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Kidneys with heavy proteinuria show fibrosis, inflammation, and oxidative stress, but no tubular phenotypic change

ARVI-MATTI KUUSNIEMI, RISTO LAPATTO, CHRISTER HOLMBERG, RIITTA KARIKOSKI, JUHANI RAPOLA, and HANNU JALANKO

Hospital for Children and Adolescents and Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

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Background. Sustained proteinuria is a major factor leading to kidney fibrosis and end-stage renal failure. Tubular epithelial cells are believed to play a crucial role in this process by producing mediators leading to fibrosis and inflammation. Congenital nephrotic syndrome of the Finnish type (NPHS1) is a genetic disease caused by mutations in a podocyte protein nephrin, which leads to constant heavy proteinuria from birth. In this work we studied the tubulointerstitial changes that occur in NPHS1 kidneys during infancy.

Methods. The pathologic lesions and expression of profibrotic and proinflammatory factors in nephrectomized NPHS1 kidneys were studied by immunohistochemistry, Western blotting, and cytokine antibody array. Oxidative stress in kidneys was assessed by measurement of gluthatione redox state.

Results. The results indicated that (*1*) severe tubulointerstitial lesions developed in NPHS1 kidneys during infancy; (*2*) tubular epithelial cells did not show transition into myofibroblasts as studied by the expression of vimentin, α-smooth muscle actin (α-SMA), collagen, and matrix metalloproteinases 2 and 9 (MMP-2 and -9); (*3*) the most abundant chemokines in NPHS1 tissue were neutrophil activating protein-2 (NAP-2), macrophage inhibiting factor (MIF), and monocyte chemoattractant protein-1 (MCP-1); (*4*) monocyte/macrophage cells expressing CD14 antigen were the major inflammatory cells invading the interstitium; (*5*) the arteries and arterioles showed intimal hypertrophy, but the microvasculature in NPHS1 kidneys remained quite normal; and (*6*) excessive oxidative stress was evident in NPHS1 kidneys.

Conclusion. Heavy proteinuria in NPHS1 kidneys was associated with interstitial fibrosis, inflammation, and oxidative stress. The tubular epithelial cells, however, were resistant to proteinuria and did not show epithelial-mesenchymal transition.

Sustained heavy proteinuria is believed to lead to progressive kidney damage [1]. High urinary protein content may elicit proinflammatory and profibrotic effects that contribute to chronic tubulointerstitial damage and loss of renal function [2]. Several in vitro studies have demonstrated that urinary proteins stimulate tubular epithelial cells to secrete factors, such as monocyte chemoattractant protein-1 (MCP-1), that promote inflammation [3]. This is associated with an influx of inflammatory cells to interstitium as well as activation and proliferation of interstitial fibroblasts. The major profibrotic factor implicated in this process is transforming growth factor-β (TGF-β) [4]. Tubular epithelial cells can also directly promote fibrosis by producing extracellular matrix (ECM) components. Tubular epithelial cells may undergo epithelial-mesenchymal transition (EMT) into myofibroblasts, which may invade interstitium [5]. With increased fibrosis and inflammation the number of functioning nephrons progressively declines and renal failure ensues.

The data on the pathophysiologic lesions caused by proteinuria are mainly based on animal models and cell culture studies. In this work we evaluated the tubulointerstitial changes in kidneys with congenital nephrotic syndrome of the Finnish type (CNF, NPHS1). NPHS1 is a recessively inherited renal disease caused by mutations in the nephrin gene [6]. Nephrin is a podocyte-specific protein located at the slit diaphragm of kidney glomerulus. The two most common mutations in the nephrin gene, Fin-major and Fin-minor, lead to a severe disorder characterized by lack of nephrin in the kidney glomerulus and massive proteinuria (up to 100 g/L) starting already during the fetal period [7]. Children with NPHS1 are nephrectomized as infants and their kidneys serve as a unique material for studying the consequences of continuous, heavy proteinuria. Since the basic defect in NPHS1 only affects the glomerular filter, all tubulointerstitial lesions observed are secondary to the protein leakage.

In this work, we used immunohistochemistry, Western blotting, cytokine array, and glutathione analysis to evaluate the nature of the tubulointerstitial lesions, possible changes in tubular epithelial cell phenotype, cortical cytokine production, and oxidative state in NPHS1 kidneys.

Key words: nephrin, proteinuria, tubulointerstitial fibrosis, NAP-2, oxidative stress.

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METHODS

Tissue samples

A total of 52 kidneys were nephrectomized from children with NPHS1 between 1986 and 2003. The age of the patients at the time of nephrectomy ranged from 4 to 44 months. Before nephrectomy these children were treated with daily albumin infusions to supplement the continuous heavy proteinuria. Routine formalin-fixed paraffin-embedded samples were taken from the kidneys and the rest of the renal cortex was snap-frozen in liquid nitrogen and stored at −70°C.

As controls we used eight normal adult kidneys (age 47 to 58 years) removed for transplantation. These kidneys had proved unsuitable for transplantation mainly because of vascular abnormalities. Formalin-fixed paraffin-embedded sections and snap-frozen samples were collected. The cadaver kidneys had to be used as controls, since fresh tissue samples from normal infant kidneys were not possible to obtain. In the cytokine array, porcine kidney samples were used as additional controls, since brain death may affect the cytokine expression. Porcine kidney samples were obtained from two newborns and one 6-month-old pig. They were snap-frozen and stored at −70°C.

The study protocol was approved by the ethical committee of the Hospital for Children and Adolescents of the University of Helsinki.

Antibodies

The following antibodies were purchased from DakoCytomation (Glostrup, Denmark): CD20cy (M0755), CD68 (M0876), pan-Ig (against IgA, IgG, and IgM) (P0212), α-smooth muscle actin (α-SMA) (M0851), and vimentin (M7020). Antibodies acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were heat shock protein 27 (HSP27) (sc-1048), NAP-2 (sc-19224), pan-cytokeratin (sc-8018), megalin (sc-16476), TGF-β1 (sc-146), HSP27 (sc-1048) and vascular endothelial growth factor (VEGF) (sc-7269). Antibodies against CD3 (ab828), CD14 (ab8679), and collagen type I (ab6308) were bought from Abcam (Cambridge, Cambridgeshire, UK). Anticollagen type VI (MAB3303) was bought from Chemicon International (Temecula, CA, USA), and anti-CD13 (MS-1079-S) and anti-myeloperoxidase (MPO) (RB-373-A) from NeoMarkers (Fremont, CA, USA). In addition we had two anti-MCP-1 antibodies: ALX-804–465 from Alexis Biochemicals (Lausen, Switzerland) and AF-279-NA from R&D Systems (Minneapolis, MN, USA). All these primary antibodies were unconjugated, except for horseradish peroxidase (HRP)-conjugated pan-Ig antibodies, and the dilutions used ranged from 1:10 to 1:250 in immunohistochemistry and from 1:1000 to 1:3000 in Western blotting.

We used the following markers for different cell types: CD13 for monocytes, CD68 for macrophages, CD14 for monocytes and macrophages and some of their subpopulations, MPO for granulocytes and monocytes, CD3 for T lymphocytes, CD20cy for B lymphocytes, mast cell tryptase (MCT) for activated mast cells and pan-immunoglobulin (pan-Ig) antibody for immunoglobulin producing plasma cells.

Light microscopy

The histologic lesions in NPHS1 kidneys were evaluated by light microscopy from paraffin-embedded tissue sections stained with hematoxylin and eosin or periodic acid silver methenamin (PASM). In order to quantitate the changes, interstitial fibrosis, inflammatory cells, tubular cysts, and glomerular sclerosis were graded from 0 to 3. The total histologic score was the sum of these values and ranged from 0 (normal) to 12 (severely damaged). The samples were scored independently by two experienced renal pathologists.

Immunohistochemistry

For the immunofluorescence stainings, the cryosections (5 μm) of the kidney samples were fixed with 3.5% paraformaldehyde or acetone, depending on the antibody used. The stainings were performed in a traditional way. Sections used as negative control were incubated in phosphate-buffered saline (PBS) instead of a primary antibody.

Immunoperoxidase stainings were performed on the sections of formalin-fixed, paraffin-embedded renal samples in a conventional way. To improve antibody penetration, microwave treatment in 10 mmol/L citric acid for 10 minutes was performed or Dako Target Retrieval Solution (S1699) (DakoCytomation) was used depending on the antibody. Amplification of the primary antibody reaction was achieved by incubating the sections with biotinylated secondary antibody (Vector Elite ABC Kit) (Vector Laboratories Inc., Burlingame, CA, USA). Immunoperoxidase staining of cryosections was performed similarly. The sections were fixed with 3.5% paraformaldehyde or acetone depending on the antibody used.

Light microscopy was performed with a standard Leica DM RX light microscope equipped with an Olympus DP70 digital camera. To calculate the area fraction of a particular immunostained component images were imported to the freeware image analysis program NIH ImageJ 1.32j (National Institutes of Health, Bethesda, MD, USA). Sequential gray-scale images were grabbed using the ×10 objective. The presence of glomeruli in the fields was ignored and a threshold was applied to each image at a constant level that distinguished between the stained component and the background. The proportion of black