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Angiotensin converting-enzyme inhibition restores glomerular glycosaminoglycans in rat puromycin nephrosis

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ABSTRACT

Background: Aberrant glomerular polyanionic charge of glycosaminoglycans (GAGs) and sialic acid expression has been observed in proteinuric human and experimental glomerular diseases. Angiotensin-converting enzyme inhibitors (ACEI) lower proteinuria and amend renal function deterioration via hemodynamic mechanisms. We tested the hypothesis that ACEI modulate proteinuria additionally by modifying glomerular GAGs.

Methods: In this study, we explored the effects of the ACEI enalapril on proteinuria and GAG synthesis in puromycin aminonucleoside (PAN)-treated rats. We employed cationic colloidal gold (CCG) localization in glomerular basement membranes (GBM) to identify GAGs by electron microscopy and determined sialic acid residues by immunohistochemical staining with lectins. To clarify ACEI effects on GAG production *in vitro*, we studied *de novo* GAG synthesis into newly synthesized proteoglycans in podocytes and mesangial cells using ^{35}S incorporation. Cells were incubated with or without PAN, and with increasing doses of the ACEI enalaprilat.

Results: PAN rats developed severe proteinuria that was significantly improved by enalapril treatment. In non-treated PAN rats GBM GAGs were reduced, whereas in the enalapril-treated group GBM GAGs were significantly increased to control levels. Enalapril did not affect glomerular sialic acid. Furthermore, in cultured podocytes and mesangial cells PAN decreased *de novo* GAG synthesis, an effect which was significantly ameliorated by enalaprilat treatment.

Conclusion: Treatment with ACEI improves permselectivity properties of the glomerular capillary wall by maintaining its GAG content. This finding provides an additional new mechanism, whereby ACEI exert anti-proteinuric effects.

1. Introduction

Proteinuria is associated with altered permselectivity of the glomerular capillary wall (GCW), which is regulated by a filtration barrier conferred by shape-, size- and charge-selective properties (Haraldsson et al., 2008). The charge-selectivity is related mainly to the polyanionic charge of glycosaminoglycans (GAGs) of heparan sulfate (HS) proteoglycan (HSPG) and sialic acid residues. HSPG are localized in the glomerular basement membrane (GBM) (Kanwar and Farquhar, 1979), and on the podocyte and glomerular endothelial cell surfaces (Raats et al., 2000; Rops et al., 2004), while sialic acid is present on podocytes (Kerjaschki et al., 1984) and on glomerular endothelial cells (Satchell, 2013). Podocytes are crucial for maintaining the glomerular filtration barrier, and in cell culture express mRNA and protein for HSPGs, which are secreted into the GBM (Bjornson Granqvist et al., 2006; Boerries et al., 2013; Chen et al., 2010). Decreased GCW HSPG exists in a variety of human proteinuric diseases including diabetic nephropathy and systemic lupus erythematosus, as well as in experimental

Abbreviations: ACEI, angiotensin converting-enzyme inhibitors; CCG, cationic colloidal gold; EM, electron microscopy; GAG, glycosaminoglycan; GBM, glomerular basement membrane; HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; PAN, puromycinaminonucleoside; RAS, renin-angiotensin system.

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proteinuric models such as puromycin aminonucleoside (PAN) nephrosis (Raats et al., 2000; Rops et al., 2004).

We have employed cationic colloidal gold (CCG) to localize GAGs in the kidney using electron microscopy (EM) (Weinstein et al., 1997), and have demonstrated reduced glomerular polyanions in nephrotic rats with adriamycin nephropathy (Skutelsky et al., 1995).

Detailed classical *in vivo* studies in both animals and humans have characterized the sieving properties of the GCW, which confers both size- and charge-selectivity. By using dextran molecules of varying charge it was demonstrated that neutral particles are restricted from the urinary space, whereas anions have a fractional clearance that approaches zero (Pollak et al., 2014). Studies with charge-modified tracers demonstrated that the GBM contains anionic sites associated with HSPG (Kanwar and Farquhar, 1979; Kanwar et al., 1983). HSPG consists of a core protein to which HS chains are covalently linked; hence, the large number of N- and O-sulfations yields very strong anionic charge (Kanwar and Farquhar, 1979). HSPG may also contribute to the GCW size-dependent permeability by virtue of their presence on cell surfaces and involvement in cell-cell and cell-matrix adhesion (Farquhar, 2006). In various glomerular diseases, the negative charge is diminished in the glomerular capillary filter, including the GBM (Raats et al., 2000; Rops et al., 2004), the endothelium (Satchell, 2013) and the surface of podocytes (Sugar et al., 2014). Furthermore, the composition and organization of the GBM affects the organization of the podocyte foot processes and epithelial slits (Farquhar, 2006).

However, the charge-selective properties of the GCW are controversial. The concept that GBM HSPGs provide charge selectivity stemmed from classic studies in which negatively charged macromolecules were less permeable than neutral or cationic counterparts of equivalent size (Kanwar et al., 1980). This concept has been challenged following establishment of engineered mouse models with a disturbed HS expression in the glomerular filtration barrier that show no signs of permeability defects (Chen et al., 2008; Goldberg et al., 2009; Harvey and Miner, 2007). Yet recently we have shown that up-regulation of heparanase, which degrades HSPG, is associated with proteinuria and disease progression in transplanted kidneys (Shafat et al., 2012) and this has also been demonstrated in diabetic nephropathy (Garsen et al., 2014; Gil et al., 2012; Rops et al., 2012; Shafat et al., 2011). Beyond charge selectivity, GBM HSPG also serves as an important reservoir for growth factors and enzymes, preventing their degradation (McCarthy and Wassenhove-McCarthy, 2012).

Progressive kidney disease is linked to activation of the renin-angiotensin system (RAS), thus inhibitors of the RAS are mainstays of renal protection therapy. RAS inhibitors exert their effect by both hemodynamic and non-hemodynamic mechanisms such as modulation of extracellular matrix components (Taal and Brenner, 2000).

The purpose of the present study was to investigate the functional charge barrier in proteinuric rats with PAN nephrosis, which is a prototype model for human minimal change disease (Pippin et al., 2009). Since the charge-selectivity relates to the properties of GAGs and sialic acid, we tested the hypothesis that the angiotensin converting-enzyme inhibitor (ACEI) enalapril may modulate glomerular anionic charge. GBM GAGs were determined by using CCG staining, and immunohistochemistry using the lectins *Peanut agglutinin* (PNA) and *Maakialeukoagglutinin* (MAL) determined sialic acid content. To further clarify the effect of ACEI on glomerular cells *in vitro*, GAG synthesis was determined in cultured podocytes and mesangial cells. Cells were incubated with PAN in the presence of increasing doses of enalaprilat, and *de novo* GAG synthesis was studied by metabolic labeling using ^{35}S incorporation into newly synthesized cell-associated proteoglycans.

2. Methods and materials

2.1. In vivo

Twenty-six male Wistar rats (Belinson, Israel), 198–261 gr were housed in individual metabolic cages with free access to standard diet and water. After 3 days of acclimatization, rats were randomized into three groups: six controls received 0.9% NaCl, and 20 were injected with PAN (75 mg/kg) (Sigma, St. Louis, Missouri), all by the tail vein. Ten of the latter were treated with enalapril (50 mg/L, MSD, Israel) in their drinking water 3 days prior to PAN injection and throughout the study. On day 10, 24-h urine protein collections were completed (Beckman Array-Protein system), rats were weighed, anesthetized, and blood was collected for measurement of serum albumin, creatinine and cholesterol (Autoanalyzer, Hitachi, Japan). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All rat experiments were in accordance with the Rabin Medical Center Institutional Guidelines, and approved by the Ethical Committee for Animal Experimentation. Blood collection and animal euthanasia was performed under intra-peritoneal injection of 200 mg/kg sodium pentobarbital, and all efforts were made to minimize animal suffering.

2.2. Tissue preparation

The kidneys were removed rapidly and placed on ice. Slices were fixed in freshly prepared 0/5% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4. For EM histochemistry, cortices were separated from the medulla. One-mm^3 blocks were washed with PBS, dehydrated in ethanol and embedded in LR-White resin (Polysciences, Washington PA). For morphology, similar blocks were post-fixed with 1% OsO_4 in Veronal-acetate buffer, pH 7.4 for 1 h at 4 °C, dehydrated in ethanol and propylene oxide, and embedded in araldite (Polysciences).

2.3. Histochemistry

Longitudinal slices were embedded in paraffin. Five μm sections were mounted on slides, and deparaffinized with xylene and descending ethanol series. Following rehydration with PBS, sections were treated with 2% H_2O_2 for 10 min at 37 °C, to quench endogenous peroxidase activity. The lectins PNA and MAL were used to localize sialic acid. PNA binds mainly to βGalβ1,3GalNAc, which is exposed after removal of sialic acid by neuraminidase, as we have shown previously (Weinstein et al., 1997). Sections were incubated for 1 h at 37 °C with 1 U/ml neuraminidase (Type 5, *Clostridium perfringes* origin, Sigma) in acetate buffer, pH 6.5. Control sections were incubated with PNA without neuraminidase pretreatment. MAL binds mainly to α2,3-linked sialic acid. To block non-specific binding, sections were covered for 10 min with 1% bovine serum albumin/PBS, and incubated with 50 μg/ml biotin-labeled lectin in PBS (Vector, Burlingame) for 1 h at 25 °C, followed by avidin-biotin-peroxidase complex for 30 min. After washing, horseradish peroxidase was activated by 8 min incubation with 3,3′-diaminobenzidine and H_2O_2/PBS reaction solution and counterstained with 0.2% methyl green. Sections were washed in tap water, dehydrated with ethanol and xylene and coverslipped with Merkoglass (Merk, Darmstadt, Germany). Negative controls omitted the lectin. Evaluation of staining intensity was carried out in a blinded manner by two examiners using a scale from 0 to +++.