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Inhibition of Fibrogenesis upon Hydralazine-induced DNA Demethylation

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Abstract

Chronic progressive kidney disease (CKD) remains an unsolved problem in clinical Nephrology as neither biomarkers to predict progression towards end-stage renal failure in individual patients nor specific drugs to inhibit decline of kidney function are available in the clinic yet. In this regard, the prototypical epigenetic mechanism of CpG island promoter methylation has emerged as modulator of disease progression with utilities both as biomarker and as therapeutic target. Recent studies demonstrated the potential of hydralazine, a long-established antihypertensive drug with de-methylating activity, to reverse aberrant CpG island promoter aberration and ameliorate progression of chronic kidney disease. Here we review contribution of aberrant promoter methylation to progression of fibrogenesis and mechanisms underlying hydralazine's demethylating activity and discuss possible translational implications.

Keywords: Kidney; Fibrosis; Epigenetics; Methylation; Tet3; Fibroblast; Hydralazine

Introduction

While progression of chronic kidney disease remains an unsolved challenge, recent pre-clinical studies revealed that that the epigenetic mechanism of DNA-hypermethylation contributes to progression of fibrosis and that fibrogenesis in the kidney could be ameliorated through administration of the de-methylating compound hydralazine. Importantly, such de-methylating anti-fibrotic activity was achieved at doses which were substantially lower than standard anti-hypertensive regimen, which could be also confirmed by retrospective analysis of patients with nephrosclerosis. Because hydralazine has been in clinical use as anti-hypertensive drug for over 50 years, we here discuss an opportunity to address a pressing clinical problem by re-purposing of an old and safe drug.

Contribution of renal fibrosis to progression of chronic kidney disease

Renal fibrosis, also referred to as tubulointerstitial fibrosis (TIF) is of major importance for clinical nephrology as it is the common pathway leading to end-stage renal failure in patients [1,2]. Currently, there are no specific treatments available in the clinic to inhibit (or even reverse) progressive renal fibrosis [3,4]. Furthermore, there is a paucity of biomarkers available to predict the individual risk of patients with renal disease to develop end-stage renal failure [1]. Renal fibrosis – just like fibrosis in other organs – is a pathologic scarring process [5-7]. A hallmark of fibrosis is that – as opposed to physiologic wound healing - the active scarring does not cease once the initial insult has been contained, but it becomes an autonomous process [5].

Activated fibroblasts are commonly considered main mediators of renal fibrosis 5 (just like in any other organ) [8,9]. The term "activated" refers to the observation, that fibroblasts associated with wound healing or fibrosis display an increased proliferative activity and increased production of extracellular matrix (ECM) constituents and formation of stress fibers [5]. It is well established that activated fibroblasts isolated from fibrotic kidneys maintain their activated state when cultured *in vitro*, as primary "fibrotic" fibroblasts are characterized by an increased proliferation rate and increased secretion of ECM constituents [10]. It has been hypothesized that such maintenance of the activated stage reflects the failure of the fibroblasts in the fibrotic kidney to return to their resting state as they do in physiologic wound healing [10]. This phenomenon is not only observed in fibrotic kidney fibroblasts, but is also true for fibroblasts isolated from any given organ [8,11]. Furthermore, such pathological maintenance of the activated state has received increased attention in the cancer field in the case of "carcinoma-associated-fibroblasts" [12,13] (Figure 1).

Epigenetic modifications

Perpetuated disease progression of fibrosis in contrast to physiological wound repair points to a role of epigenetics in fibrogenesis [14-16]. Epigenetic modifications in principle can "stably alter gene expression without altering the primary nucleotide sequence" [17]. The two principal epigenetic mechanisms are histone modifications and DNA-methylation. In a simplified view, genes can be either "open" for transcription or "locked" [18]. "Open" genes that are accessible for polymerases are associated with un-methylated promoter regions and with histones, which are permissive for gene transcription ("activating histone marks"). "Locked" genes are associated with DNA-hypermethylation of the promoter region and enrichment of histone modifications which prevent gene transcription ("inhibitory histone marks") [17]. While understanding of the role of histone modifications in progression of chronic kidney disease is still rudimentary, there substantial is evidence from past years which demonstrates a causal role of aberrant DNA methylation in renal fibrogenesis (and in fibrosis in other organs as well) [19-23]. DNA methylation (syn. promoter CpG island methylation) is a process in which methyl groups are added to clusters of cytosine nucleotides within promoter DNA regions. DNA methylation typically acts to repress gene transcription, whereas unmethylated promoters are open for transcription. DNA methylation plays important roles in physiologic processes including cell differentiation during development, genomic imprinting, X-chromosome inactivation, and repression of repetitive elements

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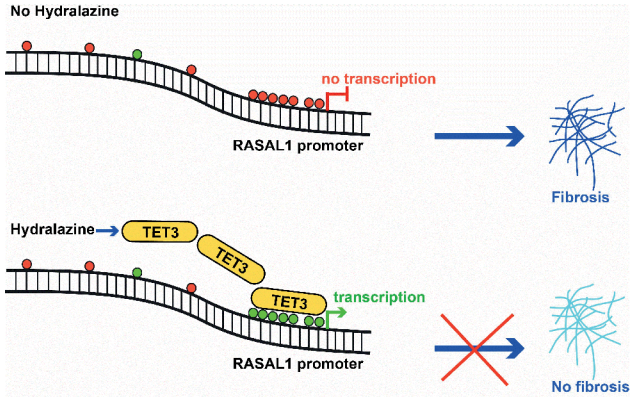

Figure 1: Schematic illustration of Hydralazine's de-methylating anti-fibrotic activity. Methylation of *RASAL1* CpG promoter islands (red circles) causes transcriptional RASAL1 suppression and subsequently contributes to fibroblast accumulation (**top picture**). Hydralazine induces expression of the enzyme TET3 which via its CXXC binding motif targets CpG promoter islands of select genes (green circles), including *RASAL1*. TET3 facilitates hydroxymethylation of methylated cytosine residues, which subsequently induces
RASAL1 transcription, CpG island promoter de-methylation and inhibits fibrogenesis.

[18,24]. Suppression of gene transcription through DNA methylation in differentiating cells is normally permanent and prevents cells from converting into a different cell type [25]. DNA methylation suppresses gene transcription by either physically blocking the binding of transcription factors, or by recruiting methyl-CpG-binding domain proteins (MBDs), which then recruit additional chromatin remodeling proteins forming heterochromatin. In humans there are four known enzymes which can methylate DNA in principle, termed DNA methyl transferases Dnmt1, Dnmt3a, DNMt3b and DnmtL [18]. During development de novo methylation is carried out by Dnmt3a, Dnmt3b and Dnmt3L, whereas Dnmt1 mostly is necessary to preserve DNA methylation during DNA replication (otherwise DNA replication would result in unmethylated daughter strands) [26,27].

Contribution of aberrant DNA methylation to fibroblast activation and fibrosis

Progression of chronic kidney disease is associated with substantial and consistent changes of renal methylation patters, providing correlative evidence for contribution to disease progression [28,29]. First causal evidence for a role of aberrant DNA methylation in kidney fibrosis stems from studies, in which fibrosis in mouse models of renal fibrosis were ameliorated by treatment with the de-methylating drug 5'Azacytidine (5'Aza) [19]. Evidence for direct causal contribution of aberrant DNA methylation was made possible by identification of genes which are selectively methylated in kidney fibrosis [19]. In this regard, our group in a genome-wide methylation screen, by comparing fibroblasts from fibrotic and non-fibrotic kidney biopsies, identified the gene RASAL1 (which encodes for Ras-Gap-like protein 1, RASAL1), an inhibitor of Ras signaling [19].

In general, the Ras superfamily of proteins is a family of structurally related small GTPase signal transduction molecules, with Ras being the prototypical member [30]. Ras stands for "Rat sarcoma", reflecting that Ras was originally discovered through studies of cancer causing viruses (*HRas* was discovered in Harvey sarcoma virus and *KRas* in Kirsten sarcoma virus). Ras is the most common oncogene (i.e., 90% of pancreatic cancers and 50% of thyroid cancers are causally linked to activating Ras mutations) and as a consequence most knowledge on Ras signaling in health and disease stems from cancer research [30]. In humans three *Ras* genes (*HRAS, NRAS, KRAS*) encode four proteins (H-, N- and K-as4A and K-ras4B which are generated through alternative splicing) [31,32]. Ras signaling is complex, as multiple effector pathways can be activated – all with established contributions in progression of chronic kidney disease [33,34]. The most prominent Ras-signaling pathway is initiated by phosphorylation of Raf, which in turn phosphorylates mitogen activated protein kinases ERK1 and ERK2, which ultimately enhance cell cycle progression [30]. In addition, Ras interacts directly with the phosphatidylinositol-3-kinases (PI3-Ks), leading to activation of the kinase phosphinositide-dependent protein kinase-1 (PDK1) and Akt kinase 30. Akt has a strong anti-apoptotic effect by phosphorylating various targets and is considered to play a major role in the survival signal which is generated by Ras proteins [30]. Ras proteins are G proteins which can be most adequately described as on-off switches: They are inactive when GDP-bound and active when GTP bound. Under physiological conditions GDP-bound Ras proteins are anchored to the cell membrane. Upon growth factor stimulation, so called guanine nucleotide exchange factors (GEFs) facilitate conversion of inactive Ras-GDP to Ras-GTP. Well-known examples of such GEFs include Son of Sevenless and cdc25 [35,36]. Ras-GTP is inactivated through hydrolysis of GTP to GDP. This is facilitated through so-called GTPase activating proteins (Ras-GAPs) [35,36]. (The name refers to the fact that Ras has its own GTPase activity, which is however insufficient to hydrolyze GTP itself and requires an extrinsic catalytic activator – the Ras-GAPs.) RASAL1 is referred to as Ras-GAP-like protein, which catalyzes Ras-inactivation by binding to GTP-Ras and catalyzing hydrolysis to GDP-Ras [37]. This means that depletion of RASAL1 through CpG island promoter methylation results in intrinsic Ras hyperactivity [38]. RASAL1 depletion in cultured renal fibroblasts recapitulates important aspects of fibroblast activation: Increased proliferative activity, increased collagen production and stress fiber formation [19]. Furthermore, administration of a broad Ras-GTP inhibitor "FTS" ameliorated renal fibrosis in mice (which had been challenged with either folic acid or nephrotoxic serum nephritis) through blunting the increased intrinsic Ras-GTP activity, which is observed in kidneys undergoing fibrogenesis [19]. Overexpression of RASAL1 in transgenic mice ameliorates renal fibrosis, providing definitive evidence that transcriptional suppression of RASAL1 causally contributes to renal fibrogenesis.

RASAL1 promoter CpG island methylation is a constituent of renal fibrosis irrespective of the underlying disease

Experimental kidney fibrosis is associated with *Rasal1* hypermethylation and decreased Rasal1 expression, including mouse models of folic acid-induced nephropathy, nephrotoxic serum nephritis [19], unilateral ureteral obstruction (UUO), diabetic nephropathy through administration of streptozotocin (DN) [39], *COL4A3*-deficient "Alport"-mice (*COL4A3KO*) and 5/6 nephrectomy (5/6 NX) [39]. In whole kidney biopsies from patient with varying degree of fibrosis and corresponding fibroblasts, severe fibrosis was associated with *RASAL1* hypermethylation and transcriptional silencing of *RASAL1* [39]. In this regard, transcriptome analysis data on larger cohorts reveal that chronic kidney disease due to minimal change nephropathy and hypertensive nephrosclerosis also correlated with decreased RASAL1 expression [39]. In summary, *RASAL1* is consistently hypermethylated

in kidney fibrosis irrespective of the underlying cause, suggesting that ensuing Ras-GTP hyperactivity might be a highly relevant pathway of renal fibrogenesis.

Consistency of RASAL1 methylation in fibrotic kidney biopsies not only corroborates contribution of RASAL1 hypermethylation to disease contribution, but also raises the possibility of RASAL1 methylation as biomarker of kidney fibrosis progression. However, the need for a renal biopsy to assess intrarenal degree of *RASAL1* methylation is prohibitive in this regard. On the other hand, the degree of renal RASAL1 methylation is reflected by levels of circulating *Rasal1* promoter DNA fragments in peripheral blood [40]. Such correlation of fibrosis, decreased renal RASAL1 expression and increased levels of methylated circulating *RASAL1* DNA fragments can be found in mice challenged kidney fibrosis models [40]. Our group also explored the possibility that circulating methylated *RASAL1* promoter fragments could similarly reflect intrarenal *RASAL1* methylation (and possibly fibrosis) in patients with CKD as they do in mice all single-center cohort of patients with varying degree of renal fibrosis at time of biopsy, renal fibrosis was inversely correlated with intrarenal RASAL1 mRNA expression levels [40]. The degree of intrarenal *RASAL1* promoter CpG island methylation correlated with the degree of renal fibrosis, irrespective of the underlying disease [39,40]. Furthermore, we observed that degrees of intrarenal RASAL1 promoter CpG island methylation correlated with levels of methylated RASAL1 promoter fragments [40]. Few patients displayed significantly increased levels of methylated RASAL1 promoter fragments at CKD stage 1, at CKD stage 4 all patients analyzed had increased levels of circulating RASAL1 DNA. Importantly, circulating *RASAL1* promoter fragment levels correlated better with degree of interstitial fibrosis at the time of biopsy was superior to that of estimated glomerular filtration rate (eGFR), serum creatinine or blood urea nitrogen (BUN) levels [40]. Current limitations include the labor intensity of currently established assays which involves immunoprecipitation of methylated DNA from blood samples and the lack of large prospective clinical studies. In summary, methylation of *RASAL1* contributes causally to progression of kidney fibrosis, is an attractive therapeutic target and there is promise of utility as biomarker for kidney fibrosis. Furthermore, with the possibility of therapeutic de-methylation, there comes the attractive possibility of a biomarker-stratified epigenetic therapy which could be monitored for therapeutic efficacy and adequate dosing.

Mechanisms of hydralazine-mediated demethylation

In general, methylated DNA can be de-methylated either through passive or active mechanisms. Passive de-methylation can occur when methylation marks are not copied on the newly synthesized strands meaning that during a first cell division, hemi-methylated DNA is generated and the methylation mark is entirely removed upon the second cell division [41]. For rapid de-methylation to occur, active de-methylation mechanisms involve oxidation of methylated cytosine (5mC) to generate 5-hydroxymethyl-cytosine (5hmC), 5-formyl-cytosine (5fC) and 5-carboxyl-cytosine (5caC); subsequent base excision and replacement with naked cytosine are a consequence [41]. This pathway is recapitulated when kidney cells are exposed to hydralazine: Hydralazine induces expression of Ten-Eleven Translocation enzyme-3 (Tet3), which catalyzes hydroxymethylation of aberrantly methylated genes (such as *Rasal1*) [40]. Such hydroxymethylation (which itself is sufficient to increase transcriptional activity of previously methylated genes) is followed by carboxylation of 5hm-cytosine residues [40]. In subsequent steps hydralazine induces replacement of hydroxymethylated cytosines with naked cytosine bases: First, hydroxymethylated cytosines are further oxidized to 5-formyl-cytosine (5fC) and then to 5-carboxyl-cytosine (5caC) before excision of 5caC by the DNA glycosylase Tdg, and restoration of cytosine via follow-on base excision repair [41]. Unlike in physiological de-methylation in germ-line cells an alternate pathway which involves 5hmC deamination by the Aid/Apobec family of cytidine deaminases before Tdg-mediated base excision repair is not being activated in kidney fibroblasts by hydralazine [41]. Although the de-methylating activity of Hydralazine is inferior to that of 5'-Azacytidine, it is sufficient to re-activate Rasal1 expression and normalize intrinsic proliferative activity of fibrotic renal fibroblasts – without inducing potentially genotoxic side effects like 5'Aza [40]. Of note, in kidney fibroblasts only Tet3 of the family of Ten-Eleven Translocation enzymes is induced, which does not rule out the possibility that other Tet-family members are involved in other organs [40]. Importantly, hydralazine-induced Tet3 does not cause uncontrolled de-methylation, but targets only select genes – including *RASAL1* – which have a CXXC motifs flanking methylated CpG islands [22,40]. Such demethylating activity of hydralazine is already being utilized, as phase III clinical trials are ongoing which test impact of hydralazine mediated-demethylation on cancer progression [42,43]. Importantly, de-methylation is induced by hydralazine by doses whch are lower than those, which are typically used to lower blood pressure: In clinical cancer trials 3 x 25 mg/day hydralazine were determined as optimum de-methylating dose, which is in line with the optimum dose of 1-2 times 25 mg/day dihydralazine which were identified in our studies (dihydralazine has a longer half life as compared to hydralazine–HCl) [42]. In contrast to hydralazine, the established de-methylating drug 5'Aza is incorporated into DNA where it inactivates Dnmts by trapping them through formation of covalent complex formation. In addition, 5'Aza incorporation directly causes DNA damage, which in turn initiates DNA repair mechanisms which also result in replacement with naked unmethylated DNA [44]. However, such DNA damage can also directly initiate cell death [44]. Hence, from an epigenetic standpoint, hydralazine provides sufficient de-methylating activity, combined with a benign side-effect profile.

Hydralazine revisited

In summary, aberrant DNA methylation of specific genes – including RASAL1 – contributes causally to progression of fibrosis in the kidney, as well as in other organs including the heart [19,22]. There is evidence that such aberrant fibrotic DNA methylation can be detected in the blood test [40]. Finally, there is evidence that such aberrant DNA methylation can be reversed by treatment with hydralazine, opening the possibility for a biomarker-stratified anti-fibrotic therapy with hydralazine [40]. In this regard, hydralazine (which is primarily used in the U.S., as Apresoline) and its derivate dihydralazine (which is primarily used in Europe as Nepresol) have in in clinical use as anti-hypertensives for more than 60 years, providing substantial evidence for their safety and anti-hypertensive effectiveness [40,45]. Hydralazine is the oldest anti-hypertensive drug which has remained on the market, with primay applications being pregnancy hypertension, hypertension in ICU settings and as third-line add-on anti-hypertensive in complicated hypertension. Due to new novel mechanistic insights, hydralazine may has to be re-visited in comparison to inhibitors of the renin-angiotensin-aldosterone system (RAAS) such as angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). Today RAAS-inhibitors are the gold-standard in therapy of hypertension, chronic heart failure and proteinuric kidney diseases, because numerous large controlled clinical trials demonstrated efficacy in reducing blood pressure, but also in improving chronic heart failure and in reducing

cardiovascular mortality and in reducing proteinuria and improving outcome of proteinuric chronic kidney diseases shortly after their introduction to clinical use [40,46-49]. In comparison, hydralazine was introduced to the clinic in 1952, before the age of modern clinical trials, and the first small controlled clinical study on efficacy of hydralazine in hypertension was not reported before 1964 [50]. Nevertheless, hydralazine as part of "standard-triple therapy" was the gold-standard for anti-hypertensive therapy [51,52] – until ACEIs emerged and their superior effectiveness to lower blood pressure and more importantly to reduce cardiovascular mortality at optimum doses was proven [53,54]. Hydralazine received renewed interest, when a fixed combination of hydralazine and isosorbit dinitrate (BiDil, marketed as new drug then as patent protection for hydralazine had long run out) showed effectiveness to improve chronic heart failure [55]. While BiDil was effective in all patients, it was only superior to ACEIs (at optimum dose) in self-declared African Americans (but not in Caucasians), and FDA approval was only granted for use in African Americans – making BiDil the first "race-based drug" [56,57]. However, hydralazine in treatment was only inferior to optimally dosed RAAS inhibitors and it may have to be considered that optimum doses of RAAS inhibitors (i.e., for treatment of heart failure 10 mg/ day Ramipril or 320 mg/ day Valsartan) are often not reached due to decrease of glomerular filtration rate, hyperkalemia or hypotension. In this regard, hydralazine does not require dose reduction it cases of low GFR, making it a possible substitute to RAAs inhibitors, even when its de-methylating efficacy is not even taken into account [58,59]. In fact, studies comparing efficacy of ACEIs to hydralazine concluded that combination of ACEIs and hydralazine should be tested – but such trials have not yet been done. To this ongoing discussion the new insights on the impact of epigenetics and de-methylating activity of hydralazine now add an entirely new aspect. When considering possibility of substituting hydralazine for RAAS in clinical scenarios when RAAS inhibitors can not be used at optimal doses, it has to be taken into account that optimal de-methylating activity of hydralazine is already achieved at doses which are below blood pressure lowering regimen. In retrospective analysis we documented effective RASAL1 de-methylation and reno-protection over a one-year period in patients with CKD stage 4 with hypertensive nephrosclerosis at dihydralazine doses of 2 × daily 12.5 mg. In these patients systolic and diastolic blood pressure was not affected by low-dose dihydralazine, confirming that observed reno-protection was independent of possible anti-hpertensive effects of dihydralazine. These results raised the possibility that dihydralazine could be used in be utilized in biomarker-stratified fashion, even without risk of hypotensive episodes and reflex tachycardia, which can be observed upon standard dose (100-200 mg) dihydralazine. Furthermore, such de-methylating effectiveness of low-dose hydralazine conceptually makes combination of hydralazine and RAAS inhibitors an attractive therapeutic combinations in patients with chronic heart and/ or chronic kidney failure, which may deserve clinical testing after all. Considering the safety of hydralazine, there may be a lot to gain at a very low risk.

Authors´ contribution

MZ wrote the manuscript. EMZ wrote the manuscript.

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