In vivo renin activity imaging in the kidney of progeroid Ercc1 mutant mice

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ABSTRACT

Changes in the renin-angiotensin system, known for its critical role in the regulation of blood pressure and sodium homeostasis, may contribute to aging and age-related diseases. While the systemic renin-angiotensin system is suppressed during aging, little is known about its regulation and activity within tissues. Yet, this knowledge is required to successively treat and/or prevent renal disease in the elderly. In this study, we tested the use of the renin activatable near-infrared fluorescent probe ReninSense680™ to facilitate non-invasive imaging of renin activity in vivo. First, we validated the specificity of the probe, by detecting increased intrarenal activity after losartan treatment and the virtual absence of fluorescence in renin knock-out mice. Second, age-related kidney pathology, tubular anisokaryosis, glomerulosclerosis and increased apoptosis was confirmed in kidneys of 12, 18 and 24-week-old Ercc1d/− mice, while initial renal development was normal. Next, we examined the in vivo renin activity in these Ercc1d/− mice. Interestingly, increased intrarenal renin activity was detected by ReninSense in Ercc1d/− compared to WT mice, while plasma renin activity was lower. Hence, this study demonstrates that intrarenal RAS activity does not necessarily run in parallel with circulating renin in the aging mouse. In addition, our study supports the use of this probe for longitudinal imaging of altered RAS signaling in aging.
INTRODUCTION

Aging is a natural biological process that is associated with diverse detrimental changes in cells and tissues, ultimately leading to loss of organ function. Progressive deterioration of the renal structure is part of the normal aging process, including loss of renal mass, loss of tubules and increase in the incidence of glomerulosclerosis and tubulointerstitial fibrosis. Besides sclerosis and loss of most of the glomeruli, the remaining glomeruli often exhibit impaired filtration ability. Accordingly, many elderly suffer from a decline in renal function, often shown as a progressive decrease in glomerular filtration rate and renal blood flow. These age-related structural and functional changes may predispose the kidney to acute kidney injury or progressive chronic kidney disease.

The renin-angiotensin system (RAS) has long been recognized for its critical role in the regulation of blood pressure and fluid homeostasis. Changes in the responsiveness and activity of the RAS have been shown to play an important role in aging, as well as in renal disease as it predisposes the elderly to acute kidney injury and chronic kidney disease. It is suggested that overexposure to the RAS hormone angiotensin (Ang) II causes DNA damage as well as cellular senescence and/or apoptosis; processes known to play a role in aging and disease. Moreover, interference in the RAS system by using RAS blockers has been proposed to extend lifespan and to prevent age-associated changes. However, not all elderly respond well to RAS blockade and related adverse events include acute kidney injury, hyperkalemia and hypotension. Thus, we need more insight into the regulation of the RAS during aging, in order to successively treat and/or prevent renal disease in the elderly population.

Although Ang II is considered to be the principal effector molecule of the RAS, renin is the rate-limiting enzyme in the cascade and plays an essential role in regulating RAS activity. Several classes of drugs blocking renin activity have been shown to have renoprotective actions. Currently, plasma renin activity is used as the clinical marker for systemic RAS activity, and previous studies have shown that circulating renin is suppressed with advancing age. However, multiple studies reported on the existence of so-called tissue RAS, which may act independently of the systemic RAS. Indeed, RAS components in the kidney did not always change in parallel with RAS components in the circulation. In fact, inappropriate activation of the intrarenal RAS might underlie the pathogenesis of hypertension and renal injury (reviewed within Kobori et al.). Thus, next to systematic plasma renin activity measurements more emphasis should be placed on quantifying tissue RAS activity. As it is difficult to measure tissue RAS components in vivo, non-invasive imaging of local renin activity would help to evaluate the possible role of tissue renin activity in disease development and progression. Moreover, the development of new non-invasive imaging methods with the use of near-infrared fluorescent (NIRF) probes could lead to better detection and treatment options in the future.
It has previously been shown that kidneys of the progeroid Ercc1d/− mouse model display severe tubular attenuation and degeneration with marked anisokaryosis.18,19 Moreover, Schermer et al.20 showed that age-related transcriptional changes were present in the glomeruli of Ercc1d/− mice, thus suggesting that the progeroid Ercc1d/− mouse model is a valuable tool to study age-related glomerular pathologies. To investigate age-related changes in the intrarenal RAS in vivo, we applied the renin activatable NIRF probe ReninSense680™ allowing non-invasive imaging of renin activity in the progeroid Ercc1d/− mouse model.21

MATERIAL AND METHODS

All animal experiments were performed under the regulation and permission of the Animal Care Committee, conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8523, revised 1985). As required by Dutch law, formal permission to generate and use genetically modified animals was obtained from the responsible local and national authorities (DEC 118-11-05 and DEC 139-12-16).

Experimental animals

Animals used in this study were male and female Ercc1d/− mutants and their wild-type Ercc1+/+ littermates (WT) in an F1 hybrid FVB/N-C57BL/6J background. The generation of nucleotide excision repair-deficient Ercc1d/− mice has been previously described.22 Ren1c homozygous null mice (RenKO; 3 females and 1 male) were generated as described before (C57BL/6J background) and sacrificed at the age of 3-6 months.23 A separate group of WT mice were divided into two groups, which were either given losartan (100 mg/kg/day) in drinking water, or drinking water only from 5 weeks of age until the age of 12 weeks when the animals were sacrificed.

All mice were housed under standard laboratory conditions (temperature 23±1°C, 12-hour light-dark cycle) and maintained on standard chow (Special Diets Services, Essex, UK) with ad libitum access to water. Since Ercc1d/− mice are smaller, water bottles with long nozzles were used and food was administered within the cages from four weeks of age.

In vivo microCT-FMT imaging of renin activity

Ercc1d/− and WT mice, treated with or losartan or placebo, were injected intravenously with ReninSense680™ (2 nmol/100µl per 25 gram bodyweight) (Perkin Elmer Inc., Akron, Ohio, USA) 24 hours post FMT imaging. Mice were anesthetized (1.5-2.5% isoflurane, O₂ 1 L/min) and depilated to minimize the interference of fur on the fluorescent signal. To improve detection of intrarenal renin activity, mice were injected with the NIRF probe...
Annexin-Vivo750™ (Perkin Elmer Inc.) 2 hours post FMT imaging to visualize the kidneys and/or imaged with the microCT to allow co-registration of anatomical data with the in vivo fluorescence. Before FMT imaging, mice were injected in the tail vein with the iodine contrast agent eXIA160 (Binitio Biomedical Inc., Ottawa, Canada) for microCT imaging. Mice were positioned in the animal imaging cassette, restrained to prevent movement during imaging and imaged by using the Quantum FX imaging system (microCT) (Perkin Elmer Inc.). After microCT imaging, mice remained under anesthesia and the cassette was transferred to the FMT 2500 fluorescence tomography in vivo imaging system (Perkin Elmer Inc.). FMT imaging was performed using 680 and 700 nm excitation and emission wavelengths, respectively, 24 hours after injection. The multimodal animal imaging cassette facilitates the co-registration of microCT and FMT data through fiducial landmarks. Fusion of microCT and FMT images was done using the TrueQuant 4.0 software (Perkin Elmer Inc.). The position of the kidney was determined by the accumulation of the fluorescence of Annexin-Vivo750™ in the kidney and/or based on the distribution of the iodine contrast visualized with the microCT, which allowed quantification of in vivo fluorescence of ReninSense680™.

**Tissue collection and ex vivo fluorescent imaging of excised kidneys**

Mice were euthanized after in vivo microCT-FMT imaging by isoflurane overdose. Blood samples were harvested by cardiac puncture, transferred to EDTA coagulation vials and centrifuged at 4600 rpm for 10 minutes to collect plasma. Next, kidneys were excised, emersion fixated in formalin and assessed for ex vivo tissue epifluorescence using the FMT system and the Odyssey® CLx imaging system (LI-COR® Biosciences, Lincoln, Nebraska, USA). A separate group of Ercc1d/- and WT mice were sacrificed, kidneys were excised, snap frozen in liquid nitrogen and stored at -80°C.

**In vitro fluorescent imaging of kidney and plasma renin activity**

Activation of ReninSense680™ was determined in plasma (pooled plasma from C57Bl/6J mice, GeneTex, Irvine, CA, USA) and kidney lysates. Frozen kidneys of 2 WT and 4 RenKO mice were homogenised in PBS using mortar-pestle method. Protein concentration was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Samples were pre-incubated in the presence or absence of different concentrations of the renin inhibitor aliskiren (10⁻¹¹ – 10⁻⁴ M) at 37°C for 30 minutes. Next, tissue fluorescence was assessed by incubation of plasma or kidney lysates with ReninSense680™ (end concentration 0.2 pmol/μl) at 37°C in a humidified incubator for 30 hours. Fluorescence was measured using the Odyssey® CLx imaging system (excitation settings 700 nm). For background subtraction, kidney lysates of RenKO mice together with denatured kidney and plasma lysates (by heating the sample for 10 min at 70°C) were incubated with and without ReninSense680™.
Plasma renin concentration measured by enzyme-kinetic assay

To determine the plasma renin concentration, Ang I generation was quantified in the presence of excess sheep angiotensinogen.24,25

Histological assessment

Emersion fixated kidneys were embedded in paraffin, sectioned at 5 µm, and mounted on Superfrost Plus slides. Cross-sections of the whole kidney including the cortex and medulla were stained for haematoxylin and eosin (HE), Periodic acid-Schiff stain (PAS), Jones 2 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). The number of TUNEL-positive cells in the kidney was determined using 40x magnification.

Urine measurements relevant to renal function

Urine was collected and urinary protein, creatinine and urea level were measured according to supplier instructions with Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA), QuantiChrome Creatinine Assay Kit (Gentaur, Brussels, Belgium) and QuantiChrome Urea Assay Kit (Gentaur, Brussels, Belgium), respectively.

Statistical analysis

Data are expressed as the mean±SEM. Differences between groups were evaluated by Student’s t-test or ANOVA, and corrected for multiple testing by post-hoc Bonferroni analysis when needed. P<0.05 was considered significant. All analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Progeroid Ercc1<sup>−/−</sup> mice display age-related kidney pathology

We first set out to confirm the age-related kidney pathology in Ercc1<sup>−/−</sup> mice, for which we examined kidneys of 12 and 18-week-old mice. Indeed, from 12 weeks onwards Ercc1<sup>−/−</sup> mice display progressive kidney pathology including tubular degeneration and anisokaryosis (Fig. 1a). In addition, they present with signs of kidney aging, shown by reduced proliferation (data not shown) and increased apoptosis (Fig. 1b) already at 12 weeks of age, which was even more pronounced at 18 weeks. Moreover, at 18 weeks of age, hyaline proteinaceous casts were present within the lumen of the tubules in kidneys of Ercc1<sup>−/−</sup> mice. Renal development of Ercc1<sup>−/−</sup> kidneys was found to be normal, as 12-week-old animals had normal kidney architecture including normal numbers of glomeruli (Fig. 1c). To rule out significant renal dysfunction due to the observed pathology, we confirmed that urinary
albumin, creatinine and urea levels were unaltered in Ercc1d−/− mice compared to WT mice (Fig. 2a-c).

**Figure 1.** Histopathological changes in the kidney of progeroid Ercc1d−/− mice. a. Haematoxylin and eosin (HE), Periodic acid-Schiff stain (PAS) and Jones 2 staining of the kidneys of 12 and 18-week-old Ercc1d−/− mice and their wild-type (WT) littermates. Histological examination showed signs of kidney aging in Ercc1d−/−, including anisokaryosis, tubular degeneration and glomerulosclerosis. Moreover, hyaline proteinaceous casts were found within the lumen of the tubules in kidneys of Ercc1d−/− mice at 18 weeks of age (indicated by the arrow in PAS staining). In all panels, scale bar = 50μm. b. TUNEL staining indicated increased apoptotic cell death in Ercc1d−/− kidneys. c. The number of glomeruli confirmed normal kidney development from birth in Ercc1d−/−. ***P<0.01 vs. WT.
ReninSense selectively detects renin activity in the kidney in vitro

To assess the ability of ReninSense680™ to detect both kidney and plasma renin, activation of ReninSense was tested in kidney lysates and plasma from WT and Ren1c homozygous null (RenKO) mice, with and without co-incubation of the renin inhibitor aliskiren. As expected, ReninSense was rapidly activated in kidney lysates of WT mice assessed by fluorescent measurements with the odyssey system. The microplate kidney extract fluorescent assay showed <5% variation between duplicate wells. Aliskiren blocked ReninSense activation in a concentration-dependent manner by maximally ≈80% (Fig. 3a). The half maximal inhibitory concentration (IC50) for aliskiren in kidney lysates was approximately 10^{-7.7} M as measured here with the ReninSense probe (Fig. 3b), i.e. close to the IC50 reported earlier for mouse renin.26 The remaining fluorescent signal in the presence of the highest concentration of aliskiren was comparable to the fluorescence seen in kidney extracts from RenKO mice and denatured kidneys, indicating that this is the background fluorescent level of the ReninSense probe, in other words the detection limit of this system. When evaluating the ReninSense probe in mouse plasma, fluorescence levels remained in this background range and were unaffected by aliskiren, indicating that the probe cannot be used to measure renin activity in plasma using the odyssey system.

In vivo imaging of renin upregulation shown by ReninSense

To address the ability of ReninSense to be cleaved and used as a readout for in vivo renin activity, ReninSense activation was examined in WT mice treated either with vehicle or with the AT1 receptor antagonist losartan, which is known to increase renin levels. In addition, ReninSense activation was measured in RenKO mice. Animals were imaged tomographically by FMT 2500 24 h after ReninSense injection. To improve detection of intrarenal renin activity, mice were injected with the NIRF probe Annexin-Vivo750™ to visualize the kidneys.

Figure 2. Functional renal changes in progeroid Ercc1-/- mice. Urinary albumin (a), creatinine (b) and urea (c) was unaltered in Ercc1-/- mice compared to WT mice at 12 and 18 weeks of age.
and, when possible, also imaged with the microCT to allow co-registration of anatomical data with the in vivo fluorescence (Fig. 4a). Losartan-treated mice showed increased in vivo (Fig. 4b) and ex vivo (Fig. 4c) activation of ReninSense in their kidneys compared to vehicle treated mice. The increase in renin activity after losartan treatment was validated by quantification of the in vivo results (Fig. 4d), increased plasma renin activity (Fig. 4e) and increased renin expression levels in the kidney (Fig. 4f). As expected, fluorescence of ReninSense could not be detected in vivo or ex vivo in RenKO mice, which do not express the renin gene. These results validate the specificity of the ReninSense probe for renin activity.

**Increased renin activity in the kidney of progeroid Ercc1d/− mice in vivo**

While it is generally accepted that circulating renin activity is suppressed during aging, little is known about the regulation and activity of renin within tissues with increasing age. In order to investigate in vivo kidney renin activity during aging, we injected progeroid Ercc1d/− mice and their WT littermates with ReninSense. Combined microCT and FMT imaging of ReninSense showed increased in vivo intrarenal renin activity in Ercc1d/− mice compared to WT mice already from 12 weeks of age onwards, which was significantly different at 24 weeks of age (Fig. 5a and b). Quantification of the in vivo fluorescence (Fig. 5b) and ex vivo imaging of the kidneys (Fig. 5c) confirmed these results. We found no differences in in vivo renin activity between male and female mice (data not shown). Remarkably, plasma renin activity in the Ercc1d/− mice was significantly lower compared to WT mice at 24 weeks of age (Fig. 5d), while normal plasma renin activity levels were found at 6 weeks of age.
Figure 4. *In vivo* activation of ReninSense in kidneys of WT mice, with and without losartan treatment. 

a. Mice were imaged tomographically by FMT 2500 and microCT 24 h after ReninSense injection. MicroCT imaging and FMT imaging of Annexin-Vivo allowed accurate localization of the kidneys. Combined microCT and FMT imaging of Annexin-Vivo and ReninSense showed *in vivo* renin activity in the kidneys and bladder (clearance of probe). b. Losartan-treated mice showed increased *in vivo* intrarenal renin activity, which was confirmed by quantification (c). d. *Ex vivo* imaging of the kidneys by the Odyssey® system confirmed activation of the ReninSense probe in losartan-treated mice. Fluorescence of ReninSense could not be detected *in vivo* of *ex vivo* in RenKO mice. e. Losartan treatment increased plasma renin activity. f. Increased expression levels of renin in the kidney were found in losartan-treated mice. ND, not detectable. Data are mean±SEM of n=3. *P<0.05, ***P<0.01 vs. WT.
DISCUSSION

Changes in the RAS are associated with the pathophysiology of various cardiovascular and renal diseases, and therefore targeting the RAS seems a logical therapeutic approach in treatment of these diseases. Indeed, pharmacological RAS blockade has been shown to effectively slow down the progression of renal disease. However, it is important to note that not all patients, e.g. elderly, respond well to RAS blockade. While the systemic RAS is suppressed with advancing age, the regulation and activity of tissue RAS during aging is not well defined. As such, previous reports showed that although the circulating RAS is suppressed during normal aging, some components of the intrarenal RAS are elevated. Varying tissue RAS activity might, at least in part, explain why elderly respond
unpredictable to RAS blockade. Therefore, in this study we aimed to evaluate the use of the renin activatable near-infrared fluorescent probe ReninSense to facilitate non-invasive imaging of renin activity in vivo. In addition, we investigated the activity of plasma as well as intrarenal renin in progeroid Ercc1d/− mice with accompanying age-related kidney pathology. First, we showed that ReninSense specifically detects renin activity, as fluorescence of the probe was increased after losartan treatment, while virtually no fluorescence could be detected in RenKO mice. Secondly, this study demonstrated that intrarenal renin activity does not necessarily run in parallel with circulating renin in the progeroid aging Ercc1d/− mice.

It is important to note that most of the clinical studies supporting the beneficial effects of RAS inhibition do not include participants older than 75 years of age or elderly patients that are frail with a high comorbidity burden.30, 31 Not all elderly respond well to RAS blockade, and related adverse events include acute kidney injury, hyperkalemia, hypotension and a further decline in glomerular filtration rate.3, 11, 12, 32, 33 Additionally, combination therapy with ACE inhibitors and angiotensin receptor blockers in patients with cardiovascular complications is linked to an increased risk of adverse renal outcomes with higher rates of hyperkalemia, hypotension, renal dysfunction and no observed benefit with respect to overall mortality.34-37 The occurrence of these side effects might be worse in the elderly population, as they are prone to develop acute kidney injury and hyperkalemia due to the risk of complete RAS inhibition as they already have low plasma renin levels. Therefore, caution and close monitoring are recommended when using these drugs in elderly patients with kidney dysfunction and the optimal RAS inhibition with respect to end organ protection has yet to be determined in the elderly.38 In this respect, it would be interesting to see how RAS inhibition would affect the aging kidney alone. In other words, study the effect of RAS inhibition in kidney-specific Ercc1 mutant mice, which would represent a healthy mouse with aging kidneys. This might answer important questions on how the RAS is regulated in the aging kidney, and whether this is a systemic effect or not.

Controversy remains as to whether all RAS components that are required to generate Ang II locally are produced locally, or are taken up from the circulation.15, 39, 40 In the present study, the opposing findings on intrarenal and plasma renin in progeroid Ercc1d/− mice supports an independent upregulation of intrarenal RAS. This might be very similar in the elderly as their circulating renin is lower with increasing age.7, 14 It remains to be seen whether kidney renin levels are increased with age in the elderly population. Interestingly, low plasma renin levels with increased kidney renin levels have also been found in diabetic patients.13, 41 Animal models of early diabetic nephropathy identically showed decreased plasma renin activity and increases in kidney renin.42-45 Epidemiologic studies showed that with age, the incidence and susceptibility of abnormal glucose levels and diabetic disease increases, however the mechanisms linking aging and diabetes are not well understood.46, 47 It is suggested that increased intrarenal renin is responsible for the
development and progression of nephropathy in diabetes, through increased intrarenal AT1 receptor signaling. Therefore, it would be interesting to investigate whether diabetes is responsible for this increased intrarenal renin and accompanying kidney injury, or rather that this increased intrarenal RAS, like diabetes, is in fact an concomitant result of the aging process.

As the circulating RAS does not necessarily reveal the responsiveness of the RAS within tissues, there is a need for reliable methods to assess the RAS within tissues. Whether urinary angiotensinogen reflects intrarenal RAS activity is doubtful. In addition, renal plasma flow responses to infused Ang II are used as an indirect measure of intrarenal RAS activation in humans, as it correlates inversely with endogenous RAS activity. However, all these methods are indirect measurements of intrarenal RAS activity and currently there is no method to directly assess intrarenal RAS activity in humans. Thus, non-invasive imaging of the ReninSense probe holds considerable promise to improve the detection and localization of local renin activity, including intrarenal renin. Determining local renin activity would help to evaluate the complexity of RAS biology and the possible role of local renin activity in disease development and progression. Moreover, this method enables longitudinal imaging of altered RAS signaling, consequently, disease progression can be monitored over time and the effect of (new) interventions can be studied non-invasively.

In the present study, the fluorescence levels of the ReninSense probe in mouse plasma remained in the background range and were unaffected by aliskiren, indicating that the probe cannot be used to measure renin activity with the odyssey system. These results are consistent with the results demonstrated by Zhang et al., as ReninSense fluorescence in mouse plasma in their hands was also unaffected by renin inhibition. Only when mice were treated with low salt diet, ReninSense fluorescence increased over time and L-810 treatment in these mice reduced the fluorescence to a level similar to the fluorescence levels in untreated mouse plasma, indicating that these measured fluorescence in normal mouse plasma actually represented background. We did however, observe that ReninSense was rapidly activated in kidney lysates of WT mice and that aliskiren blocked ReninSense activation by maximally \( \approx 80\% \). The remaining fluorescent signal in the presence of the highest concentration of aliskiren was comparable to the fluorescence seen in kidney extracts from RenKO mice and denatured kidneys. This implies that the remaining fluorescent signal either represents the background fluorescent level of the ReninSense probe, or represents activation of the probe ReninSense by renin-like enzyme (e.g. cathepsins), which might also be capable of reacting with the angiotensinogen sequence of the probe. Nevertheless, when comparing in vivo and ex vivo kidney activation of ReninSense in RenKO mice, fluorescence did not reach the threshold value an thus could not be detected, while losartan significantly increased kidney fluorescence levels in vivo as well as ex vivo, verifying the specificity of the probe to measure renin activity in the kidneys of small animals.
In conclusion, we have demonstrated that the NIRF probe ReninSense can be used to non-invasively visualize and measure intrarenal renin activity. By using this method to identify local RAS activity, we might gain important insights into the changes in the RAS that occur with age as well as in other (age-related) diseases. Although further study is warranted, our observations in the progeroid Ercc1d/- mouse model provide evidence that circulating RAS activity does not necessarily run in parallel with intrarenal RAS activity during aging, which has important clinical consequences. As this increased intrarenal RAS activity, might contribute to the disturbed kidney pathology observed in these mice, future investigations should examine the effect of the observed age-dependent changes in intrarenal renin activity on kidney deterioration.

REFERENCES


