Obstructive nephropathy: insights from genetically engineered animals.

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Short title

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Abstract

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Congenital obstructive nephropathy is the primary cause for end stage renal disease 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)fibroblasts 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 pathological role for angiotensin II (AII) and transforming growth factor 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 anti-fibrotic) for AII, for ECM degrading enzymes metalloproteinase 9 and tissue plasminogen activators, for plasminogen activator inhibitor 1 and for the adhesion molecule osteopontin in obstructive nephropathy. Further use of these animals, especially in combination with pharmacological tools should help to better identify potential anti-fibrotic strategies in obstructive nephropathy.

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 60-65 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 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 human, 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 (Figure 1). These different features of the pathology appear rapidly, all within around one week after the induction of the pathology and are highly reproducible from one experiment to another.

UUO: the pre-genetic engineering era

Before the use of genetically modified mice to study obstructive nephropathy, the UUO model, using gene expression analysis and pharmacological 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 (AII)

was identified, acting via transcription factor nuclear factor (NF)-κB, tumor necrosis factor (TNF)α and transforming growth factor β (TGFβ), as an important cause of fibrosis. Consequently blockage of the renin angiotensin system 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 (AT_1) receptor stimulation can also be beneficial in UUO [18]. Similarly, endogenous inhibition of extracellular matrix degrading enzyme activity was incriminated for a long time as an important cause for ECM accumulation [19] in UUO but the use of knockout 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) 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 biological 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 pharmacological 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 (8 to 10) 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 pathological insults. Finally, it is preferable to examine the kidney structure of genetically engineered animals before undertaking UUO. Indeed, some knockout animals including the angiotensinogen, angiotensin type 1 [22] or type 2 [8] receptor knockout mice generate animals with impaired renal development and histological 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 usual or non-expected results obtained using genetically modified animals. The paper is divided into the different stages of the development of obstructive nephropathy (Figure 1). Furthermore there are two additional sections on two "molecules" currently identified as the main actors in the fibrotic process: the renin-angiotensin-system (RAS) and TGFβ. 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 the Table.

Cellular infiltration (Figure 2)

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 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 to discriminate 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 upregulated in UUO [25]. UUO using OPN null mutants ($OPN^{-/-}$) 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 TGFβ 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 directly 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 apoptosis and decreased tubulointerstitial fibrosis compared to wild-type mice [31].

Another group of adhesion molecules, selectins, were studied for their involvement in cellular infiltration during 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 expressed on endothelial cells and platelets and L-selectins is expressed on leukocytes. Triple selectin ($EPL^{-/-}$) and L-selectin ($L^{-/-}$) knockout mice displayed significantly less (56% and 32%, respectively) macrophage infiltration 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 ligand [33]. The expression of sulfatide is modified during obstructive nephropathy. In the normal kidney, sulfatide is expressed in distal tubules however no leukocyte traffic in this area is observed, but during UUO, sulfatide relocates to the interstitium and peritubular capillaries, where monocytes are considered to extravasate into the interstitium. The use of sulfatide knockout mice confirmed the interaction between sulfatide and L-selectin and the role of this interaction in ureteral obstruction induced macrophage infiltration [34]. Sulfatide deficiency reduced by 50% macrophage infiltration but unfortunately the effect on tubulointerstitial fibrosis was not studied. Blockade of the interaction between sulfatide and L-selectin could thus be a potential useful strategy to block cellular infiltration in obstructive nephropathy.

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 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 macrophages 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 AT_1 receptor in UUO (see below in the RAS section).

Other than confirming the role of a number of molecules involved in cellular infiltration in UUO and attributing an anti-apoptotic function to the adhesion molecule osteopontin, the knockout animals showed that knockout 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 MCP-1, ICAM-1 and 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 dependant 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 $p21^{WAF/CIP}$, $p27^{KIP1}$ and $p57^{KIP2}$ and inhibits cyclin-CDK complexes during both G_1 and S phases of the cell cycle. $p21^{WAF/CIP}$ and $p27^{KIP1}$ 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 $p27^{KIP1}$ 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, $p21^{WAF/CIP}$, but in these mice interstitial myofibroblast proliferation was temporarily 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 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 compartment specific and time-dependent effects and

the large number of mediators of cell proliferation and apoptosis make the interpretation of these data obtained with knockout mice difficult. More studies on different knockouts or double knockouts combined with pharmacological tools, if possible, should give more insight into these early and important events of obstructive nephropathy.

Fibroblast accumulation

Irrespective of the initial cause, the development of tubulointerstitial fibrosis is characterized by the appearance of activated (αSMA-positive) fibroblasts (also called myofibroblasts). It is widely accepted that these cells are the principal actors responsible for extracellular matrix deposition [49]. The appearance of myofibroblasts is also a prominent feature in the obstructed kidneys during ureteral obstruction [49]. While the pro-fibrotic 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 (Figure 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 epithelial-mesenchymal transition (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 (FSP1) as a fibroblast marker, it was shown that interstitial kidney fibroblasts arise from both EMT (36%) and bone marrow (14-15%) 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 (Figure 3): i) loss of epithelial adhesion properties, ii) de novo αSMA expression and actin reorganization, iii) disruption of the tubular basement membrane and iv) enhanced cell migration and invasion capacity. TGFβ 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 TGFβ inhibition, EMT and subsequent renal fibrosis.

Extracellular matrix accumulation

Tubulointerstitial fibrosis is the result of an imbalance between extracellular matrix (ECM) synthesis and degradation. The pathological tubular extracellular matrix 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 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 extracellular matrix 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 (Figure 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 (Figure 3, step 3 in the EMT process described in the preceding section) and thus inhibiting EMT. In addition, tPA deficiency did nor 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 μM) 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 physiological inhibitor 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 TGFβ and procollagens I and III. This suggests that in this model PAI-1 is playing a pro-inflammatory rather than an antiproteolytic 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 MMP9 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 anti-fibrotic 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, -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, basal membrane degradation) than about their role in interstitial ECM degradation.

Renin Angiotensin System

A number of renal diseases and their progression to ESRD involve angiotensin II (AII) making it an important target in progressive renal disease. The different components of the renin angiotensin system (RAS) are present in the kidney [71] and as described above, AII and the RAS have been known for a long time to be involved in the etiology of obstructive nephropathy [72, 73]. Angiotensin 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 AT_1 and AT_2 receptor. AII signaling through the AT_1 receptor results in vasoconstriction, cell proliferation and fibroblast activation while its counterpart, the AT_2 receptor, generally results in vasodilatation, inhibition of cell proliferation and increased apoptosis [74]. It is generally accepted that stimulation of the AT_1 receptor is inducing the deleterious effects of the RAS. AT_1 and AT_2 knockout and genetically engineered mice with 0 to 4 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 0 to 4 copies of the angiotensinogen gene, UUO-induced renal interstitial collagen content increased linearly with increasing angiotensin expression (up to 2 copies and reaching a plateau above 2 copies of the angiotensinogen gene; [75]). This effect seems to be mediated by the AT_1 receptor since AT_{1a} receptor (the predominant form of the AT_1 receptor in mice) knockout mice develop significantly less UUO-induced tubulointerstitial fibrosis [676]. In contrast, AT_2 knockout display accelerated tubulointerstitial collagen deposition after UUO [77]. The deleterious effects of AII are thus most probably mediated by the AT_1 receptor. It appears that many of the effects of AT_1 receptor stimulation in tubulointerstitial fibrosis are mediated by TGF-β1, TNFα, and activation of NF-κB (Figure 5; [78]). Angiotensinogen gene expression is stimulated by NF-κB and since AT_1 receptor stimulation induces NF-κB activation this provides a positive feedback loop to probably maintain high AII levels under pathological conditions [79]. NF-κB is also strongly involved in TNFα induction during UUO [78] while TNFα 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 AII and TNFα was studied in more detail using a combination of AT_1a and TNF receptor (TNFR1 and TNFR2) knockout mice and pharmacological tools [81]. It was shown that the double TNFα receptor knockout displayed around 40% less tubulointerstitial fibrosis than the wild-type mice comparable to what was observed observed for the AT_1a knockout mice. This level of fibrosis in double TNFα receptor knockout mice was further lowered with about 25% by enalapril treatment (an ACE inhibitor) suggesting both AII dependent and independent activation of the TNFα system. By studying TGFβ expression during UUO, these analyses combining knockouts and pharmacology also showed that levels of this cytokine are almost exclusively controlled by AII and do not involve TNFα dependent pathways.

The above studies using classical knockout mice on the RAS system confirmed the role for the AT_1 receptor as an important mediator of extracellular matrix accumulation during UUO. However an elegant study using bone marrow transplantation from AT_1 knockout mice to wild type mice showed that AT_1 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 AT_1 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 AT_1 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 AT_1 receptor knockout bone marrow derived macrophages are at the origin of this increased interstitial fibrosis by decreased phagocytic clearance of extracellular matrix components and apoptotic cells in these mice. This combined knockout and bone marrow transplantation approach has thus allowed to dissociate between macrophage infiltration and renal fibrosis which is not possible in "simple" knockouts and presents a drawback of classical non temporal and non spatial knockouts to study complex pathological 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 AI into AII, 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 (Figure 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 2 fold higher in bradykinin B2 receptor 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 (tPA and MMP2 activities) in the obstructed kidneys of B2 receptor knockout compared to wild type mice. This suggested that the cascade bradykinin/B2 receptor/tPA/MMP2 was involved in the lower level of tubulointerstitial fibrosis in wild type compared to B2 receptor knockout mice although in the light recent discoveries described in the above section on extracellular matrix degradation this hypothesis might be revised soon. 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 treated wild type and B2 receptor knockout mice. In contrast to the above study, it was recently shown in a model of cyclosporine-induced interstitial fibrosis that bradykinin was responsible for the anti-fibrotic effects of ACE-inhibitor benazepril which was accompanied by increased plasmin activity [85, 86]. Further studies on local renal bradykinin concentrations during UUO and other interstitial fibrosis models, which might be modified since UUO has been shown to increase ACE activity and decrease kallikrein expression [87], or effects on the bradykinin B1 receptor are necessary to confirm the absence of a role for bradykinin in the protective effects of ACE-inhibitors in experimental hydronephrosis.

TGFβ related effects

As mentioned above, and long before the use of genetically engineered animals, TGFβ was incriminated as a key molecule in fibrosis (Figure 6; [14]). Its expression is increased in numerous fibrotic conditions [88] including experimental and human hydronephrosis [89]. TGFβ1 (the main TGFβ isoform) knockout mice are not viable and die at mid-gestation [90]. TGFβ synthesis is strongly induced by AII and the profibrotic actions of TGFβ 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 occurred is not clear. UUO in knockout mice of molecules interacting with TGFβ or involved in TGFβ signaling helped in better understanding of these mechanisms.

TGFβ activates transmembrane receptor serine/threonine kinases which transduce downstream signals to cytoplasmic latent transcription factors called Smads. Smad 2 and 3 are directly phosphorylated by one of these receptors after which they bind to Smad 4 before nuclear translocation were they act as transcriptional regulators [91]. Unlike, TGFβ and Smad2, Smad 3 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 TGFβ signalling in experimental hydronephrosis [93]. Smad 3 knockout mice are protected against UUO-induced tubulointerstitial fibrosis which is accompanied by reduced monocyte influx, EMT and collagen deposition [93]. The results on Smad 3 null mice and complementary experiments *in vitro* show that it is probably the effect of Smad 3 on EMT that is the main profibrotic factor. Furthermore, Smad 3 is essential for TGFβ amplification since TGFβ levels are extremely low after UUO in Smad 3 null mice compared to wild type mice. Finally it was shown that the effect of Smad 3 on monocyte accumulation further stimulated EMT. This results in a TGFβ/Smad3 induced

profibrogenic myofibroblast population which is probably responsible for the larger part of UUO-induced tubulointerstitial fibrosis.

TGFβ 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 TGFβ, PAI-1 and collagen I, III mRNA levels suggesting that interruption of TGFβ activation can protect against tubulointerstitial fibrosis. However the authors of this study also explored a possible TGFβ independent pathway to tubulointerstititial fibrosis by treating the αVβ6 integrin knockout mice with AII, which increased the collagen concentration to levels comparable to obstructed wild type mice. Interestingly, AII infusion only mildly increased active TGFβ levels and did not change Smad 2 levels in αVβ6 integrin knockout mice but significantly induced PAI-1 expression. These data thus suggest TGFβ independent profibrotic effects of AII with both pathways triggering PAI-1 expression and resulting in increased interstitial fibrosis. The AII induced TGFβ independent pathway for PAI-1 induction in UUO remains to be elucidated.

Other, small molecules interacting with TGFβ 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-Perspective

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 TGFβ and AII in macrophage infiltration,

(myo)fibroblast accumulation and ECM accumulation, the use of genetically engineered animals has also modified our vision of things that were accepted to be true in obstructive nephropathy (*i.e.* the beneficial *and* deleterious role of AII, the deleterious role of MMP9, role for PAI-1 in macrophage recruitment etc…).

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 pharmacological 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 pharmacological 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 pharmacological 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 pro-fibrotic TGFβ production but one probably also decreases macrophage clearance of apoptotic cells and ECM fragments beneficial in the development of hydronephrosis. Similarly, MMP9 stimulates on one hand EMT and on the other interstitial ECM degradation. These studies thus showed that future pharmacological 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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Table

Effects of UUO on the different stages of development of obstructive nephropathy.

	Inflammation	Proliferation	EMT	Apoptosis	Tubular atrophy	Fibrosis	Ref.
Cellular infiltration							
Osteopontin-KO	-			+		-	[29]
CD44-KO	-	=	=	+	+	-	[31]
E,P,L-selectin-KO*	-			-	=(day 5) -(day 14)	= (day 5) -(day 14)	[32]
L-selectin-KO*	-			=	=(day 5) -(day 14)	= (day 5) -(day 14)	[32]
Sulfatide- KO	-						[35]
CCR1	-		-		=	-	[36]
CCR5	=		=		=	=	[36]
Proliferation apoptosis							
P21CIP1/WAF1-KO	=	=	+	=		=	[42]
P27kip1-KO		+		+			[41]
P53-KO				-	-	-	[48]
ECM							
Plasminogen-KO††	-		=			-	[97]
tPA-KO			-			-	[21]
PAI-1-KO	-		-			-	[20]
uPAR-KO†	-	-	+	+		+	[66]
TIMP-1-KO						=	[70]
RAS-related							
Angiotensinogen-KO*					=	-	[75]
Angiotensinogen-1 to 4 gene copies*					=	+ with increasing copies	[75]
AT1 receptor-KO	-					-	[76]
AT2 receptor-KO	=	=	+	=		+	[77]
Double TNFα receptor-KO			-			-	[81]
Bradykinin B2 receptor-KO	-	=	=			+	[84]
Kallikrein overexpressing rats						-	[84]
TGFβ							
Smad 3-KO	-		-			-	[93]
Integrin αvβ6-KO	=	-				-	[94]
Decorin-KO	+			+	+	+	[95]
Endoglin-KO (+/-)						=	[96]

Observations on day 5 or 7 after UUO compared to wild-type mice; =, no difference; - decrease; +, increase; †, 14 days after UUO; ††, 21 days after UUO; * results after 28 of UUO.

Figure legends

Figure 1. Overview of the different stages of the development of obstructive nephropathy. Experimental ureteral obstruction (UUO) induces after a few hours cellular infiltration in the tubulointerstitium. These infiltrating cells (mainly macrophages) secrete growth factors and cytokines inducing a disequilibrium between apoptosis and proliferation of tubular cells, as well as inducing fibroblast activation and proliferation. Fibroblasts either infiltrate from the circulation into the interstitium, appear by EMT or appear by proliferation of the few resident fibroblasts. Activated fibroblasts secrete the ECM that is starting to accumulate into the interstitium as soon as myofibroblasts appear. As the obstruction continuous, ECM deposition becomes massive and uncontrolled apoptosis of tubular cells results in tubular atrophy. The involvement of different molecules in these stages of obstructive nephropathy, studied using genetically modified animals, is described in the text and in the subsequent figures.

Figure 2. Hypothetic representation of cellular infiltration in UUO based on data obtained with knockout mice. UUO induces macrophage infiltration in the tubulointerstitium. In UUO, the interaction between L-selectin and sulfatide seems to mediate the initial contact (rolling) between macrophages and the vascular 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 important molecules (including ICAM-1, VCAM-1 and 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.

Figure 3. Proposed role of EMT in 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 (14-15%) 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.

Figure 4. The role and connections between the renin-angiotensin and kinin-kallikrein system and proteolytic systems in UUO. The profibrotic role of angiotensin II (AII) in UUO (see Figure 5) is counterbalanced by the production of bradykinin. It is proposed that during UUO, bradykinin is activating the G-protein coupled B2 receptor which activates the proteolytic plasminogen activator (PA, combined tissular and urokinase plasminogen activator activity) system (either directly or by inhibition of PAI-1) which in turn switches on metalloproteinases (MMPs) promoting matrix degradation (bold dashed line). Angiotensin converting enzyme (ACE)-inhibition thus blocks production of profibrotic AII and promotes accumulation of the antifibrotic peptide bradykinin. The role of the proteolytic enzymes in matrix degradation in UUO is depicted in the grey area in figure. Both pro and antifibrotic properties of this system have now been proposed: i) in UUO it was shown that plasmin can generate both profibrotic TGFβ and antifibrotic hepatocyte growth factor (HGF), ii) activation of MMPs degrades the extracellular matrix but stimulates EMT and iii) PAI-1 has been shown, besides its role to inhibit

PA activity, to stimulate cellular infiltration in UUO. The dotted lines show other controversial effects PA activation TGFβ and inhibition of cellular infiltration by PAI-1 observed in other models of nephropathies (refs). Abbreviation: PAI-1, PA-inhibitor 1.

Figure 5. The central role for NF-κB in angiotensin II profibrotic effects in UUO. Angiotensin II (AII) accumulation during UUO stimulates two G-protein couples receptors, the AT_1 and AT_2. Although AT_2 knockout mice display less tubulointerstitial fibrosis stimulation of this receptor and the AT_1 receptor induces NF-κB family member activation which translocates to the nucleus and activates a vast panel of proinflammatory and profibrotic cytokines leading to sustained inflammation and matrix production. NF-κB activation is also leading to at least 2 positive autocrine regulation loops by the increased expression of TNFα and angiotensinogen. Abbreviations: IκB, inhibitor of κB; NF-κB, nuclear factor - κB; TNFα, tumor necrosis factor α; AT_1 and AT_2 receptor, angiotensin type 1 and 2 receptor.

Figure 6. Proposed schematic representation of the role of TGFβ in UUO. TGFβ has been shown to be an important regulator of many of the processus leading to tubulointerstitial fibrosis including EMT, cellular infiltration and apoptosis leading to tubular atrophy and interstitial accumulation of extracellular matrix proteins. Binding of TGFβ to the transmembrane serine/threonine kinase receptor type II (RII) leads to the recruitment-phosphorylation and activation of the type I receptor kinase (RI) which in turn, phosphorylates Smad2 and Smad 3 proteins thus able to bind Smad4 and translocate to the nucleus where they regulate the transcription of target genes by binding to their specific promoter-sequences. In addition to this classical TGFβ signaling pathway there is now increasing evidence that Smad-independent pathways like activation of RhoA and p38MAPKs are involved in EMT. Smad3 knockout mice

are protected against UUO-induced tubulointerstitial fibrosis and showed the existence of a TGFβ autocrine loop during UUO. Furthermore, UUO on knockout mice showed that the production of active TGFβ is controlled by integrins, plasmin and decorin.