Obstructive nephropathy: Insights from genetically engineered animals

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Obstructive nephropathy: Insights from genetically engineered animals. Congenital obstructive nephropathy is the primary cause for end-stage renal disease (ESRD) in children. An increasingly used animal model of obstructive nephropathy is unilateral ureteral obstruction (UUO). This model mimics, in an accelerated manner, the different stages of obstructive nephropathy leading to tubulointerstitial fibrosis: cellular infiltration, tubular proliferation and apoptosis, epithelial-mesenchymal transition (EMT), (myo)fibroblast accumulation, increased extracellular matrix (ECM) deposition, and tubular atrophy. During the last decade genetically modified animals are increasingly used to study the development of obstructive nephropathy. Although the use of these animals (mainly knockouts) has highlighted some pitfalls of this approach (compensation by closely related gene products, absence of temporal knockouts) it has brought important information about the role of specific gene-products in the pathogenesis of obstructive nephropathy. Besides confirming the important pathologic role for angiotensin II (Ang II) and transforming growth factor-β (TGF-β) in obstructive nephropathy, these animals have shown the complexity of the development of tubulointerstitial fibrosis involving a large number of closely functionally related molecules. More interestingly, the use of these animals has led to the discovery of unexpected and contradictory roles (both potentially pro- and antifibrotic) for Ang II, for ECM degrading enzymes matrix metalloproteinase 9 (MMP-9) and tissue plasminogen activators (PAs), for plasminogen activator inhibitor 1 (PAI-1), and for the adhesion molecule osteopontin (OPN) in obstructive nephropathy. Further use of these animals, especially in combination with pharmacologic tools, should help to better identify potential antifibrotic strategies in obstructive nephropathy.

Obstructive nephropathy and obstructive uropathy are often used names to describe renal disease leading to hydronephrosis caused by obstruction of the urinary tract. This pathology is common in infants due to congenital abnormalities of the urinary tract, most frequently posterior urethral valves and uteropelvic junction obstruction [1]. In children, obstructive nephropathy is the primary cause for end-stage renal disease (ESRD) and represents 16.1% of all pediatric transplantations in North America [2] while obstructive nephropathy is at the origin of 0.3% ESRD patients in the total North American population [3]. The incidence of obstructive nephropathy declines after childhood and re-appears around the age of 60 to 65 years, particularly in men caused by prostate disease [4]. In the adult kidney, ureteral obstruction leads to interstitial fibrosis and tubular atrophy, while urinary tract obstruction in the maturing kidneys also permanently impairs renal development [5].

Different animal models were used in attempts to study the pathophysiology of congenital obstructive uropathy (see [6] for an extensive review). Briefly, engineered mutation of genes such as Pax-2 [7], and angiotensin type 2 (AT2) receptor deficiency [8] or spontaneously appearing genetic modifications in rat [9] or mouse strains [10] can lead to hydronephrosis associated to tubular atrophy and interstitial fibrosis. Unfortunately, the cause of the hydronephrosis in these models was found not to be necessarily due to hydrostatic obstruction. Fetal ureteral obstruction can also be induced by surgery but experiments have to be performed using big animals such as sheep or monkeys. A promising study used fetal ureteral obstruction in the rhesus monkey [11] and analyzed renal function recovery as a function of obstruction time and/or the severity but also the level of the obstruction. The major, obvious, drawbacks of the use of big animals are the handling and housing problems and their price. Finally, partial ureteral obstruction (i.e., the situation that is most frequently encountered in humans) can be obtained by burying the left ureter into the psoas muscle, but this kind of surgery leads to a poorly reproducible obstruction making it hard to obtain homogenous animal groups.

Besides these congenital or partial obstruction models, an increasingly used and popular animal model (mainly rat and mice) of obstructive nephropathy is complete unilateral ureteral obstruction (UUO) which can be performed either in newborn [12] or in adult rats and mice.
Performing UUO in newborn rat and mice allows studying the effect of obstruction on renal development since in mice and rats, in contrast to humans, only 10% of nephrons are formed at birth with the remainder developing in the first postnatal week [13]. One can regret the limited number of studies using the UUO model in engineered animals in the first day of life.

The UUO maneuver leads to acute and complete obstruction of the ureter which as mentioned above is rarely found in humans but it has the advantage that it mimics, in an accelerated manner, the different stages of obstructive nephropathy leading to tubulointerstitial fibrosis: cellular infiltration, tubular proliferation and apoptosis, epithelial-mesenchymal transition (EMT), (myo)fibroblast accumulation, increased extracellular matrix (ECM) deposition, and tubular atrophy (Fig. 1). These different features of the pathology appear rapidly, all within around 1 week after the induction of the pathology and are highly reproducible from one experiment to another.

UUO: THE PREGENETIC ENGINEERING ERA

Before the use of genetically modified mice to study obstructive nephropathy, the UUO model, using gene expression analysis and pharmacologic tools, had already largely proven its usefulness by identifying a number of important molecules and processes involved in the pathogenesis of obstructive nephropathy [14]. For example, production of angiotensin II (Ang II) was identified, acting via transcription factor nuclear factor NF-κB (NF-κB), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β), as an important cause of fibrosis. Consequently, blockage of the renin-angiotensin system (RAS) by angiotensin-converting enzyme (ACE) inhibition is significantly reducing renal fibrosis in this obstructive nephropathy model [15–17]. However, as discussed later, recent evidence using genetically engineered mice has shown that angiotensin type 1 (AT1) receptor stimulation can also be beneficial in UUO [18]. Similarly, endogenous inhibition of ECM degrading enzyme activity was incriminated for a long time as an important cause...
**Table 1.** Effects of unilateral ureteral obstruction (UUO) on the different stages of development of obstructive nephropathy

<table>
<thead>
<tr>
<th>Inflammation</th>
<th>Proliferation</th>
<th>EMT</th>
<th>Apoptosis</th>
<th>Tubular atrophy</th>
<th>Fibrosis</th>
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<td>E, P, L-selectin knockoutra</td>
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Abbreviations are: tPA, tissue plasminogen; PAI-1, plasminogen activator protein-1; uPAR, urokinase plasminogen activator receptor; TIMP, tissue inhibitors of matrix metalloproteinase; AT1, angiotensin type 1; AT2, angiotensin type 2; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; =, no difference; −, decrease; +, increase. Observations on day 5 or 7 after UUO compared to wild-type mice.

*a*Results after 28 of UUO; *b*21 days after UUO; *c*14 days after UUO.

for ECM accumulation [19] in UUO but the use of knock-out mice has changed this vision [20, 21].

**UUO: INSIGHTS FROM GENETICALLY ENGINEERED ANIMALS**

In the current review we attempt to describe the contribution of genetically engineered animals (mainly gene-knockouts (see Table 1) but also some animals over-expressing specific proteins) to the current understanding of the development of interstitial fibrosis in obstructive nephropathy.

A major drawback in knockout mice is the apparent redundancy of biologic molecules. Possible compensation during development and during the induction of the pathology might false the interpretation of data obtained using knockout mice. Effects of knockouts that are observed in the initiation phase of the pathology but fade out when the pathology progresses are quite frequent and might be explained by adaptation. It is therefore important to confirm observations, if possible, by pharmacologic knockouts. Another important issue is that one should compare the results of the knockout mice with adequate control strains (either control mice obtained by repeated (eight to ten) backcrosses to obtain the genetic background of one of the parent strains or control mice obtained by parallel breeding of both the control and the knockout strain on a mixed genetic background) since the different mouse strains are not likely to produce identical responses to the pathologic insults. Finally, it is preferable to examine the kidney structure of genetically engineered animals before undertaking UUO. Indeed, some knockout animals, including the angiotensinogen, AT1 [22], or AT2 [8] receptor knockout mice generate animals with impaired renal development and histologic lesions that look like to those obtained after an UUO.

Rather than simply reporting all published data, we have tried to describe, when possible, the more unusual or unexpected results obtained using genetically modified animals. The paper is divided into the different stages of the development of obstructive nephropathy (Fig. 1). Furthermore, there are two additional sections on two “molecules” currently identified as the main actors in the fibrotic process: the RAS and TGF-β. For a quick
appreciation of the effect of a specific knockout on a
typical parameter during the development of obstructive
nephropathy, the reader is referred to Table 1.

CELLULAR INFILTRATION

Macrophages are rare in the healthy renal cortex [23].
However, a few hours after ureteral obstruction a large
number of blood-derived macrophages accumulate in
the tubulointerstitial space [4]. This cellular infiltration
(Fig. 2) is preceded by local expression of chemokines [24,
25], chemokine receptors [24], and adhesion molecules
[26, 27]. Despite strong correlative data, no functional
studies describing the role of these molecules in UUO-
induced cellular infiltration have been described. The use
of knockout mice allowed discrimination between simple
fortuitous induction and genuine involvement in cellular
recruitment of some of these molecules in UUO-
induced cellular infiltration have been described. The use
of knockout mice allowed discrimination between simple
fortuitous induction and genuine involvement in cellular
recruitment of some of these molecules in UUO-induced
nephropathy.

Adhesion molecules: Osteopontin and selectins

Osteopontin (OPN) is an adhesion molecule that binds
to macrophages and mediates their adhesion in vitro.
Injection of OPN neutralizing antibody in rats or mice
significantly reduces macrophage infiltration induced by
a chemotactic peptide [28]. Furthermore, OPN is up-
regulated in UUO [25]. UUO using OPN null mu-
tants (OPN−/−) confirmed the important role of OPN in
macrophage recruitment since these mice have around
75% less tubulointerstitial macrophages than their wild-
type littermates [29]. This difference was translated into
significantly less TGF-β and tubulointerstitial collagen
expression. A surprising observation in this study was
the increased number of interstitial and tubular apoptotic
cells in OPN−/− mice. However, no correlation could be
observed between the number of macrophages and the
number of apoptotic cells suggesting that OPN can di-
rectly act as a cell survival factor. This newly discovered
inhibitory effect of OPN on apoptosis was confirmed on
cultured renal epithelial cells. The effects of OPN might
be mediated by the CD44 receptor since OPN is one of
the major ligands of this receptor which is found on
macrophages and lymphocytes and is, as OPN, involved in
cell migration [30]. Indeed CD44 knockout mice (CD
−/−) responded in a similar way to UUO: reduced cellular
infiltration (around 25% reduction), increased apo-
ptosis, and decreased tubulointerstitial fibrosis compared to
wild-type mice [31].

Another group of adhesion molecules, selectins, were
studied for their involvement in cellular infiltration dur-
ing the development of obstructive nephropathy in 2-day-
old mice [32]. Three types of selectins exist: E-selectin
which is expressed on endothelial cells, P-selectin is ex-
pressed on endothelial cells and platelets, and L-selectins
is expressed on leukocytes. Triple selectin (EPL−/−)
and L-selectin (L−/−) knockout mice displayed signifi-
cantly less (56% and 32%, respectively) macrophage in-
filtration in the obstructed kidney than wild-type mice.
However, in contrast to OPN knockouts, the degree of
macrophage infiltration was correlated to the degree of
apoptotic tubular cells in these selectin knockout mice.
Both macrophage infiltration and apoptotic tubular cells
were lower in EPL−/− and L−/− mice than in wild-type
mice resulting at day 14 post-UUO in reduced tubular
atrophy and interstitial fibrosis. Another study showed
the importance of L-selectin in macrophage recruitment
but this time knockout mice for sulfatide were used, a
sulfated glycolipid that is an important L-selectin lig-
and [33]. The expression of sulfatide is modified during
obstructive nephropathy. In the normal kidney, sulfatide
is expressed in distal tubules; however, no leukocyte traf-
fic in this area is observed, but during UUO, sulfatide

Fig. 2. Hypothetic representation of cellular
infiltration in unilateral ureteral obstruction
(UUO) based on data obtained with knock-
out mice. UUO induces macrophage inflilt-
ration in the tubulointerstitium. In UUO,
the interaction between L-selectin and sul-
fatide seems to mediate the initial contact
(rolling) between macrophages and the vascu-
lar endothelium. These rolling macrophages
are exposed to adhesion molecules like
chemokines and osteopontin expressed on
endothelial cells. The chemokines bind to
chemokine receptors (in UUO mainly the
CCR1) on the macrophages. Osteopontin
binds to the CD44 receptor. This results in
firm adhesion and transendothelial migration
of macrophages. The role of other impor-
tant molecules (including intercellular adhe-
sion molecule-1 (ICAM-1), vascular cell ad-
hesion molecule-1 (VCAM-1), and monocyte
chemotactrant protein-1 (MCP-1)]) involved
in cellular infiltration shown to be induced in
UUO has not been studied yet and should give
a more complete picture of cellular infiltration
in UUO.
Chemokine receptors

CCR1 and CCR5 are chemokine receptors [35] which are induced by ureteral obstruction [24]. CCR1 and CCR5 knockout mice have helped to establish that the CCR1 receptor is responsible for leukocyte recruitment after UUO and not the CCR5 receptor [36]. UUO in CCR1 knockout mice resulted in reduced leukocyte recruitment, reduced EMT, and significantly lower tubulointerstitial fibrosis than their control littermates. The use of an antagonist confirmed the effect of the CCR1 receptor is responsible for leukocyte recruitment after UUO and not the CCR5 receptor [36]. UUO in CCR1 knockout mice resulted in reduced leukocyte recruitment, reduced EMT, and significantly lower tubulointerstitial fibrosis than their control littermates. The use of an antagonist confirmed the effect of the CCR1 knockout. An interesting application of knockout mice involved the transfer of cells between knockout and wild-type strains to show the involvement of CCR receptors in macrophage and T-cell recruitment in the inflammatory phase of experimental hydronephrosis. Purified F4/80 macrophage and CD8 T cells of wild-type, CCR1−/− and CCR5−/− mice were prepared and injected in the circulation of obstructed wild-type mice. Three hours after injection, in vivo renal cell recruitment was reduced for CCR1-deficient cells compared to CCR5-deficient or wild-type cells confirming the importance of this CCR1 cellular recruitment. A similar cell transfer between wild-type and knockout animals also revealed an interesting feature of the AT1 receptor in UUO (see below).

Other than confirming the role of a number of molecules involved in cellular infiltration in UUO and attributing an antiapoptotic function to the adhesion molecule OPN, the knockout animals showed that knock-out of various molecules reduced but never completely eliminated cellular infiltration showing that different, independent pathways, are additive in leukocyte recruitment during UUO. Other macrophage recruiting molecules, including monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) have been shown to be induced during ureteral obstruction [37, 38].

PROLIFERATION/APOPTOSIS

Tubulointerstitial disease is associated with cell proliferation and apoptosis; however, over time an imbalance between these two processes leads to unchecked apoptosis resulting in progressive cell loss and tubular atrophy [39]. Cyclins and their catalytic partners cyclin-dependent kinases (CDK) positively control cell proliferation. Negative regulators of the cell cycle include the cyclin-dependent kinase inhibitors (CKI). CKIs inhibit the cell cycle at multiple checkpoints through inactivation of cyclin-CDK complexes [40]. Two families of CKIs exist: the INK4 family and the CIP/KIP family. The latter includes p21WAF1/CIP1, p27KIP1, and p57KIP2 and inhibits cyclin-CDK complexes during both G1 and S phases of the cell cycle. p21WAF1/CIP1 and p27KIP1 knockout mice are available and were used to explore the role of these CKIs in cell proliferation and apoptosis in experimental obstructive nephropathy [41, 42]. As expected for a CKI knockout, UUO-induced tubular cell proliferation was significantly higher in p27KIP1 knockout mice at day 3 than in the wild-type animals [41]. Surprisingly, UUO-induced tubular cell proliferation was not modified in the other CKI knockout, p21WAF1/CIP1, but in these mice interstitial myofibroblast proliferation was temporally higher compared to wild-type mice [42]. However, no effect of both CKIs knockout on UUO-induced tubulointerstitial fibrosis was observed. These minor effects of both CKI might be explained by cell or renal compartment specific CKI effects (e.g., epithelial vs myofibroblasts or differential effects of UUO on renal compartments as was shown by a recent UUO study on neonatal mice [43]) and functional redundancy (gene-knockout compensation) as discussed above.

Before the use of genetically engineered animals, apoptosis was considered to be a crucial lesion in UUO [39, 44, 45]. The transcription factor p53, a well-known tumor suppressor gene, is induced in UUO [46]. Activated p53 can induce either cell cycle arrest or apoptosis. After DNA damage, p53 blocks the progression of the cell cycle until the DNA can be repaired, or, if damage is extensive the cells enter into p53-induced apoptosis [47]. p53 knockout mice were used to determine the role of this gene and apoptosis in the development of tubulointerstitial fibrosis in obstructive nephropathy [48]. p53 knockout mice (homo- and heterozygote) displayed between 50% and 70% less tubular and interstitial cell apoptosis which was accompanied by a reduced interstitial volume. As was observed with knockout mice for cellular infiltration described above, the p53 knockout exhibited attenuation not elimination of apoptosis, suggesting that UUO-induced renal cell apoptosis proceeds through both p53-dependent and -independent pathways. Interstitial volume was lower in p53-deficient mice at day 7; however, no difference was observed at day 15 to 30, although tubular and interstitial cell apoptosis were still different at day 15. In contrast, tubular dilatation was correlated with reduced apoptosis.

These above studies on the role of cell proliferation and apoptosis using gene knockout animals were all but conclusive about the role of these processes in the development of tubulointerstitial fibrosis. The cell or renal
Fig. 3. Proposed role of epithelial-mesenchymal transition (EMT) in ureteral obstruction. Unilateral ureteral obstruction (UUO) induces EMT which contributes significantly (36%) to the appearance of interstitial myofibroblasts. Myofibroblasts are the main source of profibrotic and inflammatory cytokines leading to matrix accumulation. The remainder of the fibroblasts comes from bone marrow (14% to 15%) and probably local fibroblast proliferation and activation [98]. EMT involves four key events leading to epithelial cell migration and invasion into the interstitium and transition into fibroblasts [49]. Interestingly one of these events, tubular basement membrane degradation, is blocked in tissue plasminogen activator (tPA) knockout mice (in contrast to what was expected for a tPA knockout) thereby blocking EMT and subsequent tubulointerstitial fibrosis. Abbreviations are MMP-9, matrix metalloproteinase-9; TGF-β, transforming growth factor-β; α-SMA, α-smooth muscle actin.

**FIBROBLAST ACCUMULATION**

Irrespective of the initial cause, the development of tubulointerstitial fibrosis is characterized by the appearance of activated [α-smooth muscle actin (α-SMA)-positive] fibroblasts (also called myofibroblasts). It is widely accepted that these cells are the principal actors responsible for ECM deposition [49]. The appearance of myofibroblasts is also a prominent feature in the obstructed kidneys during ureteral obstruction [49]. While the profibrotic role of myofibroblasts in renal fibrosis is widely accepted, their origin(s) remains largely unclear. Three hypotheses have been proposed regarding the origin of these cells during fibrogenesis (Fig. 3). One hypothesis proposes that marrow stromal cells are the progenitors for tissue fibroblasts that, via the circulation, populate peripheral organs [50]. The second hypothesis suggests that the myofibroblasts are derived from local activation of resident interstitial fibroblasts [49]. Finally, the third hypothesis proposes EMT for the generation of local interstitial fibroblasts [51]. Tubular EMT is a process in which renal tubular cells lose their epithelial phenotype and acquire new features characteristic of mesenchyme [49]. Using a combination of bone marrow chimeras, transgenic reporter mice and the fibroblast-specific protein-1 (FSP-1) as a fibroblast marker, it was shown that interstitial kidney fibroblasts arise from both EMT (36%) and bone marrow (14% to 15%) while the rest of the fibroblasts are most likely contributed by local fibroblast proliferation during UUO. [52]. Thus EMT appears to be an important contributor to the appearance of fibroblasts and consequently fibrogenesis in obstructive nephropathy and an interesting target in treatment of tubulointerstitial fibrosis.

EMT involves four key events (Fig. 3): (1) loss of epithelial adhesion properties, (2) de novo α-SMA expression and actin reorganization, (3) disruption of the tubular basement membrane, and (4) enhanced cell migration and invasion capacity. TGF-β is currently the sole factor described able to induce all four steps [53]. Two molecules: hepatocyte growth factor (HGF) [54] in the UUO-model and bone morphogenic protein-7 (BMP-7) [55] in a model of tubulointerstitial fibrosis induced by nephrotoxic serum have been shown to reverse (that is, after establishment of renal fibrosis), via TGF-β inhibition, EMT, and subsequent renal fibrosis.

**ECM ACCUMULATION**

Tubulointerstitial fibrosis is the result of an imbalance between ECM synthesis and degradation. The pathologic tubular ECM is composed of collagen I, III, and IV, V, VII, XV, laminin and fibronectin [56]. ECM degradation is thought to be largely dependent on the plasminogen system [plasminogen, plasminogen activators]...
(PAs), and plasmin) primarily via the activation of latent matrix metalloproteinase (MMPs). The serine protease plasmin is generated from its precursor plasminogen by two different types of PAs: urokinase type (uPA) and tissue type (tPA). Plasmin can directly degrade matrix proteins fibronectin, laminin [57], proteoglycan [58], and type IV collagen [59] and activates pro-MMP-1 (interstitial collagenase [60]) and pro-MMP-3 (stromelysin-1) [61]. MMP-3 then activates MMP-9. Independently of plasmin, uPA was shown to activate MMP-2 via membrane type 1 (MT)-MMP [62]. In theory, the combined activity of these enzymes is sufficient to degrade the ECM but knockout mice for some of the components of the ECM degrading enzyme pathways showed other, important, roles for these proteins in the pathology of ureteral obstruction (Fig. 4, gray area).

Deficiency in tPA, one of the two main plasmin-forming enzymes should, in theory, aggravate obstruction induced tubulointerstitial fibrosis; however, tPA-deficient mice showed decreased ECM deposition in the tubulointerstitium [21]. By a yet unknown mechanism, deficiency of tPA was able to selectively block EMT via reduced expression of MMP-9 in renal interstitial fibroblasts thereby most probably reducing damage of the tubular basement membrane (Fig. 3) and thus inhibiting EMT. In addition, tPA deficiency did not modify plasmin nor uPA activity in control and obstructed kidneys, suggesting absence of an effect of the knockout on the ECM degrading capability of these mice. The absence of a decreased plasmin activity in these tPA knockout mice might be explained by the fact that plasminogen is present in high concentrations in plasma (2 l mol/L) and that about 40% of this plasminogen is present in extravascular sites [63]. Therefore the plasminogen transforming activity of one PA (i.e., uPA in the tPA knockouts) might be sufficient to maintain wild-type plasmin levels. Thus, in contrast to what was expected, the tPA knockouts unmasked a potential aggravating role for tPA in EMT but did not provide information about the role of this enzyme in plasmin generation for ECM degradation in the pathogenesis of tubulointerstitial fibrosis.

Another study seems to confirm the absence for a role of plasmin in ECM degradation. This time it was not the PAs that were studied but their main physiologic inhibitor plasminogen activator protein-1 (PAI-1) [64]. PAI-1 is up-regulated in almost all known renal diseases, including UUO [65] showing a casual relationship between the pathogenesis of renal disease and this molecule. Classically, it was thought that inhibition of the PA activity by
PAI-1 reduced plasmin production and thus ECM degradation. Indeed, UUO in PAI-1 knockout mice showed that these mice had significantly lower tubulointerstitial fibrosis than their control littermates [20]. But neither the mean renal PAs nor plasmin activities were increased in PAI-1−/− mice compared to PAI-1+/+ mice. Surprisingly, it was the number of interstitial macrophages that was dramatically lower in PAI-1 knockout mice which was accompanied by less interstitial myofibroblasts and significant reductions in mRNA levels for TGF-β and procollagens I and III. This suggests that in this model PAI-1 is playing a proinflammatory rather than an anti-inflammatory role.

The uPA receptor (uPAR) is a multifunctional protein that localizes pericellularly and binds components of the urokinase plasmin activation system, including uPA and PAI-1. Binding of PAI-1 to the uPA-uPAR complex promotes internalization and degradation of both uPA and PAI-1 [64]. The uPAR receptor might play a role in the generation of active plasmin and thus in the renal fibrogenic response. uPAR knockout mice were less resistant to UUO-induced renal tubulointerstitial fibrosis than wild-type mice which was accompanied by reduced renal uPA, tPA and possibly MMP-9 activity [66]. Surprisingly plasmin activity was not lower in uPAR knockouts, which was attributed in this paper to improper assay conditions, which seems likely especially since plasmin activity was also not modified after obstruction in these experiments in contrast to earlier experiments of this laboratory [20]. An interesting observation in these experiments is decreased HGF expression in after obstruction in uPAR−/− mice compared to uPAR+/+ mice. uPA is known to activate HGF [67]. The well-known antifibrotic activities of HGF might also contribute, independently of an effect of the uPAR knockout on plasmin, to the observed differences.

Another control point of the ECM-degrading machinery is at the level of MMP activity inhibition. This is established by tissue inhibitors of MMP (TIMPs). TIMPs, especially TIMP-1, are up-regulated during UUO-induced hydronephrosis [68, 69]. However, knockout mice for TIMP-1, the inhibitor of MMP-9, did not attenuate UUO-induced tubulointerstitial fibrosis [70] although MMP-9 activity was higher in kidneys of obstructed TIMP-1 knockout mice. Compensation by other TIMPs (TIMP-2 and -3) or PAI-1 or the possibility that inhibition of intrinsic MMP activity does not constitute a profibrogenic event (i.e., MMP-9 inhibition resulting in less EMT as described above) in the kidney might explain the absence of an effect of the TIMP-1 knockout.

In conclusion, studying knockout mice for the “ECM degrading enzyme system” has taught us more about less conventional roles of these proteins (inflammation and basal membrane degradation) than about their role in interstitial ECM degradation.

RAS

A number of renal diseases and their progression to ESRD involve Ang II making it an important target in progressive renal disease. The different components of the RAS are present in the kidney [71] and as described above, Ang II and the RAS have been known for a long time to be involved in the etiology of obstructive nephropathy [72, 73]. Ang II, formed from its precursor angiotensinogen by the subsequent action of renin and ACE (the major angiotensin I to II transforming enzyme), activates two G protein–coupled receptors the AT1 and AT2 receptor. Ang II signaling through the AT1 receptor results in vasoconstriction, cell proliferation, and fibroblast activation while its counterpart, the AT2 receptor, generally results in vasodilatation, inhibition of cell proliferation and increased apoptosis [74]. It is generally accepted that stimulation of the AT1 receptor is inducing the deleterious effects of the RAS. AT1 and AT2 knockout and genetically engineered mice with zero to four copies of the angiotensinogen gene exist. These animals were explored to determine the role of the RAS in experimental hydronephrosis and the results of these studies have in general confirmed the important role of the RAS in the pathology of obstructive nephropathy. In mice with zero to four copies of the angiotensinogen gene, UUO-induced renal interstitial collagen content increased linearly with increasing angiotensin expression (up to two copies and reaching a plateau above two copies of the angiotensinogen gene [75]). This effect seems to be mediated by the AT1 receptor since AT1a receptor (the predominant form of the AT1 receptor in mice) knockout mice develop significantly less UUO-induced tubulointerstitial fibrosis [676]. In contrast, AT2 knockout display accelerated tubulointerstitial collagen deposition after UUO [77]. The deleterious effects of Ang II are thus most probably mediated by the AT1 receptor. It appears that many of the effects of AT1 receptor stimulation in tubulointerstitial fibrosis are mediated by TGF-β1, TNF-α, and activation of NF-κB (Fig. 5) [78]. Angiotensinogen gene expression is stimulated by NF-κB and since AT1 receptor stimulation induces NF-κB activation this provides a positive feedback loop to probably maintain high Ang II levels under pathologic conditions [79]. NF-κB is also strongly involved in TNF-α induction during UUO [78] while TNF-α itself is a strong inducer of NF-κB expression [80] providing an additional positive feedback loop in maintaining high NF-κB activity during UUO. The interplay between Ang II and TNF-α was studied in more detail using a combination of AT1a and TNF receptor (TNFR1 and TNFR2) knockout mice and pharmacologic tools [81]. It was shown that the double TNF-α receptor knockout displayed around 40% less tubulointerstitial fibrosis than the wild-type mice comparable to what was observed observed for the AT1a knockout mice. This level of fibrosis in double TNF-α receptor
knockout mice was further lowered with about 25% by enalapril treatment (an ACE inhibitor) suggesting both Ang II–dependent and –independent activation of the TNF-α system. By studying TGF-β expression during UUO, these analyses combining knockouts and pharmacology also showed that levels of this cytokine are almost exclusively controlled by Ang II and do not involve TNF-α–dependent pathways.

The above studies using classic knockout mice on the RAS system confirmed the role for the AT1 receptor as an important mediator of ECM accumulation during UUO. However, an elegant study using bone marrow transplantation from AT1 knockout mice to wild-type mice showed that AT2 receptors can also participate in the reduction of renal fibrosis in obstructive nephropathy [18]. When the bone marrow of wild-type mice was replaced by bone marrow of AT1 receptor knockout mice, these mice developed, in contrast to what was expected, more severe interstitial fibrosis than non transplanted wild-type mice. Mice transplanted with AT1 receptor knockout bone marrow had significantly lower numbers of peripheral blood monocytes and macrophage progenitors in bone marrow with impaired phagocytic capabilities compared to wild-type macrophages. It is suggested that the reduced phagocytic capabilities of the AT1 receptor knockout bone marrow–derived macrophages are at the origin of this increased interstitial fibrosis by decreased phagocytic clearance of ECM components and apoptotic cells in these mice. This combined knockout and bone marrow transplantation approach has thus allowed dissociation between macrophage infiltration and renal fibrosis which is not possible in “simple” knockouts and presents a drawback of classic nontemporal and nonspatial knockouts to study complex pathologic processes.

ACE inhibition has shown long before the use of genetically engineered animals to be very efficient in reducing tubulointerstitial fibrosis in experimental hydronephrosis [15–17]. Besides blocking the conversion of Ang I into Ang II, ACE inhibitors also act on the kinin-kallikrein system by inhibiting the degradation of bradykinin [82], which suggests a role for bradykinin in the reduction of UUO-induced renal fibrosis and has opened research into the role of bradykinin and related peptides in obstructive nephropathy (Fig. 4). Bradykinin acts on a G protein–coupled receptor, the bradykinin B2 receptor. Bradykinin B2 receptor knockout mice and transgenic rats expressing increased endogenous bradykinin levels [TGR (hKLK1) rats, overexpressing the bradykinin producing enzyme kallikrein] were used to determine the role of bradykinin and its B2 receptor in experimental hydronephrosis [83]. UUO-induced tubulointerstitial fibrosis was twofold higher in bradykinin B2 receptor

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**Fig. 5. The central role for nuclear factor-xB (NF-xB) in angiotensin II profibrotic effects in unilateral ureteral obstruction (UUO).** Angiotensin II (AII) accumulation during UUO stimulates two G protein–coupled receptors, the angiotensin type 1 (AT1) and angiotensin type 2 (AT2). Although AT2 knockout mice display less tubulointerstitial fibrosis, stimulation of this receptor and the AT1 receptor induces NF-xB activation which translocates to the nucleus and activates a vast panel of proinflammatory and profibrotic cytokines leading to sustained inflammation and matrix production. NF-xB activation is also leading to at least two positive autocrine regulation loops by the increased expression of tumor necrosis factor-α (TNF-α) and angiotensinogen. IκB is inhibitor of NF-xB.
knockout mice compared to wild-type mice and significantly lower in transgenic TGR (hKLK1) than in wild-type rats. This suggested that bradykinin via B2 receptor activation was antifibrotic. No effects of the knockout were observed on cell proliferation and fibroblast activation and on gene expression of a large number of genes involved in the development of tubulointerstitial fibrosis. However, the activity of proteolytic enzymes was significantly lower (PAs, tPA and uPA, and MMP-2 activities) in the obstructed kidneys of B2 receptor knockout compared to wild-type mice. This suggested that the cascade bradykinin/B2 receptor/PA/MMP-2 was involved in the lower level of tubulointerstitial fibrosis in wild-type compared to B2 receptor knockout mice. Finally, the bradykinin receptor knockout mice were used to determine the role of bradykinin in the beneficial effects of ACE inhibition on experimental hydronephrosis [84]. Surprisingly, although ACE inhibition lowered significantly renal fibrosis, no difference was observed between the degree of tubulointerstitial fibrosis in ACE inhibitor-
including experimental and human hydronephrosis [89]. TGF-β1 (the main TGF-β isoform) knockout mice are not viable and die at midgestation [90]. TGF-β synthesis is strongly induced by Ang II and the profibrotic actions of TGF-β are thought to involve reduction of MMP expression, increased TIMP and PAI-1 expression, and increased synthesis of matrix proteins. However, the mechanism by which this occurs is not clear. UUO in knockout mice of molecules interacting with TGF-β or involved in TGF-β signaling helped in better understanding of these mechanisms.

TGF-β activates transmembrane receptor serine/threonine kinases which transduce downstream signals to cytoplasmic latent transcription factors called Smads. Smad2 and 3 are directly phosphorylated by one of these receptors after which they bind to Smad4 before nuclear translocation where they act as transcriptional regulators [91]. Unlike, TGF-β and Smad2, Smad3 knockout mice are viable although with a reduced life span up to 6 months due to impaired mucosal immunity [92] and has allowed to study the role of TGF-β signaling in experimental hydronephrosis [93]. Smad3 knockout mice are protected against UUO-induced tubulointerstitial fibrosis which is accompanied by reduced monocyte influx, EMT, and collagen deposition [93]. The results on Smad3 null mice and complementary experiments in vitro show that it is probably the effect of Smad3 on EMT that is the main profibrotic factor. Furthermore, Smad3 is essential for TGF-β amplification since TGF-β levels are extremely low after UUO in Smad3 null mice compared to wild-type mice. Finally, it was shown that the effect of Smad3 on monocyte accumulation further stimulated EMT. This results in a TGF-β/Smad3–induced profibrogenic myofibroblast population which is probably responsible for the larger part of UUO-induced tubulointerstitial fibrosis.

TGF-β is maintained in a latent form and can be activated by binding to αvβ6 integrin. Experimental hydronephrosis in αvβ6 integrin null mice resulted in significantly lower tubulointerstitial fibrosis [94]. This decrease in interstitial fibrosis was accompanied by lower TGF-β, PAI-1, and collagen I and III mRNA levels suggesting that interruption of TGF-β activation can protect against tubulointerstitial fibrosis. However, the authors of this study also explored a possible TGF-β independent pathway to tubulointerstitial fibrosis by treating the αvβ6 integrin knockout mice with Ang II, which increased the collagen concentration to levels comparable to obstructed wild-type mice. Interestingly, Ang II infusion only mildly increased active TGF-β levels and did not change Smad2 levels in αvβ6 integrin knockout mice but significantly induced PAI-1 expression. These data thus suggest TGF-β independent profibrotic effects of Ang II with both pathways triggering PAI-1 expression and resulting in increased interstitial fibrosis. The Ang II–induced TGF-β independent pathway for PAI-1 induction in UUO remains to be elucidated.

Other, small molecules interacting with TGF-β such as decorin or endoglin can modify its action and knockout out mice for both molecules have shown a role for decorin [95] but not for endoglin [96] in the development of tubulointerstitial fibrosis.

CONCLUSION

Genetically modified animals have made a major contribution to our current understanding of the pathogenesis of obstructive nephropathy. These animals, when subjected to UUO, have yielded important information about the beneficial and deleterious (or, controversially, both “beneficial and deleterious”) role of a specific gene product. Besides confirming the role of key-mediators in obstructive nephropathy, such as TGF-β and Ang II in macrophage infiltration, (myo)fibroblast accumulation and ECM accumulation, the use of genetically engineered animals has also modified our current vision of concepts that were accepted to be true in obstructive nephropathy (i.e., the beneficial and deleterious role of Ang II, the deleterious role of MMP-9, role for PAI-1 in macrophage recruitment).

The use of knockout mice has also shown the limitations of genetically modified animals. Especially compensation during development by closely related gene products makes interpretation of the results with knockout mice difficult. This problem might be circumvented by temporal knockouts (i.e., Cre-Lox systems) although a pharmacologic knockdown is often easier to achieve. Another problem of genetically modified animals is the fact that these animals do not allow to study reversibility of the pathology. This is an important issue since renal fibrosis is often already present in an advanced state when renal disease is detected. Again temporal knockouts or pharmacologic knockdown are good alternatives.

The use of genetically modified animals, mainly knockout of a single gene, has also underlined the complexity and the interconnection of the different events that lead to hydronephrosis. It shows that pharmacologic treatment when aiming at one molecule at a time is probably not always the good strategy for treatment. These studies have taught us that when blocking the RAS, one decreases profibrotic TGF-β production but one probably also decreases macrophage clearance of apoptotic cells and ECM fragments slowing down in the development of hydronephrosis. Similarly, MMP-9 stimulates on one hand EMT and on the other interstitial ECM degradation. These studies thus showed that future pharmacologic approaches to treat hydronephrosis should consider both these deleterious and beneficial effects of one molecule on the different stages of obstructive nephropathy.
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