Towards an integrated approach on RAAS-blockade

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 8

RENAL ACE2 EXPRESSION IN HUMAN KIDNEY DISEASE

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Inge Hamming
Harry van Goor
Gerjan Navis
Abstract

Angiotensin-converting enzyme 2 (ACE2) is a recently discovered homologue of angiotensin-converting enzyme (ACE) thought to counterbalance ACE. ACE2 cleaves angiotensin I and angiotensin II into the inactive angiotensin 1-9 and the vasodilator and anti-proliferative angiotensin 1-7, respectively. ACE2 is known to be present in human kidney, but no data on renal disease are available so far.

Renal biopsies from 58 patients with diverse primary and secondary renal diseases were studied (hypertensive nephropathy n=5, IgA glomerulopathy n=8, minimal change nephropathy n=7, diabetic nephropathy n=8, focal glomerulosclerosis n=5, vasculitis n=7, and membranous glomerulopathy n=18), 17 renal transplants and 18 samples from normal renal tissue. Immunohistochemical staining for ACE2 was scored semi-quantitatively.

In control kidneys ACE2 was present in tubular and glomerular epithelium and in vascular smooth muscle cells and endothelium of interlobular arteries. In all primary and secondary renal diseases and kidney transplants we found neoexpression of ACE2 in glomerular and peritubular capillary endothelium. There were no differences between the various renal disorders, or between acute and chronic rejection and control transplants. ACE inhibitor treatment did not alter ACE2 expression.

In primary and secondary renal disease and in transplanted kidneys neoexpression of ACE2 occurs in glomerular and peritubular capillary endothelium. Further studies should elucidate the possible protective mechanisms involved in the de novo ACE2 expression in renal disease.
Introduction

Angiotensin converting enzyme (ACE), a key enzyme of the renin angiotensin system, plays a crucial role in renal (patho-)physiology. It converts angiotensin I to angiotensin II, a potent vasoconstrictor, growth modulator and pro-inflammatory peptide. Renal ACE is primarily a membrane-bound protein residing on the surface of epithelial and endothelial cells [1;2]. Recently, a homologue of ACE, angiotensin converting enzyme (ACE2), has been identified in humans and rodents [3-5]. ACE2 cleaves angiotensin I and angiotensin II into the inactive angiotensin 1-9 and the vasodilator angiotensin 1-7, respectively. Therefore, it is thought to counterbalance the effects of ACE [6]. In contrast to ACE, ACE2 activity is not inhibited by ACE inhibitors [3].

Recently, we reported the localization of ACE2 in the normal human kidney. Abundant staining was present in the vascular endothelium and vascular smooth muscle cells [7]. Moreover, we observed glomerular visceral and parietal epithelial ACE2 staining, but no endothelial or mesangial staining. In the interstitium strong staining was found in the proximal tubular brush border, a weak cytoplasmatic staining in the collecting duct, and distal and proximal tubulus. Considering the role of the renin-angiotensin system in renal disease, the localization of ACE2 in human kidney disease would be of interest [6;8]. However, so far no data are available on ACE2 in human renal disease. Therefore, in the present study we investigated the expression of ACE2 in various primary and secondary renal disorders and after renal transplantation.

Methods

Kidney specimens

All procedures and use of (anonymized) tissue were performed according to national ethical guidelines. Kidney samples were obtained by percutaneous renal biopsy from patients undergoing diagnostic evaluation. Clinical data were obtained from a renal biopsy database linked with a biopsy number. Control specimens (n=18) were taken from normal parts of kidneys from patients who underwent renal oncological surgery.

Renal tissue was fixed in 4% paraformaldehyde and processed for paraffin embedding according to standard procedures. Routine morphology was evaluated by hematoxylin and eosin-stained sections by a qualified pathologist. Control specimens were only used if characterized as non-diseased.

Biopsies from 58 subjects with diverse primary and secondary renal diseases were investigated. Diagnoses included: hypertensive nephropathy (n=5), IgA glomerulopathy (n=8), minimal change nephropathy (n=7), diabetic nephropathy (n=8), focal glomerulosclerosis (n=5), vasculitis (n=7), and membranous glomerulopathy (n=18). Moreover, biopsies of 17 renal transplant recipients, with acute
(n=5) or chronic rejection (n=6) and control transplants (n=6) without morphological signs of rejection on examination were investigated.

**Immunohistochemistry**

Renal biopsies were first dewaxed and subjected to heat induced antigen retrieval by overnight incubation in 0.1 M Tris/HCl (pH 9) buffer at 80°C. Endogenous peroxidase was blocked with 0.075% H2O2 in phosphate-buffered saline (PBS, pH 7.4) for 30 minutes. Antibody dilutions were made in PBS supplemented with 1% bovine serum albumin.

A polyclonal rabbit anti-ACE2 antiserum (Millenium Pharmaceuticals, Inc, Cambridge, MA) diluted in PBS and supplemented with 1% bovine serum albumin was used at a concentration of 1:1000 for 1 h at room temperature. Antibody binding was detected using sequential incubations with peroxidase-labelled goat anti-rabbit and peroxidase labelled anti-goat (GARPO/RAGPO; Dako, Glostrup, Denmark). Human AB serum (1%) was added to the secondary antibodies. Peroxidase activity was developed by using 3,3'-diaminobenzidine tetrachloride (DAB) for 10 min. Counterstaining was performed using Mayer’s haematoxylin. Three kind of control test were performed to determine the specificity of the antibody. First, control sections were incubated with anti-ACE2 antibody solutions which had been pre-incubated with the synthetic peptide to which the antibody was raised (peptide sequence: NTNITEENVQMNNAGDKW aa 51-69; Pepscan Systems BV, Lelystad, The Netherlands); second, sections were incubated with unrelated rabbit polyclonal antibodies (anti-alpha 1 inhibitor 3 or anti-nitrotyrosine); and third, sections were incubated with PBS without the primary antibodies. These control sections did not reveal any staining (Figure 1F).

A qualified pathologist analyzed the staining for structures positive for ACE2. The staining was analyzed semi-quantitatively. The combined intensity and distribution of immunostaining were determined on a scale of 0 to 3+ (0: absent; 1+: low intensity; 2+: moderate intensity; 3+: high intensity) for different parts of the glomerulus (mesangium, endothelium, visceral epithelium and parietal epithelium) and interstitium (tubular epithelium, vascular smooth muscle cells, vascular endothelium, peritubular capillaries).

**Data analysis**

Clinical data are presented by a break-up according to diagnosis and expressed as median values and range. Data on immunostaining are expressed semiquantitively as described above. As no apparent differences were present between the various native renal disorders, the immunohistochemistry data are presented together. The data on membranous glomerulopathy - the largest category of patients – were analyzed by a break-up according to the use of ACE-inhibition.
Table 1 | Clinical data

<table>
<thead>
<tr>
<th>Category</th>
<th>M/F</th>
<th>Age (year)</th>
<th>MAP (mmHg)</th>
<th>Serum creatinine (ug/L)</th>
<th>Proteinuria (g/day)</th>
<th>n treated with ACEi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>9/9</td>
<td>58 (2-73)</td>
<td>88 (65-113)</td>
<td>90 (44-145)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Primary and secondary renal diseases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertensive nephropathy</td>
<td>3/2</td>
<td>45 (25-73)</td>
<td>102 (93-140)</td>
<td>218 (134-817)</td>
<td>0.8 (0.2-6.3)</td>
<td>1</td>
</tr>
<tr>
<td>IgA glomerulopathy</td>
<td>6/2</td>
<td>46 (23-83)</td>
<td>103 (90-116)</td>
<td>131 (87-264)</td>
<td>2.2 (0.0-6.3)</td>
<td>2</td>
</tr>
<tr>
<td>Minimal change nephropathy</td>
<td>6/1</td>
<td>24 (6-67)</td>
<td>89 (76-111)</td>
<td>98 (35-124)</td>
<td>6.3 (4.2-8.9)</td>
<td>2</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>2/6</td>
<td>56 (42-70)</td>
<td>117 (80-132)</td>
<td>135 (89-359)</td>
<td>5.3 (0.1-12.5)</td>
<td>7</td>
</tr>
<tr>
<td>Focal glomerulosclerosis</td>
<td>5/0</td>
<td>76 (39-80)</td>
<td>107 (100-113)</td>
<td>273 (76-896)</td>
<td>7.7 (1.8-10.9)</td>
<td>3</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>2/5</td>
<td>50 (27-70)</td>
<td>97 (73-100)</td>
<td>174 (97-550)</td>
<td>2.8 (0.5-6.1)</td>
<td>2</td>
</tr>
<tr>
<td>Membranous glomerulopathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-ACEi</td>
<td>4/4</td>
<td>45 (30-68)</td>
<td>105 (80-120)</td>
<td>96 (75-248)</td>
<td>6.9 (4.0-19.6)</td>
<td>0</td>
</tr>
<tr>
<td>ACEi</td>
<td>5/5</td>
<td>51 (43-78)</td>
<td>107 (68-135)</td>
<td>134 (69-390)</td>
<td>8.2 (3.8-21.3)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Transplanted kidney</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No rejection signs</td>
<td>3/3</td>
<td>39 (28-69)</td>
<td>95 (85-110)</td>
<td>160 (104-954)</td>
<td>0.5 (0.0-0.7)</td>
<td>0</td>
</tr>
<tr>
<td>Acute rejection</td>
<td>2/3</td>
<td>49 (23-63)</td>
<td>97 (61-110)</td>
<td>440 (112-1021)</td>
<td>2.3 (0.2-18.2)</td>
<td>0</td>
</tr>
<tr>
<td>Chronic rejection</td>
<td>0/6</td>
<td>32 (29-55)</td>
<td>106 (101-113)</td>
<td>192 (110-328)</td>
<td>0.7 (0.3-9.0)</td>
<td>0</td>
</tr>
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</table>

Abbreviations are: M/F, male-to-female ratio; MAP, mean arterial pressure; ACEi, ACE-inhibition.
MAP, serum creatinine and proteinuria are expressed as median and range

Results

Clinical data

Table 1 summarizes clinical data of the control subjects and the different patient categories. The control subjects were older than the patients, with a mean age of 64 ± 6 versus 49 ± 18 year (p<0.05). The male-to-female ratio of all subjects was 47/46. The patients had a variety of renal disorders, most with primary glomerular involvement. A wide range of blood pressure, renal function, and proteinuria was present across the different groups. Among the patients with membranous glomerulopathy, no differences in renal function and proteinuria were present between subjects with and without ACE-inhibitor.

ACE2 expression in normal renal tissue

Control kidneys were all normal on routine morphological examination, except for some arteries with moderate atherosclerosis, compatible with the age of these subjects.

In the glomeruli weak glomerular visceral ACE2 staining was observed, whereas the parietal epithelial cells were moderately positive (Figure 1A, table 2). Despite clear endothelial staining of the
vessels, the mesangium and glomerular endothelium were negative for ACE2 (Figure 1A, 1D). Abundant staining was present in the brush border of the proximal tubular cells, whereas the cytoplasm of these cells was weakly positive (Figure 1B, 1C). Epithelial cells from the distal tubules and collecting ducts showed weak cytoplasmic staining (Figure 1E).

In cortical radial arteries (interlobulair) ACE2 staining was present in endothelium and smooth muscle cells (Figure 1D). Moreover, ACE2 staining was present in endothelium of medium-sized and small veins but absent in endothelium of peritubular capillaries (Figure 1C). Finally, linear staining was present on the urothelium lining the renal pelvis and ureter. This staining pattern was uniform across all investigated subjects.

### ACE2 expression in primary and secondary renal diseases

ACE2 expression was remarkably uniform in the various primary and secondary renal disorders. These data are therefore presented as a single group (Table 2). In tubuli and blood vessels ACE2 expression was similar to that in healthy controls, except for additional staining in the intima fibrosis of blood vessels (Figure 2B). In the glomerular visceral and parietal epithelium, on the other hand, ACE2 expression was enhanced as compared to controls (Figure 2A and 2C). Moreover, neoexpression of ACE2 was observed in the glomerular capillary endothelium and peritubular capillaries (Figure 2C, 2D and 2F). The glomerular mesangium showed subtle staining in more than half of the biopsies (Figure 2C). In membranous glomerulopathy, the number of patients allowed a comparison between subjects

| Abbreviations are: ACEi, ACE-inhibition; SMC, smooth muscle cells |

### Table 2 | ACE2 immunolocalization in control, native kidney disease, influence of ACEi and transplantation |

<table>
<thead>
<tr>
<th></th>
<th>Mesangium</th>
<th>Endothelium</th>
<th>Epithelium</th>
<th>Tubular epithelium</th>
<th>Vascular</th>
<th>Peritubular capillaries</th>
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<tr>
<td>Controls</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+++</td>
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<td>-</td>
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<tr>
<td>Native kidney disease</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Membranous glomerulopathy</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>non-ACEi</td>
<td></td>
<td></td>
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<tr>
<td>ACEi</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Transplantation</td>
<td>No rejection signs</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Acute rejection</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Chronic rejection</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
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</table>

Abbreviations are: ACEi, ACE-inhibition; SMC, smooth muscle cells
A-F: Representative example of ACE2 staining in healthy control tissue.
A/B: In the glomerulus, ACE2 is slightly expressed in the visceral (arrow-head) and parietal epithelium (arrow). B/C: In the tubulus, strong staining in the brush border (arrow) and weak cytoplasmatic staining is present. D: Positive ACE2 staining is seen in renal vessels; in the endothelium (arrow) and smooth muscle cells (arrow-head) ACE2. E: Cytoplasmatic staining is observed in the distal tubulus (arrow-head) and collecting ducts (arrow). F: Control section stained with anti-ACE2 in the presence of the synthetic ACE2 peptide show no staining of the renal tissue.
Figure 2 | ACE2 staining in human kidney disease.
A: Glomerulus with membranous glomerulopathy. Strong ACE2 staining is observed in parietal epithelium (arrow). B: Vessel of a diabetic patient. In all diseased vessels ACE2 is expressed in intima fibrosis (arrow), smooth muscle cells (arrow-head) and endothelium (short-arrow). C/D: In diabetic glomeruli, ACE2 neoexpression is found in the glomerular endothelium (arrow) and mesangium (short-arrow). Enhanced ACE2 staining of the visceral epithelium (arrow-head) is observed. E: The glomerulus of a transplanted kidney with acute rejection shows enhanced staining in the parietal epithelium (arrow) and neoexpression of ACE2 in the glomerular endothelium (arrow-head). F: Tubuli of a kidney with membranous glomerulopathy. In all diseased peritubular capillaries (arrow-heads) neoexpression of ACE2 is found.
treated with ACE inhibition versus those without ACE inhibition: no differences in ACE2 expression however, could be detected between these two groups. Finally, ACE2 expression was not related to the severity of the renal involvement (as estimated from serum creatinine or proteinuria) across the different diagnosis groups. No relationship between ACE2 expression, gender or age could be detected either.

**ACE2 expression in the transplanted kidney**

Presence of acute or chronic rejection was assessed according to the Banff criteria on routine morphology. All transplant biopsies showed similar localization of ACE2 expression (Figure 2E and table 2) without differences between acute or chronic rejection and control transplants. ACE2 expression in tubules, blood vessels, glomerular visceral epithelium and mesangium was similar to that in healthy controls. Glomerular parietal epithelial ACE2 staining, on the other hand, was enhanced (Figure 2E). In addition, subtle staining of the glomerular capillary endothelium and peritubular capillaries was present. Compared to the primary and secondary renal diseases, the glomerular changes were less prominent in renal transplants, and no mesangial neoexpression of ACE2 was detected. ACE2 expression was not related to the severity of renal function impairment, proteinuria, age or gender.

**Discussion**

This study is the first to provide immunohistochemical data on renal ACE2 expression in human renal disease. Under pathological conditions and in transplanted kidneys we found neoexpression of ACE2 in glomerular and peritubular capillary endothelium and enhanced expression in visceral and parietal glomerular epithelium. We found no differences between the various renal disorders, or between acute and chronic rejection and control transplants. In accord with the reported insensitivity of ACE2 to ACE-inhibitors, ACE-inhibition had no discernable effect on ACE2 expression [5].

Our population was selected to represent a variety of renal disorders, and a range of severity of renal involvement. As the number of patients per diagnosis category was relatively small our data are not well-suited to conclude on diagnosis-related factors. However, our population is well-suited to detect changes in ACE2 expression that are robust across different renal conditions. Interestingly, neoexpression of ACE2 in glomerular and peritubular endothelium was consistently observed in diseased kidneys across the different diagnosis categories, and across the range of severity of renal function impairment and proteinuria, thus providing such a robust finding.

Our control samples were obtained from subjects that underwent renal oncological surgery, representing a wide age-range, with age-related changes in the control kidneys of elderly subjects. ACE2 expression in the kidneys with intrinsic disease was clearly distinguishable from that in control samples, reinforcing the assumption that the observed neo-expression of ACE2 is linked to presence
of intrinsic renal disease, as distinct from age-related renal changes. The latter assumption is supported by the similarity of ACE2 expression over a wide age range in the control subjects.

In addition to biopsies with primary and secondary renal diseases we also investigated renal transplants. In transplants without signs of rejection subtle glomerular endothelial expression was observed, that was enhanced as compared to the controls, but less prominent than in primary and secondary renal diseases. These changes might reflect the fact that, despite the absence of morphological signs of rejection, these transplant kidneys are not equivalent to healthy kidneys, as also apparent from the renal function impairment. No additional effects of acute or chronic rejection were observed.

The pathophysiological significance of the renal endothelial ACE2 neoexpression in primary and secondary renal diseases and renal transplants is very intriguing, since these changes are in line with those in a report on endothelial neoexpression of ACE in diseased human kidneys [2]. Local upregulation of the ACE enzyme is generally associated with enhanced angiotensin II formation resulting in increments of renal damage. The concomitant de novo expression of ACE2 at the same localization suggest a protective counteracting mechanism aiming at reducing local angiotensin II levels.

Experimental data support the pathophysiological relevance of ACE2. Genetic inactivation of ACE2 in mice resulted in severe cardiac dysfunction, which is prevented by a concomitant knockout for ACE [9] suggesting a counterbalancing role of the two enzymes. Cardiac ACE2 gene expression was reduced in three models of hypertension [9]. Recently, upregulation of ACE2 was shown after myocardial infarction by blockade of the angiotensin II type-I receptor [10]. Upregulation of ACE2 in failing human ventricles supports the relevance to human pathophysiology [11].

Few data are available on the functional role of ACE2 in the kidney. Experimental diabetic nephropathy and hypertension are associated with reduced (renal) ACE2 expression [9;12]. On the other hand, mice with an early stage of diabetes had increased ACE2 protein in renal cortical tubulus [13]. The authors suggest that these data are consistent with the assumption that neoexpression of ACE2 may reflect a protective mechanism. Recent data by Broshihan et al., showing that intrarenal distribution of ACE2 is associated with angiotensin 1-7 expression, can be taken to support the functional impact of intrarenal ACE2 [14]. Finally, data published in abstract form showed that ACE2 knockout mice develop glomerulosclerosis [15]. Taken together the available data obtained in experimental models suggest that ACE2 may be relevant to renal pathophysiology. Our present data add to this by demonstrating that ACE2 expression is consistently altered in human renal disease. Functional studies, preferably including intervention, are needed to further explore the role of ACE2 in experimental and human renal disease.
In conclusion, renal ACE2 expression is altered in human kidney disease. Neoexpression of ACE2 is found in glomerular and peritubular capillary endothelium in various renal disorders and in renal transplants. Further studies should elucidate the pathophysiological significance of these changes in renal ACE2 expression, and explore its role as a possible protective mechanism.

Acknowledgements

We thank M Donoghue and S Acton (Millenium Pharmaceuticals, Inc, 75 Sidney St, Cambridge, MA 02139, USA) for their kind gift of the ACE2 antibody.
References