Mice deficient in PAPP-A show resistance to the development of diabetic nephropathy

Jessica R Mader, Zachary T Resch, Gary R McLean², Jakob H Mikkelsen³, Claus Oxvig³, Ronald J Marler¹ and Cheryl A Conover

Endocrine Research Unit, Division of Endocrinology, Mayo Clinic, 200 First Street SW, 5-194 Joseph, Rochester, Minnesota 55905, USA

¹Department of Comparative Medicine, Mayo Clinic, Collaborative Research Building, 13400 E Shea Boulevard, Scottsdale, Arizona 85259, USA

²Cellular and Molecular Immunology Research Centre, London Metropolitan University, London, UK

³Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10C, DK-8000 Aarhus C, Denmark

Correspondence should be addressed to C A Conover
Email
conover.cheryl@mayo.edu

Abstract

We investigated pregnancy-associated plasma protein-A (PAPP-A) in diabetic nephropathy. Normal human kidney showed specific staining for PAPP-A in glomeruli, and this staining was markedly increased in diabetic kidney. To assess the possible contribution of PAPP-A in the development of diabetic nephropathy, we induced diabetes with streptozotocin in 14-month-old WT and *Papp-A* knockout (KO) mice. Renal histopathology was evaluated after 4 months of stable hyperglycemia. Kidneys from diabetic WT mice showed multiple abnormalities including thickening of Bowman's capsule (100% of mice), increased glomerular size (80% of mice), tubule dilation (80% of mice), and mononuclear cell infiltration (90% of mice). Kidneys of age-matched non-diabetic WT mice had similar evidence of tubule dilation and mononuclear cell infiltration to those of diabetic WT mice, indicating that these changes were predominantly age-related. However, thickened Bowman's capsule and increased glomerular size appeared specific for the experimental diabetes. Kidneys from diabetic *Papp-A* KO mice had significantly reduced or no evidence of changes in Bowman's capsule thickening and glomerular size. There was also a shift to larger mesangial area and increased macrophage staining in diabetic WT mice compared with *Papp-A* KO mice. In summary, elevated PAPP-A expression in glomeruli is associated with diabetic nephropathy in humans and absence of PAPP-A is associated with resistance to the development of indicators of diabetic nephropathy in mice. These data suggest PAPP-A as a potential therapeutic target for diabetic nephropathy.

Key Words

- pregnancy-associated plasma protein-A
- kidney
- diabetes
- Bowman's capsule

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Introduction

Pregnancy-associated plasma protein-A (PAPP-A) was initially identified as one of four proteins found at high concentrations in the circulation of pregnant women,

hence its name (Lin *et al.* 1974). Subsequently, PAPP-A was discovered to be a novel zinc metalloprotease expressed by a variety of cell types and tissues unrelated to pregnancy

(Lawrence *et al.* 1999, Conover *et al.* 2006, Qin *et al.* 2006). *In vitro* and *in vivo* studies indicate that PAPP-A functions to enhance the growth-stimulating actions of local insulin-like growth factors (IGFs) through cleavage of inhibitory IGF binding proteins (reviewed in Conover 2012). Although a benefit in many tissues in early life, PAPP-A (and IGFs) can have detrimental effects in later life with promotion of aging and age-related disease (Conover *et al.* 2004, Conover 2010). Indeed, *Papp-A* knockout (KO) mice live 30–40% longer than their WT littermates with reduced incidence and severity of degenerative diseases of age, such as nephropathy (Conover *et al.* 2010). These histopathological data suggesting a role for PAPP-A in the aging kidney, the renal expression of multiple components of the IGF1 system (Vasyleva & Ferry 2007), and the reported effects of IGF1 in promoting matrix production and proliferation of kidney cells (Horney *et al.* 1998, Lupia *et al.* 1999) prompted us to investigate the role of PAPP-A in the development of diabetic nephropathy, the leading cause of end-stage renal disease worldwide (US Renal Data System Annual Report 2008). There were two aims of this study: i) to assess PAPP-A expression in kidneys from normal and diabetic subjects by immunohistochemistry and ii) to determine the effect of *Papp-A* gene deletion on the development of nephropathy in an experimentally induced mouse model of type 2 diabetes. The latter was of particular interest based on a study by Swindell *et al.* (2010) looking at differential gene expression in WT vs *Papp-A* KO mice that highlighted the kidney as a tissue in which moderated IGF signaling could have favorable effects.

Materials and methods

PAPP-A immunohistochemistry human kidney

Fresh-frozen normal and diabetic kidney sections were obtained from AMSBIO (Abingdon, UK). Sections were fixed in 100% methanol, rehydrated in PBS, and probed for PAPP-A protein expression using recombinant anti-PAPP-A MAB (J H Mikkelsen & C Oxvig, unpublished observations) and detected with a FITC-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). PAPP-A staining was visualized using a LSM 510 Confocal Laser Scanning Microscopes (Carl Zeiss Micro-Imaging, Oberkochen, Germany) at 40× magnification with DAPI used as a nuclear counterstain. Mouse IgG2α was used as an isotype control.

Mice

Adapting the protocol of Wu *et al.* (2010), 14-month-old female PAPP-A KO and WT mice received i.p. injections of streptozotocin (50 µg/g per injection) freshly dissolved in 137 mM Na citrate, pH 4.5, every 2–3 days for a total of five to seven injections in order to induce stable hyperglycemia. Mice were placed in clean cages and fasted for 4 h prior to each injection. Body weight and blood glucose levels were measured prior to the initial streptozotocin injection and weekly for 4 months afterwards, in each instance after a 4-h fast. Mice were considered diabetic when their blood glucose level was sustained at ≥250 mg/dl. There was <10% mortality, and dead or killed animals were excluded from further study. Mice were not treated with insulin at any time during the study. After 4 months of stable diabetes, body and kidney weights were recorded, blood was collected, and right and left kidneys were prepared for fixation. All procedures were approved by Mayo Clinic's Institutional Animal Care and Use Committee.

Renal histology

The right kidney was cut in cross section and the left kidney was cut longitudinally, rinsed in PBS, and fixed overnight in Bouin's solution. Fixed tissues were flushed for 10 min with tap water and stored at 4 °C in PBS until shipping to the Animal Histology Core at Mayo Clinic (Scottsdale, AZ, USA) for sectioning and staining with hematoxylin–eosin. Kidney sections were qualitatively assessed by an American College of Veterinary Pathologists board-certified veterinary pathologist (R J M). A blinded histopathology report was based on standard descriptions and terminology (see legend to Table 1), and given one of four subjective grades of minimal, mild, moderate, and marked. Sections of kidney were also stained with periodic acid-Schiff (PAS) to measure mesangial area within each glomerulus using Adobe PhotoShop Software (Adobe Systems, Inc.). At least 300 glomeruli from three mice were assessed per group.

Immunohistochemistry

De-paraffinized sections of mouse kidney were stained for macrophages using F4/80 as primary antibody. Slides were rinsed with Tris-buffered saline and 0.01% Tween 20 for 3 min, treated with peroxidase blocking reagent (Dako, Carpinteria, CA, USA) for 5 min, rinsed with wash buffer (Dako), and treated with Rodent Block M (Biocare Medical, Concord, CA, USA) for 30 min. Slides were then incubated with primary antibody (F4/80 CI:A3-1, rat monoclonal, AbD

Table 1 Diabetic kidney histology. Numbers represent the percentage of mice with the indicated observations, assessed in a blinded fashion as described in the Materials and methods and Results sections. Fisher's exact test was used to compare proportions. Mononuclear cell infiltration: aggregates of primarily lymphocytes that were prominently noted around arterioles in the cortex, but also seen around larger vessels in the pelvis. The blood vessels within the aggregates of mononuclear cells were often prominent. Dilation of cortex/medulla tubules increased: dilated tubules that often contained eosinophilic (proteinaceous) filtrate. Cytoplasmic alteration of cortex tubules: characterized by small groups of tubules with subtle cytoplasmic granularity. The tubules stained lightly eosinophilic to lightly basophilic. Thickened Bowman's membrane: noticeable thickening of the membrane in several glomerular tufts. Increased glomerular size: the change was characterized by an increase in mesangial size that was often associated with an increase in cellularity, mesangial matrix, and/or prominent juxtaglomerular area. Other: amyloid: small aggregates of homogeneous, eosinophilic material within an area of mononuclear cell infiltrate. Presumptive amyloid. Metaplasia: small bone spicule in left kidney. None of the changes in any of the tissue section were graded greater than mild and most were minimal in severity

	Diabetic		Non-diabetic
	WT (n = 10)	<i>Papp-A</i> KO (n = 13)	WT (n = 12)
Mononuclear cell infiltration (%)	90	77	100
Tubule dilation (%)	80	0 ^a	75
Tubule cytoplasmic changes (%)	10	0	8
Thickened Bowman's (%)	100	< 1 ^a	7 ^b
Increased glomerular size (%)	80	23	17
Two or more abnormalities (%)	100	23	58
Other abnormalities (%)	40	0	58

^aSignificant difference between 18-month-old diabetic *Papp-A* KO and diabetic WT mice.

^bSignificant difference between 18-month-old non-diabetic and diabetic WT mice.

Serotec, Raleigh, NC, USA) for 60 min at room temperature at a concentration of 1:200 (diluted in Background Reducing Diluent (Dako)), followed by incubation with secondary antibody (Rat on Mouse Probe, Biocare, pre-diluted) for 15 min at room temperature. Slides were rinsed three times in wash buffer, incubated with tertiary reagent (Rat on Mouse HRP, BioCARE, Tempe, AZ, USA) for 15 min at room temperature, rinsed in wash buffer, and then stained for visualization with diaminobenzidine (Biocare) for 5 min. Counterstaining with hematoxylin, followed by dehydration in increasing concentrations of ethyl alcohol and xylene, was performed prior to cover slipping.

Blood chemistry

Blood urea nitrogen (BUN) measurements were performed using a Urea Nitrogen assay kit purchased from Pointe Scientific, Inc. (Canton, MI, USA). Serum IGF1 was measured using a mouse IGF1 ELISA kit generously provided by ImmunoDiagnostic Systems (Fountain Hills, AZ, USA).

Statistical analysis

Fisher's exact test was used to compare proportions of mice between groups. Two-tailed ANOVA and Tukey's

tests were used for multiple comparisons of group means. $P < 0.05$ was considered statistically significant.

Results

PAPP-A expression in human kidney

PAPP-A immunohistochemistry of sections from a normal human kidney and a kidney from a patient with diabetic nephropathy is presented in Fig. 1. In normal kidney, there was specific staining for PAPP-A within and around the glomerulus, the structural unit responsible for filtration of the plasma that is eventually processed into urine. In comparison, diabetic kidney had manifestly more intense staining in the glomerulus as well as in association with the thickened Bowman's capsule, the latter being a prominent feature of diabetic nephropathy.

General characteristics of diabetic and non-diabetic mice

There was a significant difference in body weight between WT and *Papp-A* KO mice, as reported previously (Conover *et al.* 2004), but there was no significant difference in body weight in the diabetic mice (WT and *Papp-A* KO) when compared with their respective non-diabetic controls

Figure 1

PAPP-A expression in human kidney. Sections of human kidney from a normal healthy subject and from a patient with diabetic nephropathy stained with primary antibody to PAPP-A or with isotype control, IgG2 α . Green, PAPP-A; blue, nuclei.

(Table 2). There was a significant difference in the kidney weight/body weight ratio between diabetic WT and *Papp-A* KO mice. However, this was not caused by the diabetic phenotype, as a similar difference was seen in non-diabetic mice. There was no significant difference in the harvest blood glucose level between WT and *Papp-A* KO mice, although levels were significantly elevated in the diabetic mice compared with non-diabetic mice as expected from this model. There was no significant difference in BUN levels between diabetic WT and *Papp-A* KO mice, although they were slightly elevated in diabetic compared with non-diabetic mice. These characteristics suggest changes in kidney function with 4 months of hyperglycemia,

although urine was not collected for measurement of creatinine or albumin. Furthermore, serum IGF1 levels were not significantly different between diabetic WT and *Papp-A* KO mice or between diabetic and non-diabetic WT mice, confirming previous characterization of *Papp-A* KO mice as a model of autocrine/paracrine vs endocrine regulation of IGF action (Conover *et al.* 2004).

Renal histology

Diabetes induced by streptozotocin in aged mice results in glomerular, tubulointerstitial, and vascular kidney lesions with many features of human diabetic nephropathy (Fioretto & Mauer 2007, Wu *et al.* 2010). These are morphologically characterized by thickening of the Bowman's capsule, glomerular hypertrophy with expansion of the extracellular matrix surrounding mesangial cells, and thickening of the basement membrane underlying proximal tubular epithelial cells.

WT diabetic mice developed thickening of Bowman's capsule, tubular changes, and interstitial infiltrates (Fig. 2 and Table 1). The glomeruli were enlarged in 80% and the basement membrane of Bowman's capsules was thickened in 100% of WT diabetic kidneys. Tubular dilation was noted in 80% of the kidneys and mononuclear infiltrates noted in 90%. Forty percent of the kidneys had additional abnormalities and all WT diabetic kidneys had two or more abnormalities. In contrast, diabetic *Papp-A* KO kidneys showed little or no changes in glomerular size, Bowman's capsule, and tubular dilation. However mononuclear cell infiltration was not different between diabetic WT and *Papp-A* KO mice. To distinguish age-related vs hyperglycemia-induced effects on renal histology, we also examined 12 age-matched non-diabetic WT kidneys and found little or no thickening of Bowman's membrane or increase in glomerular size, although mononuclear cell

Table 2 General characteristics of diabetic and non-diabetic WT and *Papp-A* KO mice. Data for 18-month-old diabetic and non-diabetic mice are presented as mean $\pm$ S.E.M. with the number of mice for each group in parentheses. Analysis was by ANOVA and Tukey's tests

Measurements	Diabetic		Non-diabetic	
	WT (n=10)	<i>Papp-A</i> KO (n=13)	WT (n=8)	<i>Papp-A</i> KO (n=6)
Body weight (g)	34.2 $\pm$ 2.05	22.0 $\pm$ 1.19*	37.0 $\pm$ 3.17	24.4 $\pm$ 3.71*
Kidney:body weight ratio (mg/g)	10.0 $\pm$ 0.55	11.6 $\pm$ 0.13	10.5 $\pm$ 0.68	12.1 $\pm$ 1.68*
Blood glucose (mg/dl)	277 $\pm$ 44	310 $\pm$ 28	130 $\pm$ 6.0 [†]	143 $\pm$ 4.5 [†]
Blood urea nitrogen (mg/dl)	22 $\pm$ 2.6	29 $\pm$ 1.5	19 $\pm$ 2.0	22 (n=1)
Serum IGF1 (ng/ml)	500 $\pm$ 40	475 $\pm$ 59	538 $\pm$ 66	ND

* $P < 0.05$ *Papp-A* KO vs WT and [†] $P < 0.05$ diabetic vs non-diabetic. ND, no data.

Figure 2

Renal histology of diabetic WT and *Papp-A* KO mice. Representative H&E staining of kidney sections. Thin black arrows delineate a glomerulus. Broad white arrow indicates thickened Bowman's capsule.

infiltration and tubular dilation was similar to what was seen in diabetic WT kidneys (Table 1).

Expansion of mesangial matrix that is strongly PAS-positive is a hallmark pathological feature of diabetic nephropathy (Steffes *et al.* 1989). Mesangial area was quantitated in PAS-stained diabetic kidneys and there was a significant shift to larger area in WT compared with *Papp-A* KO mice (Fig. 3). Mean mesangial area in WT and *Papp-A* KO was 3587 ± 79 and $2518 \pm 132 \mu\text{m}^2$ respectively ($P=0.002$). This was not just due to the overall larger body size of the WT mice because mesangial area in non-diabetic WT kidney was similar to what was seen in diabetic *Papp-A* KO kidney (data not shown).

Macrophage immunohistochemistry

Immunostaining for F4/80, a classical macrophage-restricted surface glycoprotein, is specific for macrophages. Macrophages were prominent in kidneys of diabetic WT mice and appeared to be diminished in kidneys of diabetic *Papp-A* KO mice (Fig. 4). Intensity of staining around the glomeruli was graded as 1, 2, or 3 (3 being the highest intensity). Non-diabetic control kidneys were grade 1. Assessment of WT diabetic kidneys ($n=9$) classified 22% as grade 1, 22% as grade 2, and 56% as grade 3. In comparison, assessment of *Papp-A* KO kidneys ($n=13$) classified 62% as grade 1, 38% as grade 2, and none as grade 3.

Discussion

Diabetic nephropathy is the leading cause of end-stage renal disease world-wide (US Renal Data System Annual Data Report 2008). Therefore, there is tremendous interest in identifying factors regulating the development of diabetic nephropathy with the intent to prevent or delay

the associated morbidity and mortality. Based on previous findings that suggest a role for PAPP-A in age-related nephropathy (Conover *et al.* 2010), we investigated the role of PAPP-A in diabetic nephropathy. The two major and novel findings of this study were as follows: i) PAPP-A expression is markedly elevated in glomeruli of human diabetic kidneys and ii) mice deficient in PAPP-A are resistant to the development of diabetic nephropathy.

Human diabetic nephropathy

It is important for the study of PAPP-A in diabetic nephropathy to ascertain where PAPP-A is expressed in the kidney and if there is site-specific regulation in diabetes. Although mouse kidney has been reported to have high levels of PAPP-A mRNA (Hourvitz *et al.* 2002, Conover *et al.* 2004), these studies were performed in whole kidney and there were/are no antibodies available that recognize murine PAPP-A. Thus, our data are the first, to our knowledge, to demonstrate specific immunolocalization of PAPP-A to glomeruli. Furthermore, the kidney of a patient with confirmed diabetic nephropathy had markedly elevated PAPP-A associated with the glomerulus as well as the surrounding Bowman's capsule. This was of particular interest as the glomerulus is a major target of renal injury, especially in diabetes (Schlondorff 2008). It is not clear from immunohistochemistry which cells produce PAPP-A because it is a secreted protein that can associate with neighboring cells as well as the cells of origin (Conover *et al.* 2007). In this way, PAPP-A can act as an autocrine/paracrine regulator of IGF action. A clinical study indicated the prognostic value of circulating PAPP-A

Figure 3

Mesangial area. Kidney sections from diabetic WT (black bars) and *Papp-A* KO (grey bars) mice were stained with PAS and mesangial area measured as described in the Materials and methods section.

Figure 4

Macrophage immunohistochemistry of diabetic WT and *Papp-A* KO kidney. Macrophage staining for F4/80 (red brown) of kidney sections from WT diabetic (grade 3) and *Papp-A* KO diabetic (grade 1) mice.

with respect to increased all-cause mortality in type I diabetic patients with nephropathy (Astrup *et al.* 2007). However, it is not known whether this increase in circulating PAPP-A reflected changes in the kidney.

Mouse diabetic nephropathy

In order to investigate the physiological and pathophysiological role of PAPP-A in the kidney, we used WT and *Papp-A* KO mice and induced stable hyperglycemia in these animals with a regimen of low-dose streptozotocin injections, following the model developed by Wu *et al.* (2010). In the original report, diabetes was induced in mice starting at 17 months, and these mice demonstrated severe nephropathy associated with elevated markers of oxidative stress, endoplasmic reticular stress, and macrophage-derived inflammation at 22 months (Wu *et al.* 2010). Induction of diabetes in younger mice (4 months old) did not produce kidney lesions after a similar level and duration of hyperglycemia. We modified this model to start the streptozotocin injections in 14-month-old mice and assessed kidney histopathology after 4 months of stable hyperglycemia (~18 months old mice). We chose this younger age to minimize potential confounding effects of age-related cancers that become prominent, especially in WT mice, after 18 months (Conover *et al.* 2010). In our protocol, mice with visual signs of tumor at harvest would be removed from the study and there would be no further analyses; no animals were removed in this cohort due to tumors. Under this modified model, diabetic WT mice developed several features of nephropathy, i.e. thickening of Bowman's capsule, and mesangial expansion, albeit not to the level of severity seen in the study by Wu *et al.* (2010). They also had increased macrophage immunostaining especially around glomeruli. These were significantly reduced in diabetic *Papp-A* KO mice.

To distinguish diabetes-induced vs age-related nephropathy, we also analyzed kidneys of non-diabetic WT and PAPP-A KO mice. There was no evidence of thickened Bowman's capsule, increased glomerular size, or mesangial expansion. However, tubule dilation and mononuclear cell infiltration occurred to a similar extent. Inflammation and oxidative stress are important contributors to kidney aging. Diabetes accelerates the aging phenotype in the kidney because of the additional hyperglycemia-induced stress (Wu *et al.* 2010). PAPP-A KO mice show enhanced resistance to inflammatory and oxidative stress (Conover 2010), which may explain, in part, the decrease in age-related kidney tubule dilation and relative resistance to the development of diabetic nephropathy.

Proposed role of IGF1 and PAPP-A in diabetic nephropathy

The renal IGF system appears to be a major contributor to the development of diabetic nephropathy (Rabkin & Schaefer 2004, Vasyleva & Ferry 2007, Bach 2012). IGF1 is synthesized and secreted by mesangial cells and has been shown to bind specific receptors on glomerular cells (Abrass *et al.* 1988, Tack *et al.* 2002). Furthermore, IGF1 synthesis and receptor expression are increased in mesangial cells from diabetic mice (Oemar *et al.* 1991, Tack *et al.* 2002) and experimental diabetes increases IGF1 receptor expression in the kidney (Werner *et al.* 1990). Exposing mesangial cells to IGF1 increases cell proliferation and matrix production while suppressing matrix degradation. Mesangial cell proliferation and matrix accumulation are mediated in part by increased mesangial cell sensitivity to IGF1 in the presence of high glucose (Horney *et al.* 1998). Thus, IGF1 action increases in diabetic kidney in an autocrine/paracrine manner that promotes matrix production and mesangial cell proliferation. We propose PAPP-A as another regulatory component, enhancing local IGF1 bioavailability. PAPP-A expression has been shown to increase in response to tissue injury (Conover 2012). Moreover, PAPP-A expression is potentially stimulated by macrophage-derived proinflammatory cytokines, tumor necrosis factor- α , and interleukin 1 β in fibroblasts and vascular smooth muscle cells and by transforming growth factor- β in human osteoblasts (Ortiz *et al.* 2003, Resch *et al.* 2004, Conover *et al.* 2006). These factors are also involved in injury response in kidney. Further *in vitro* studies are necessary to determine whether there are similar effects on PAPP-A expression and IGF action in mesangial cells.

The studies herein advance our understanding of PAPP-A in the kidney and provide a scientific rationale

for targeting PAPP-A as a way to inhibit the development and/or progression of kidney pathology in diabetes and aging.

Declaration of interest

J R M, Z T R, and R J M declared no conflict of interest. G R M is employed by London Metropolitan University and travel/accommodation/meeting expenses unrelated to activities listed covered by British Society of Immunology. J H M obtained a grant from Ansh Labs (Webster, TX, USA), and patents for selective exosite inhibition of PAPP-A activity against IGFBP4. C O obtained a grant from Ansh Labs, Danish Research Council, and patents for selective exosite inhibition of PAPP-A activity against IGFBP4. C A C obtained grants, NIH AG028141 and HL074871, from the Ted Nash Long Life Foundation, and from Ansh Labs; patents for marker for inflammatory conditions (issued), IGF binding protein 4 protease (issued), transgenic mouse lacking PAPP-A activity (issued), and treatment of osteoporosis (issued); and royalties for marker for inflammatory conditions.

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Author contribution statement

J R M designed and performed the mouse experiments, contributed to the draft manuscript, and reviewed and edited the revised versions of the manuscript. Z T R developed and performed PAPP-A immunohistochemistry and reviewed and edited the manuscript. G R M provided Ig expression plasmids and reviewed the manuscript. J H M generated the PAPP-A MAB and reviewed the manuscript. C O generated the PAPP-A MAB and reviewed and edited the manuscript. R J M assessed histopathology and immunohistochemistry and reviewed and edited the manuscript. C A C designed the experiments, analyzed data, prepared tables and figures, drafted the manuscript, and approved the final version of the manuscript.

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