albert Chenevier, Service de Néphrologie, Créteil, France

Correspondence and offprint requests to: Mario Ollero; E-mail: mario.ollero@inserm.fr

ABSTRACT

Anti-cancer therapeutic approaches targeting the vascular endothelial growth factor (VEGF) ligand (anti-VEGF) or inhibiting its receptors (RTKI) have recently been developed. In spite of the promising results achieved, a serious drawback and dose-limiting side effect is the development, among others, of renal complications. This encompasses two glomerular pathological entities, namely minimal change/focal segmental glomerulosclerosis and thrombotic micro-angiopathy, involving two distinct cell types, podocytes and endothelial cells, respectively. The mechanisms that link anti-cancer therapy by RTKI to podocyte dysfunction and nephrotic level proteinuria are still poorly understood. Nevertheless, recent findings strongly suggest a central role of RelA, the master subunit of NF-κB and c-mip, an active player in podocyte disorders. RelA, which is up-regulated following anti-VEGF therapy, is inactivated by RTKI, leading to c-mip over-expression in the podocyte. This results in severe alterations in the architecture of podocyte actin cytoskeleton and subsequent severe proteinuria. Hence, clarifying the mechanisms linking c-mip and RelA as key pathogenic factors represents a critical goal in the understanding of different glomerulopathies. In the context of VEGF-targeted anti-cancer therapy, the study of these mechanisms along with the molecular cross-talk between podocyte and endothelial cell constitutes the basis for the emerging field of onconephrology.

Keywords: c-mip, nephrotic syndrome, NF-κB, podocyte, receptor tyrosin kinase

VEGF TARGETING IN ANTI-CANCER THERAPY

Angiogenesis is acknowledged as a key process in the progression of solid tumours. Therefore, it is believed that inhibition of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) signalling pathways would lead to anti-angiogenic effects and prevent tumour progression. This has led to these two growth factors in becoming current targets of anti-cancer therapies [1, 2].

VEGF was identified as a heparin-binding peptide exerting mitogenic effects specifically on vascular endothelial cells [3]. VEGF is up-regulated in response to hypoxia, oncogene activation and cytokine stimulation, and its expression is associated with poor prognosis in several types of cancer [4, 5]. The biological functions of VEGF are mediated by its binding to one of the VEGF receptor tyrosine kinases (RTK), which include VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). The VEGF family comprises seven members, namely VEGF-A, -B, -C, -D, -E and placenta growth factor 1 and 2 (PlGF1 and PlGF2). VEGF-A (also referred to as VEGF) binds to VEGFR-1 and -2, while VEGF-C and -D bind to VEGFR-2 and VEGFR-3. VEGFR-2 has been recognized as the predominant receptor in angiogenic signalling [6, 7].

Structurally, RTKs consist of an extracellular ligand-binding domain, a trans-membrane region, and an intracellular kinase domain that mediates downstream signal transduction. Upon ligand binding, RTKs dimerize and undergo phosphorylation on their kinase domain, leading to the recruitment of adaptor
proteins that trigger intracellular signalling cascades participating in processes that regulate cell proliferation and survival, migration and metabolism [8]. Dysregulation of RTK signalling by mutation or by ectopic receptor or ligand overproduction has been connected to several aspects of tumour progression, including cell proliferation, survival, angiogenesis and tumour dissemination [9].

Therapeutic approaches targeting the VEGF ligand (anti-VEGF) or inhibiting its receptors (RTKI) have recently been developed. Several antagonists of VEGF signalling are being tested in clinical trials, including Bevacizumab (anti-VEGF monoclonal antibody), Aflibercept (VEGF trap) and RTKI such as Sunitinib, Imatinib, Sorafenib, Dasatinib and Quizartinib [10, 11]. Although RTKI are widely used as inhibitors of VEGF receptors, they also interfere with those recognized by other growth factors. These include PDGFRs, stem-cell factor receptor (c-kit), FMS-like tyrosine kinase-3 (Flt-3), b-raf and Bcl-Abl, characterized by a split kinase domain. Consequently, they are commonly named as multi-targeted RTKI and widely used in medical oncology practice [12–14]. In spite of the promising results achieved by this type of therapy, a serious drawback and dose-limiting side effect of anti-VEGF and RTKI is the development, among others, of renal complications representing 10–20% of total adverse consequences.

**RenaL SIDE EfFECTS OF VEGF-Targeted TherAPy**

The association between therapeutic inhibition of VEGF signalling and the occurrence of proteinuria and hypertension is widely established [15–18]. These dose-dependent side effects of VEGF-targeted therapy are often concomitant, and consequently proteinuria is considered as an independent risk factor for cardiovascular disease. In some cases, this proteinuria reaches the nephrotic-range level. Concerning the pathological entities associated, nephrotic proteinuria cases can be subdivided into minimal change nephrotic syndrome (MCNS), which may evolve to focal and segmental glomerulosclerosis (FSGS), whereas thrombotic micro-angiopathy (TMA) is found in glomerular and peri-tubular capillaries [18–21] (Figure 1). MCNS/FSGS has been extensively reported in patients treated with RTKI [22–25]. Interestingly, TMA lesions have been found more frequently in anti-VEGF than in RTKI therapies [11, 18, 21, 26], while MCNS/FSGS has been associated with both approaches, but less frequently in Bevacizumab-treated patients [27]. In our recent study, TMA was observed in 11 of 15 patients following Bevacizumab and VEGF-Trap therapies, while most of those receiving RTKI (Sorafenib, Sunitinib or Axitinib) developed MCNS/FSGS (Table 1). Remarkably, in this study, no patients developed both entities concomitantly. These lesions are reversible, as in most cases both proteinuria and biological parameters of TMA were resolved after therapy arrest, while renal function returns to basal level. In addition, the severity of these adverse effects seems dependent of the cumulative dose [18].

Moreover, podocyte modifications and endothelial lesions were, surprisingly, characterized by two distinct protein expression signatures [18]. On the one side, glomerular TMA resulting from anti-VEGF therapy was highlighted by increased expression of the RelA master subunit of the NF-κB transcription factor complex in both podocytes and endothelial cells, concomitant with no detection of c-mip, an active player in podocyte disorders [28]. On the other side, glomeruli from MCNS/FSGS individuals showed c-mip over-expression in podocytes, while RelA was not detected [18].

In summary, the two-faced nature of VEGF targeting-associated side effects in the glomerulus is double, in terms of both pathological entities and injured cell types. In addition, recent data point at c-mip expression and NF-κB-associated signalling as key elements in the understanding of the whole picture.

**A VIEW FROM THE ENDOTHELIAL SIDE**

A valuable approach to obtain mechanistic information about the consequences of inhibiting VEGF-associated signalling was the construction of a podocyte-specific VEGF deletion model. Mice engineered this way developed TMA in adulthood, confirming the role of podocyte-expressed VEGF in glomerular endothelium function [15]. In this model, thrombotic

**Figure 1:** Electron microscopy images of glomerular lesions associated with VEGF-targeted therapies. In normal glomeruli (control, left), glomerular basement membrane is thin, visceral epithelial cell foot processes are well juxtaposed and the fenestration of endothelium is well preserved. In TMA (centre), glomerular basement membrane exhibits a double contour formation, with sub-endothelial widening and cellular interposition without electron dense deposits. Circulating inflammatory cells are present in the capillary lumen. Podocyte alterations in MCNS (right), only detectable by electron microscopy, are characterized by fusion of foot processes and flattening of podocyte body, while endothelial fenestrations are preserved. Bars correspond to 1000 nm (left) and 2000 nm (center and right).
Table 1. VEGF-targeted therapies and associated lesions (from the Izzedine et al. study [18]).

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Total patients</th>
<th>MCNS/FSGS</th>
<th>TMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-VEGF</td>
<td>15</td>
<td>–</td>
<td>11</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>9</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>VEGF Trap</td>
<td>6</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>RTKI</td>
<td>15</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>11</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Axitinib</td>
<td>1</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>3</td>
<td>2</td>
<td>–</td>
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The increase in intra-renal angiotensin-II induced by proteinuria depends on NF-κB activation, according to a previous report [37]. By hindering not only the induction of pro-inflammatory cytokines but also the activation of the intra-renal renin–angiotensin system, inhibition of renal NF-κB activation may be therapeutically useful as a means of retarding the development and progression of renal damage associated with persistent proteinuria [37], as well as in the management of TMA associated with anti-VEGF therapy [18].

A SIGNAL TRANSDUCTION VIEW FROM THE PODOCYTE

The mechanisms that link anti-cancer therapy by RTKI to podocyte dysfunction and nephrotic level proteinuria are still unknown. Proteinuria has been reported as secondary to nephrectomy in renal cell cancer patients [38]. We have observed in our cohort that proteinuria occurs at least one year after nephrectomy, but immediately after VEGF-targeted therapy [18].

Since inhibition of VEGF-associated RTK seems to play a central role in the development of proteinuria, it can be inferred that VEGF signalling is a critical factor in the regulation of podocyte function. Actually, soluble VEGFR-1-induced blockade of VEGF signalling accelerates proteinuria in rats, which suggests a role of VEGF in maintaining podocyte integrity and function [39]. This was supported by the phenotypic observation of a conditional VEGF inactivation model, in which 100% of mice developed pronounced proteinuria [15].

Expression of VEGFR-1, VEGFR-2 and VEGFR-3 by human primary and cultured podocytes suggests an autocrine function of VEGF [40, 41]. However, it must be noted that VEGFR-2 expression in podocytes is still controversial. Compelling evidence suggests that VEGFR-2 is expressed in a differentiated mouse podocyte cell line [41, 42]. Though, another group has failed to detect VEGFR-2 in mouse podocytes in vivo, whereas its knocking down had no effect on glomerular development and function [43].

VEGFR-2 interacts in vitro and in vivo with the specific podocyte slit diaphragm marker nephrin, a key regulator of podocyte survival via Akt signalling [44]. Several signalling molecules have been shown to interact with VEGFR-2, including the regulatory P85 subunit of PI3-kinase [45], Fyn and Nck [46]. VEGFR-2 is rapidly phosphorylated in response to VEGF and leads to Fyn recruitment, which initiates a cascade of phosphorylation events involving Nck, PAC-2 and N-WASP, leading to actin polymerization and actin stress-fibre formation [46, 47] (Figure 2). This sequence of events seems specific of VEGFR-2 activation, as VEGF-C, the ligand for VEGF-3, reduces intracellular calcium concentration in human podocytes, induces MAPK phosphorylation but has no effect on Akt phosphorylation [48].

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Interestingly another protein, c-mip, has been found to interfere with the nephrin/Akt signalling pathway [49] (Figure 2). We have recently reported that c-mip abundance increases in podocytes of patients with acquired idiopathic nephrotic syndromes, including primary MCNS and FSGS, in which podocytes are the main target of injury [28]. Transgenic mice overexpressing c-mip in podocytes develop heavy proteinuria without any inflammatory lesions or cell infiltration. C-mip switches off podocyte signalling by preventing the interaction of nephrin with Fyn, thereby decreasing nephrin phosphorylation in vitro and in vivo. Moreover, c-mip inhibits the interaction between Nck and nephrin and between Fyn and N-WASP, potentially accounting for cytoskeletal disorganization and foot processes effacement. Furthermore, intravenous injection of a small interfering RNA targeting c-mip prevents lipopolysaccharide-induced proteinuria in mice [28]. These results suggest that c-mip plays a central role in podocyte dysfunction leading to proteinuria. This hypothesis is comforted by the increased c-mip expression upon RTKI treatment and its association with the subsequent development of MCNS/FSGS [18]. Therefore, Akt, which is up-regulated after nephrectomy in the remaining kidney as part of a compensatory mechanism, is blocked by RTKI via c-mip induction, which favours the development of proteinuria.

WHEN RELA MEETS C-MIP

We have recently shown that MCNS/FSGS lesions, which are mostly observed following RTKI therapy, are associated with high c-mip abundance, alongside with low RelA expression [18]. Evidence was provided that RelA binds to the c-mip promoter in vivo and in vitro and represses its transcription, while RelA knockdown in MEF cells releases this inhibition [18]. This could explain why TMA patients, which present increased RelA levels in both endothelial cells and podocytes, show no expression of c-mip in the latter and underlines RelA as a major negative regulator of c-mip transcription. The fact that RelA is constitutively expressed in podocytes could explain the lack of c-mip detection in normal glomeruli. Conversely, inhibition of NF-κB activity by RTKI therapy, which has also been reported by other authors [50], leads to c-mip over-expression [18] and induces MCNS/FSGS, reminding the phenotype of the transgenic c-mip mouse model [28].

A sequence of events is therefore established, starting with RTKI treatment, followed by RelA inhibition, c-mip induction and ending with podocyte alterations and severe proteinuria (Figure 3A). This raises the question of the molecular mechanisms connecting these events. Sorafenib tosylate and GW5074, two drugs targeting VEGFR-2/3, have been reported to inhibit NF-κB activation [50] by increasing IκBα levels and decreasing NF-κB phosphorylation at Ser276 in the brain cortex of a treated mouse model of Alzheimer’s disease. We found the same effect by Sorafenib on RelA phosphorylation in cultured mouse podocytes [18]. It has been hypothesized that Sorafenib could inhibit IκBα degradation by inhibiting the IκB kinase complex [50]. However, the underlying mechanism of RelA inactivation by RTKI treatment ultimately leading to c-mip induction still remains unclear.

There are other situations in which RTK inhibition leads to impaired signalling in podocytes. The slit diaphragm protein CD2AP, critical to podocyte integrity, has been shown to regulate SUMOylation-related inactivation of its parologue CIN85, which in turn down-regulates RTK activity via endocytosis [51]. Given the fact that CD2AP-deficient mice overexpress CIN85 in podocytes and develop nephrotic syndrome [52, 53], it would be tempting to hypothesize that the mechanism is shared, at least in part, by RTKI treatment. In fact, CDAP-invalidated podocytes show impaired PI3K/Akt and Erk signalling cascades [52]. Another RTK, the glial-derived
neurotrophic factor Ret, is up-regulated in podocytes in the Heymann nephritis and puromycin aminoglycoside models of proteinuria, but its knockdown leads to decreased Akt phosphorylation [54]. Likewise, inactivation of another RTK, the discoidin domain receptor 1 (DDR-1), also leads to podocyte dysfunction and proteinuria [55]. Therefore, it would be interesting to assess RelA and c-mip expression and function in these models. On the contrary, diabetic proteinuria is reversed by inhibition of VEGF signalling, suggesting dissimilar pathogenic mechanisms within the podocyte [56, 57]. Nevertheless, according to a recent report, inhibition of VEGFR-1 by a synthetic peptide aggravates diabetic nephropathy in db/db mice via disruption of the PI3 kinase/Akt axis and the FoxO3a and eNOS-NOx pathway [58].

In any case, the mechanisms down-stream of c-mip activation and resulting in cytoskeletal disruption, podocyte effacement and nephrotic proteinuria are still partially understood. Better knowledge of these mechanisms would represent a profound improvement in the understanding of nephrotic syndrome pathophysiology towards an effective therapy. The reported effects of RTKI treatment on the glomerulus show a leading way to future studies as an invaluable disease model.

**CONCLUSIONS: THE EMERGING FIELD OF ONCONEPHROLOGY**

VEGF-targeted anti-cancer therapies led to two pathological entities in renal glomeruli, namely MCSN/FSGS and TMA.
These seem to be distinctly associated with RTKI and anti-VEGF drugs, respectively (Table 1 and Figure 3B). Nonetheless, both types of lesions can be observed simultaneously with these therapies. The risk to develop MCNS/FSGS or TMA could depend on the presence of pre-existing vascular alterations as well as to nephron reduction before any of the two kinds of drugs is administered. Nonetheless, the hypothesis underlying these observations should be confirmed in a larger cohort of patients, where renal biopsies would constitute an extremely valuable material. Furthermore, other mechanisms not yet clearly identified could also be involved.

Nephrotic syndrome represents, according to some reports [8, 59], a relatively frequent renal adverse effect of RTKI that constitutes a serious complication, which may lead to drug withdrawal [15, 19, 21], in spite of their beneficial anti-cancer outcome. In addition, it contributes to the economic burden associated with renal cell carcinoma therapies.

NF-κB signalling represents another target in anti-cancer therapy. A combination of anti-NF-κB drugs and Sorafenib has been recently explored in the treatment of hepatocellular carcinoma [60]. In light of the recent findings on the cross-talk between RelA and c-mip, this type of intervention must be used cautiously. NF-κB inhibitors such as Vorinostat, Thalidomide or Bortezomib, could be administered shortly before initiation of VEGF-targeted therapy. They would probably prevent TMA by reducing the expression of cytokines and other proteins involved in pro-inflammatory pathways. The need for a thoughtful comprehension of the molecular mechanisms leading to the adverse response in glomeruli opens a field of investigation that could be named as onconephrology. In this context, it would be of capital importance to be able to detect both types of lesions at early stages after initiation of therapy but, unfortunately, no reliable biomarkers are available at present. Recent evidence suggests that RelA and c-mip levels in urine could be potential candidates. Proteomic studies in our laboratory are currently underway in this direction. Also, to clarify the role of c-mip as a key pathogenic factor at the cross-roads between MCNS and FSGS, and other potential podocytic pathologies, seems to constitute a fundamental goal of future research.

**CONFLICT OF INTEREST STATEMENT**

None declared. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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