Proteinuria and function loss in native and transplanted kidneys

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Glomerular expression profiling in spontaneously proteinuric rats reveals differential expression of genes associated with cytoskeleton and protein overload

4

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submitted for publication

Abstract

To get more insight in cause and consequences of proteinuria, we studied glomerular gene expression patterns before and after the onset of increased urinary albumin excretion (UAE) in a proteinuric rat strain.

Spontaneously proteinuric Dahl salt-sensitive rats (Dahl SS) were compared to non-proteinuric, spontaneously hypertensive rats (SHR). In Dahl SS, UAE significantly increased starting from week 5 of age. Glomerular RNA profiles of 4- and 6-week-old rats were studied using Affymetrix microarray chips. Gene expression was further studied by quantitative PCR and immunohistochemistry.

398 genes were upregulated and 210 genes were downregulated in Dahl SS compared to SHR. The data were analyzed using three main approaches, and the following results were found: 1) 115 genes, located on quantitative trait loci previously associated with proteinuria, were differentially expressed between the rat strains. These included hedgehog-interacting protein and outer dense fiber 3; 2) Several pathways related to changes in the cytoskeleton were represented in the list of differentially expressed genes. Expression of the actin-regulating protein dynamin was increased in proteinuric rats and in patients with proteinuric disease; 3) The expression pattern of a group of 13 genes, of which the expression was previously found to be regulated in tubular epithelial cells upon protein loading, was differentially regulated in glomerular samples of Dahl SS versus SHR rats ($P < 0.03$). Expression of the podocyte stress marker desmin co-localized with albumin resorption droplets in podocytes.

The list of genes differentially expressed between proteinuric and non-proteinuric rat strains is predominated by those encoding for cytoskeletal proteins. Secondly, expression differences between strains may result partly from increased protein trafficking through the glomerular filtration barrier.

Introduction

The initial step in the production of urine is glomerular filtration, in which water and small solutes are filtered but macromolecules and cells are retained within the glomerular capillaries. Loss of the glomerular filtration barrier permselectivity results in the loss of proteins into the urine, proteinuria. Proteinuria is an important symptom of various kidney diseases, and its presence is related to the progression of renal and cardiovascular diseases (1,2). Knowledge about the molecular determinants of glomerular filtration has advanced considerably through the discovery of mutations in genes that underlie hereditary proteinuric kidney diseases (3). Nevertheless, the understanding of the development of proteinuria in the majority of patients that do not have such genetic mutations remains limited.

The Dahl salt-sensitive (Dahl SS) rat strain has been used as a model for the development of salt-sensitive hypertension. Sterzel et al (4) showed that prior to development of hypertension and independent of salt loading, Dahl SS rats also show an increased urinary albumin and protein excretion. Several groups have attempted to dissect the genetic basis of the increased urinary albumin excretion (UAE) in Dahl SS rats. Using the spontaneously hypertensive rat (SHR) as a reference strain, genetic linkage analysis yielded several quantitative trait loci (QTLs) of interest for the increased UAE (5-8). In several analyses by Kreutz' group a QTL for UAE was located on rat chromosome 19 (5,6), in both high and low salt conditions. Consomic strains in which the SHR chromosome 19 was introgressed into the Dahl SS background, showed a considerable amelioration of the albuminuric phenotype (9). The identification of genomic regions is helpful in elucidating the polygenetic nature of the UAE, and can demonstrate the linkage of other genetically determined phenotypes such as hypertension to the development of proteinuria. However, the relatively low resolution of the linkage analysis impairs the identification of the genes that play a role in the development of proteinuria.

It is hypothesized that proteinuria, once it has developed, exerts a toxic effect on proximal tubular epithelial cells. Although the exact nature of the compounds that cause this effect (albumin, albumin-bound factors, or other macromolecules) is subject of discussion, several studies have indicated that upon proteinuria proximal tubular epithelial cells acquire a pro-inflammatory and pro-fibrogenic state (10,11). Some studies have indicated that an increased passage of proteins through the glomerular filtration barrier may have a similar toxic effect on podocytes (12). Indeed, proteinuria is almost always accompanied by changes in podocyte cell structure, and reorganization of the podocyte cytoskeleton (13,14).

We used a microarray approach to get insight in the gene expression differences that occur in the glomerulus during the development of proteinuria. The results of the microarray experiments were used in several ways. Combination of the microarray data and previously identified genomic

regions was used to identify genes that could be of interest for the development of the increased UAE. Next to studying single genes, we used pathway analysis to identify groups of genes that differentiate between the proteinuric and non-proteinuric rat strains. These approaches yielded further genes of interest and pathophysiological concepts that were studied in more detail.

Materials and Methods

Animal studies

We compared the spontaneously proteinuric Dahl SS rat strain with non-proteinuric spontaneously hypertensive rats (SHR). Male Dahl SS and SHR rats were obtained from colonies at the Freie Universität Berlin as reported (5). The animals were fed a low-salt diet of 0.2% NaCl by weight content to prevent early development of hypertension. For the microarray studies, we used 4- and 6-week-old rats. In follow-up experiments, groups of rats that were 2, 4, 6, 8, and 10 weeks of age were studied (n = 5 to 8 per group). Systolic blood pressure, proteinuria, and albuminuria were recorded. Following described methods (15), tissues were collected under ketamin/xylazine anesthesia, and glomeruli were isolated using perfusion with iron oxide followed by magnetic retraction. Experiments were performed in accordance with institutional guidelines.

Microarrays

For the microarray experiments Affymetrix GeneChip Rat Genome 230 2.0 arrays were used (Affymetrix, www.affymetrix.com). These high-density gene chips contain 31,042 probesets, representing approximately 28,000 different genes and ESTs, on a single chip.

We compared 4- and 6-week-old Dahl SS and SHR rats, studying two rats from each strain at each time point. Total RNA from isolated glomeruli was purified with Qiagen RNeasy mini columns (Qiagen, www.qiagen.com). Quality and purity of the RNA was controlled by gel electrophoresis and measurement of the 260/280 ratio, which was between 1.8 and 2.1 in all samples. Biotin-labeled cRNA was produced using the Ambion MessageAmp II-Biotin Enhanced kit (Ambion, www.ambion.com), according to the manufacturer's instructions. Briefly, 1µg of total RNA was reverse transcribed in the presence of T7 oligo(dT) primer, followed by second-strand cDNA synthesis. After purification, in vitro transcription of the double stranded cDNA was performed in the presence of T7 RNA polymerase and biotin-11-UTP. Following another round of purification, 20 µg of cRNA was fragmented and used for hybridization to the chips. Hybridization and scanning of the chips was performed at the Leiden Genome Technology Center (www.lgtc.nl).

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (16) and are accessible through GEO Series accession number GSE13810 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE13810)

Preprocessing and differential expression analysis

Preprocessing, differential expression analysis, and parts of the pathway analysis were performed in the R-software environment (www.r-project.org). All R-packages used are available at the bioconductor website (www.bioconductor.org).

Preprocessing of the data was performed with the GC Robust Multiarray Average (GCRMA) package. GCRMA – a modification of RMA (17) – calculates expression values using background adjustment, quantile normalization, and summarization of the probe intensities, and uses the probe sequence information (e.g., GC-content) to estimate the non-specific binding related to the background noise (18). Quality control was performed using functions in the affyPLM package in R, and included evaluation of RNA degradation plots, relative log expression, and normalized unscaled standard errors. All chips were of good quality with regard to these parameters.

To identify statistically significant differences in gene expression levels we used the Linear Models for Microarray Data (Limma) package (19,20). Limma first fits a model to each gene in the different arrays, as in a standard ANOVA approach. With a contrast matrix, the differently expressed genes can subsequently be extracted. We used Benjamin and Hochberg's method to correct for multiple testing. Comparisons of strain differences and time differences within strains were made. A differential gene expression of more than 1.5 fold, with an adjusted P-value < 0.01 in strain comparisons, and an adjusted P-value < 0.05 in time comparisons, was considered significant. In the text and tables, fold changes are given as base 2 logarithmic values.

Comparison to genomic studies

Previous genetic analysis of the strains that we studied has yielded several QTLs of importance for the development of urinary albumin excretion. To integrate these genetic analyses with the microarray data, we obtained the QTLs found by Poyan Mehr et al (5), Siegel et al (6), and Garrett et al (7,8) from the rat genome database (RGD, http://rgd.mcw.edu). Of the genes that showed a differential expression in the array experiments, we evaluated those that were located within a chromosomal location identified in the previous genetic linkage analyses, using information in the RGD and Ensemble (www.ensembl.org) databases.

Pathway analysis

For pathway analysis, we used several complimentary approaches. For the evaluation of known pathways, we used the online pathway analysis program Gene Ontology Tree Machine (GOTM, bioinfo.vanderbilt.edu/gotm)(21) and the global test. GOTM uses a hypergeometric test to evaluate whether the genes of a certain Gene Ontology (GO) category are overrepresented in the

Table 1. Primer sequences

Name	Symbol	mRNA sequence	Forward	Reverse	Amplicon size
hedgehog-interacting protein	Hhip	XM_238042	CCTTTGGTTGGTGGATTCGTTTAC	TTGCTTAGTCACTGGGCTTTGC	119
osteoglycin	Ogn	NM_001106103	TGTGCCTCCTAATTTACCAGAAAG	TACCGAGTGTCATTAGCCTTGC	105
dipeptidylpeptidase 6	Dpp6	NM_022850	CCTATGTCAGTGCTTCCTTCAGTC	CTTCTCTTATCCGTGGTATTGTGC	105
minichromosome maintenance deficient 6	Mcm6	NM_017287	GGCTGGGCTTTGCTGAATACTG	GCTGACTCATCC-TCCTCTCTTCC	85
rac GTPase-activating protein 1	Racgap1	NM_001108112	TCCAGATCCAGTGACGATGTTCC	GGCAGTCCATGTTCTCTTGTTCC	124
patched	Ptch1	NM_053566	GTGGTCATCCTGATTGCGTCTG	CATAGCCCTGTGGTTCTTGTCC	102
dynamin 1	Dnm1	NM_080689	TTGATGAGAAGGAACTGCGAAGG	AAGCGAGGTCAGGAGTGAAGAG	89
cell division cycle associated 1	Cdca1	NM_001012028	AGCAAGTAACCGCCATTAATCAAG	GATGCCCTCATGGTACTTCTCC	145
chondroitin sulfate proteoglycan 2	Cspg2	XM_001058160	ACCTGCTATCCTACTGAGACTTCC	CCGACAAGGGTTAGAGTGACATTC	104
ras homolog gene family, member A	Rhoa	NM_057132	GCACACAAGGCGGGAGTTAG	CGTCTTTGGTCTTTGCTGAACAC	121
desmin	Des	NM_022531	CCTACACCTGCGAGATTGATG	GCGATGTTGTCCTGATAGCC	114
vimentin	Vim	NM_031140	GCTGCCCCTGCGTGATGTC	ATTCGTTTGACTCCTGCTTTGC	156
filamin beta	Flnb	XM_224561	GCTGCTGACTTCCTTCTGG	GTTGCCGTTCTTCTTGATGC	183
kinesin family member 23	Kif23	XM_236313	GTGACTGAACCCA-AACCTGAGAAG	CAAGAGTTAGAGCGTCTGTGAAGC	179
rab8b	Rab8b	NM_153317	CCTGCCTCCTGTTCCGCTTCTC	GCCCGCCGTGTCCCATATCTG	133
polyamine modulate factor 1 binding protein 1	Pmfbp1	NM_134393	GGCTGGAGAGGAGTATGC	TTGACGGACGACTGTAGG	196
hypoxanthine guanine phosphoribosyl transferase	Hprt	NM_012583	GGCTATAAGTTCTTTGCTGACCTG	AACTTTTATGTCCCCCGTTGA	138
TATA box binding protein	Tbp	NM_001004198	ACCGTGAATCTTGGCTGTAAACTTG	GCAGTTGTTCGTGGCTCTCTTATTC	122
hydroxymethylbilane synthase	Hmbs	NM_013168	TGAAGGATGTGCCTACCATACTACC	GCAAGGTTTCCAGGGTCTTTCC	123

group of differentially expressed genes as defined using Limma (21). In a complementary approach, the global test was used to evaluate whether genes within a certain gene set (ie, a GO category or KEGG pathway, www.genome.jp/kegg) were differentially expressed (22). The global test assigns a p-value to this association. In addition, a so-called comparative p-value can be calculated. In this calculation, the association of the group of genes of interest is compared to that of a large number of randomly selected groups of genes of the same size. The benefit of the global test approach is that it also allows identification of pathways in which many genes are only moderately regulated.

The effect of proteinuria on gene regulation in proximal tubular epithelial cells has been extensively studied. From such studies, we extracted a group of well-substantiated genes that were shown to be regulated in response to proteinuria in mouse, rat, and human proximal tubular epithelial cells. We identified the rat homologue of these genes, and used the global test to

evaluate whether this group of genes was also differentially expressed in the glomeruli of Dahl SS and SHR rats.

RNA isolation and QPCR

RNA was isolated from the glomeruli using the TRIzol (Invitrogen) method. Total RNA (0.5µg) was reverse transcribed into cDNA using AMV reverse transcriptase (Roche Diagnostics).

Primers (Isogen Bioscience) were designed using BeaconDesigner 6.0 software (PREMIER Biosoft International). To prevent genomic contamination, all primers were chosen to span at least one splice-site. Sequences of the primers are listed in Table 1. Real-time PCR using Sybrgreen as the fluorescent dye was performed using a iCycler real-time PCR machine with iCyclerIQ 3.1 software (Bio-Rad laboratories).

Immunohistochemistry

A rabbit polyclonal anti-rat albumin antibody (Nordic Immunological Laboratories, www.nordiclabs.nl) was used for immunofluorescent localization of albumin in formalin-fixed paraffin-embedded tissue after antigen retrieval by boiling the slides in Tris/EDTA buffer for 10 minutes. A mouse monoclonal anti-desmin antibody (clone 33, diluted 1:750) was used in albumin and desmin double-labeling studies. As secondary antibodies, Alexa-conjugated anti mouse and anti-rabbit antibodies were used as appropriate. The immunofluorescent staining was evaluated using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany).

For immunohistochemical localization of dynamin in rat and human tissues, we used the mouse anti-dynamin antibody Hudy 1 (Upstate biotechnology, catalog # 05-319, www.upstate.com, diluted 1:80). As secondary antibody, we used anti-mouse Envision (Dako cytomotion, Glostrup, Denmark). Staining was performed using standard procedures as described previously (23). Specificity of the staining was tested through replacing the antibody by serum of the same species, in the same dilution as the secondary antibodies. For quantification of the staining of renal biopsies, we used both a digital image analysis and a semi-quantitative approach (scoring on a scale of 1 to 4). The results of both approaches were comparable and correlated highly with each other.

Biopsies from patients with acquired proteinuric kidney diseases

Biopsies from patients with various proteinuric kidney diseases were studied in comparison to controls. Disease categories included minimal change disease (n = 8), focal segmental glomerulosclerosis (n = 3), IgA nephropathy (n = 3), lupus nephritis (n = 6), diabetic nephropathy (n = 6), other diseases (including post-streptococcal glomerulonephritis, membranous nephropathy, light chain excretion nephropathy, n = 6). For controls, biopsies from patients with interstitial nephritis or with no glomerular pathology, tissue from kidneys unsuitable for transplantation for technical reasons, and tissue from kidneys obtained at autopsy was used (n = 16).

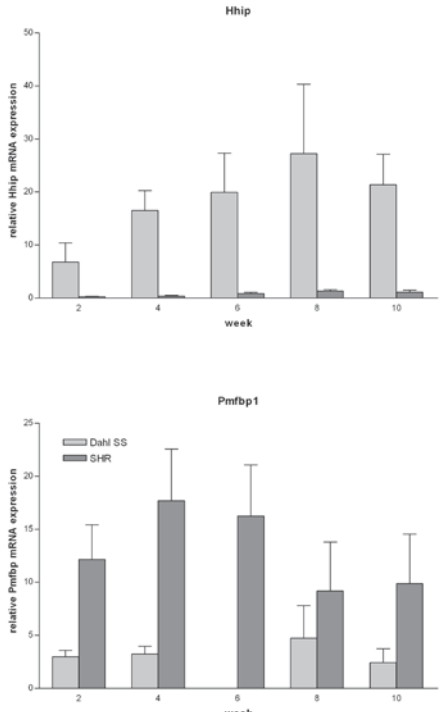


Figure 1. mRNA expression of hedgehog-interacting protein (Hhip, A) and polyamine modulated factor binding protein 1 (Pmfbp1, B) in glomeruli of Dahl SS and SHR rats of various age. Hhip was upregulated in Dahl SS rats at all time points, and also before the development of overt proteinuria (week 2). Pmfbp1 was downregulated in Dahl SS rats compared to SHR.

Animal phenotype

The animals used for the microarray experiments had normal systolic blood pressure (125 mmHg for 6-week-old SHR and DS rats). The UAE was 2.9 ± 0.0 and 32 ± 4.5 mg/24h for the 4- and 6-week-old Dahl SS rats, respectively, and < 0.1 mg/24h for the SHR rats at both time points. These values reflect those of the total group (n = 5-8) (15). Over the studied 10-week time course, the Dahl SS rats showed increasing UAE rates, while the SHR rats remained non-albuminuric.

Differential regulation of genes

We first compared the differential regulation of genes between the two rat strains, irrespective of the time points. Using a false discovery rate of < 0.01 and a 1.5 fold change as cut-off, 366 genes were found to be significantly upregulated, and 151 downregulated. The top-20 of up- and downregulated of these are listed in Table 2.

Comparing the differential regulation in time, first looking at the strains individually, we found that the gene expression levels of the Dahl rats were relatively similar at 4 and 6 weeks: only 16 and 7 genes were up- and downregulated, respectively, between 4 and 6 weeks. In contrast, the non-proteinuric SHR rat showed substantial regulation of genes between 4 and 6 weeks, with an upregulation of 397 genes and a downregulation of 210 genes at 6 weeks.

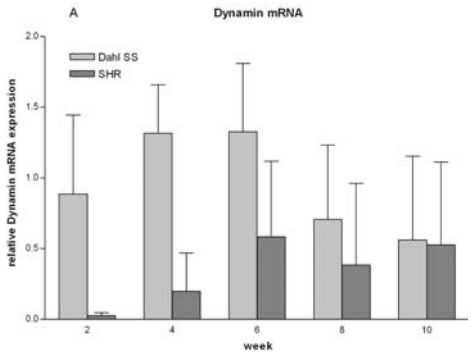
We subsequently evaluated which genes showed a different pattern of regulation in time between the two rat strains. For example, this would identify genes that over time are downregulated in the Dahl rat, while they are upregulated in the SHR. In total, 63 genes were significantly upregulated in the Dahl rat in comparison to the SHR, while 85 were downregulated.

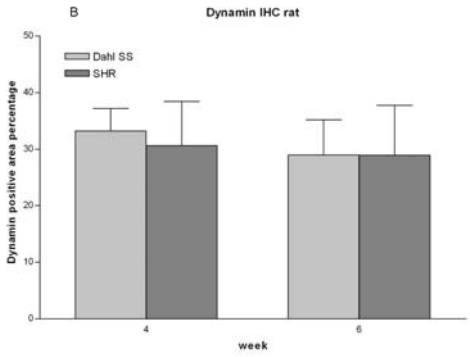
We validated the results of the microarray using QPCR in individual rats. Measurements of 16 different genes showed that the direction of changes (up- or downregulation) was always consistent. The extent of changes showed some variability between the microarray and QPCR, but there was no consistent under- or overestimation of the regulation in the array results.

Table 2. Top 20 up- and downregulated genes

DahlSS vs SHR: Upregulated			
Gene name	Symbol	RNO	Fold change
similar to hedgehog-interacting protein	RGD1564108	19q11	5.2
osteoglycin	Ogn	17p14	4.8
EST		11q11	2.3
SUMO/sentrin specific protease 5	Senp5	11q22	4.2
EST			4.0
osteoglycin	Ogn	17p14	4.4
similar to vacuolar protein sorting 13C protein	RGD1560364	8q24	3.4
Similar to teratocyte-specific carboxylesterase	RGD1564156	8q24	4.7
EST			2.1
RT1 class II, locus Bb	RT1-Bb	20p12	7.6
zinc finger homeobox 1b	Zfhx1b	3q12	3.9
RT1 class Ib, locus Aw2	RT1-Aw2	20p12	6.4
histone 2a	H2a	17q11	1.4
tetraspanin 18	Tspan18	3q24	2.7
EST		9q13	1.9
Cut-like 1 (Drosophila)	Cutl1	12q12	1.3
EST	RGD1310127	1q36	1.2
Similar to Leucine rich repeat and sterile alpha motif containing 1	RGD1564403	3p11	1.5
GRAM domain containing 3	Gramd3	18q12.1	0.6
Mindbomb homolog 1 (Drosophila)	Mib1	18p13	1.4

DahlSS vs SHR: Downregulated			
Gene name	Symbol	RNO	Fold change
aldo-keto reductase family 1, member B8	Akr1b8	4q22	-4.5
EST			-4.3
similar to interferon regulatory factor 10	RGD1562711	3q41	-3.9
EST			-3.7
acyl-Coenzyme A oxidase 2, branched chain	Acox2	15p14	-3.7
similar to RIKEN cDNA 4921520P21; DMRTC1	LOC363483	Xq31	-3.4
transmembrane protein 14A	Tmem14a	9q13	-3.0
torsin family 1, member B	Tor1b	3p12	-3.0
EST			-2.9
polyamine modulated factor 1 binding protein 1	Pmfbp1	19q12	-2.8
RT1 class Ib, locus Aw2	RT1-Aw2	20p12	-2.7
similar to hypothetical protein FLJ20647	RGD1305326	2q43	-2.6
EST			-2.5
solute carrier family 2, member 5	Slc2a5	5q36	-2.5
nuclear protein 1	Nupr1	1q36	-2.4
GNAS complex locus	Gnas	3q42	-2.4
apolipoprotein B	Apob	6q14	-2.4
spectrin alpha 1	Spna1	13q24	-2.4
protein kinase C, beta 1	Prkcb1	1q36	-2.4
EST			-2.3





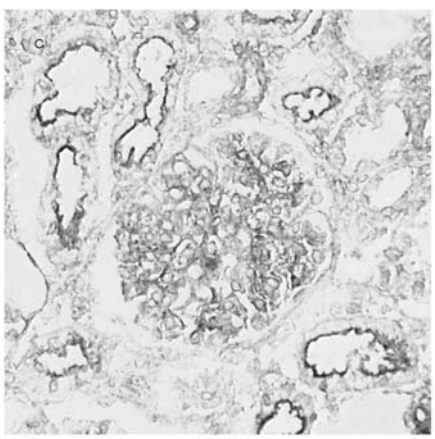


Figure 2. Dynamin expression in rat kidneys. The microarray showed a higher dynamin mRNA expression in 4- and 6-week-old Dahl SS rats compared to SHR. This was confirmed by QPCR (A). At later time points (8 and 10 weeks), dynamin upregulation was not significant. There was no clear increase in dynamin protein expression in Dahl SS rats (B). Dynamin protein was seen in the glomerulus and at the tubular brush border (C).

Relation to previously defined QTLs

Of all genes and ESTs with a known chromosomal location, which were differentially regulated between the two strains, we identified those genes that were located on QTLs know to be involved in the development of proteinuria in the studied rat strains (5-8). Of all up- and downregulated genes, 82 and 33 were located on such genomic regions. Within the UAE QTL on chromosome 19 (5,6), we identified five upregulated, and three downregulated probes. Of the five upregulated probes, three recognized hedgehog-interacting protein (Hhip) (5.2 times). The other two probes were ATP-binding cassette sub-family B (MDR/TAP) member 10 (Abcb10) (1.5 times), and integrin beta 1 (Itgb1) (0.9 times). The downregulated genes included polyamine modulated factor binding protein 1 (Pmfbp1, also called ODF3) (-2.8 times), WW domain-containing oxidoreductase isoform 2 (RGD1565791) (1.1 times), and hypothetical protein MGC3207 (RGD1307789) (-0.71 times). Of these, we validated the expression of Hhip and Pmfbp1 by QPCR. Dahl SS rats showed an increase in Hhip at all time points, while a downregulation of Pmfbp1 was seen. At week 6, levels of Pmfbp1 were undetectable in Dahl SS rats, whereas SHR rats showed a stable expression throughout the studied time course (Figure 1).

Pathway analysis

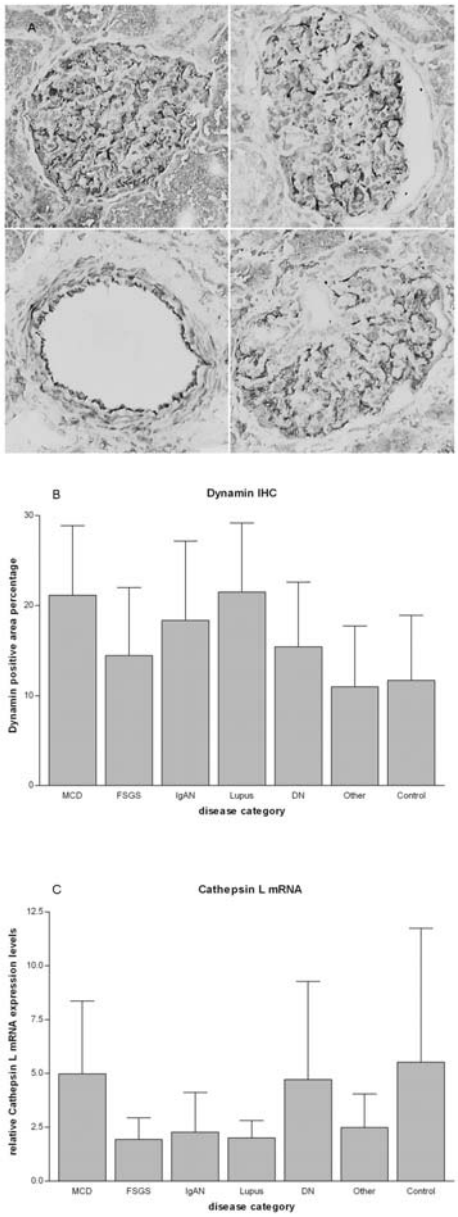
Pathway analysis was performed to get insight in the biological processes that were represented by the differentially regulated genes. GOTM analysis of the differentially regulated genes in time showed that cell-cycle associated genes were significantly enriched in the SHR rat, while

Table 3. Differential expression of cytoskeleton-related genes

Gene name	Symbol	RNO	Fold change	Function
periplakin	Ppl	10q12	2.06	intermediate filament binding
filamin, beta	Flnb	15p14	0.90	actin binding
dynamin 1	Dnm1	3p11	1.75	actin dynamics regulation
supervillin	Svil	17q12	1.39	actin binding
moesin	Msn	Xq31	1.95	actin filament -- membrane cross-linking
ARP1 actin-related protein 1 homolog A (yeast)	Actr1a	1q54	0.63	cytoskeleton organization
Rho guanine nucleotide exchange factor (GEF) 17	Arhgef17	1q32	1.07	actin cytoskeleton organization and biogenesis
CAP, adenylate cyclase-associated protein 1 (yeast)	Cap1	5q36	0.79	actin cytoskeleton organization and biogenesis
myosin Ib	Myo1b	9q22	0.99	actin binding
myosin IC	Myo1c	10q24	0.83	actin binding
tropomyosin 1, alpha	Tpm1	8q24	1.16	actin filament capping
parvin, alpha	Parva	1q33	1.39	actin cytoskeleton organization and biogenesis
microtubule-associated protein, RP/EB family, member 1	Mapre1	3q41	1.14	regulation of microtubule polymerization
caldesmon 1	Cald1	4q22	1.07	actin binding
Src homology 2 domain-containing transforming protein C1	Shc1	2q34	0.65	actin cytoskeleton organization and biogenesis
thymoma viral proto-oncogene 1	Akt1	6q32	1.44	cell projection organization and biogenesis
actin, beta	Actb	12p11	0.62	cytoskeleton organization
echinoderm microtubule associated protein like 4	Eml4	6q12	0.98	microtubule stabilization
kinesin light chain 1	Klc1	6q32	0.67	microtubule motor activity
signal-regulatory protein alpha	Sirpa	3q36	1.03	actin filament organization
mitogen activated protein kinase kinase kinase 1	Map3k1	2q14	0.83	actin filament polymerization
filamin, beta	Flnb	15p14	1.07	actin binding
actin related protein 2/3 complex, subunit 1B	Arpc1b	12p11	0.92	cytoskeleton organization
tropomyosin 4	Tpm4	16p14	1.45	actin binding
A kinase (PRKA) anchor protein 2	Akap2	5q24	0.59	actin filament organization
WD repeat domain 44	Wdr44	Xq12	1.00	
plastin 3 (T-isoform)	Pls3	Xq14	1.33	actin filament organization
polyamine modulated factor 1 binding protein 1	Pmfbp1	19q12	-2.81	cytoskeleton organization and biogenesis
spectrin alpha 1	Spna1	13q24	-2.39	cytoskeleton organization

Genes are listed with their chromosomal location and function. Fold changes are indicated as base 2 logaritmic value. A positive value indicates upregulation in Dahl SS vs SHR.

this was not the case in Dahl rats. Cell cycle related genes were also significantly enriched in the list of genes that showed a differential expression in time between SHR and Dahl SS strains.



Pathway analysis showed a relative enrichment of genes that were differentially expressed between the two strains in 49 GO categories. Nineteen of these GO categories were related to cytoskeletal changes. In a complementary approach we used the global test to evaluate KEGG pathways, as well as GO categories. This analysis also showed that the differences in gene regulation between the Dahl SS and SHR could in part be explained by regulation of cytoskeletal genes (KEGG pathway 04810, $P < 0.03$, comparative p value 0.17); GO:0005856, cytoskeleton, $P < 0.05$, comparative P value 0.21). The differentially expressed cytoskeletal genes are listed in Table 3.

Expression of dynamin in rat and human glomeruli

Dynamin is a cytoskeleton-associated gene that we identified as one of the genes that was upregulated in the Dahl SS rats compared to the SHR rat. Because recent studies showed that the actin-remodeling properties of dynamin may be important in podocytes (24), we decided to further evaluate the expression and distribution of dynamin in our model. Evaluation in individual rats confirmed the upregulation of dynamin mRNA expression in 4- and 6-week-old Dahl SS rats. At later time points upregulation of dynamin mRNA was not statistically significant (figure 2A). At the protein level, dynamin expression

Figure 3. Dynamin expression in human kidneys. The protein distribution of dynamin in human tissue (A) was similar to that in rats (figure 2C): dynamin was expressed in the glomerulus in a podocyte-like pattern (A, panel 1 and 2); the endothelial cells of large vessels were positive, while PTC and intraglomerular vessels did not show immunoreactivity (A, panel 3 and 4). Using digital image analysis, the protein expression of dynamin in various acquired proteinuric disease was quantified (B). Dynamin protein expression was increased in minimal change disease and in lupus nephritis compared to controls. The expression of cathepsin L mRNA was decreased in most acquired proteinuric diseases (C).

Table 4. Genes with differential expression in proximal tubular epithelial cells in proteinuric conditions

Model (in vivo, in vitro)	Species	Protein that evoked response	Method (RT PCR, ISH, microarray)	Genes regulated	Reference
Protein overload after uninephrectomy	Rat	proteinuria	RT PCR, in situ PCR	Ang II, ACE, renin (downregulated)	(40)
Proteinuric diseases	Human	proteinuria	ISH in biopsies	HO-1	(41)
PTEC	Human	HSA	RT PCR	ET-1, TGF-beta	(42)
PTEC, mouse protein overload	Human, mouse	HSA, mouse proteinuria	RT PCR	Fractalkine (CX3CL1)	(43)
Passive heymann nephritis, 5/6 NX	Rat	proteinuria	RT PCR	MCP-1	(44)
PTEC	Rat	BSA (delipidated)	RT PCR	MCP-1	(45)
PTEC	Rat	BSA, transferrin	RT PCR	MCP-1	(46)
Protein overload proteinuria	Rat	proteinuria	RT PCR	MCP-1	(47)
Protein overload proteinuria	Rat	proteinuria	RT PCR	MCP-1, osteopontin (ssp1)	(48)
Adriamycin nephropathy	Rat	proteinuria	RT PCR whole cortex	MCP-1, RANTES	(49)
Membranous nephropathy	Human	proteinuria	ISH	MCP-1, RANTES, TGF-beta, PDGF	(50)
Membranous nephropathy, MCD	Human	proteinuria (not albuminuria)	ISH	MCP-1, RANTES, TGF-beta	(51)
Protein overload proteinuria	Rat	proteinuria	ISH	TIMP-1	(52)
PTEC	Human	serum proteins		Fibronectin	(53)
PTEC	Human	albumin	Microarray	EGF receptor	(54)

PTEC – proximal tubular epithelial cell; MCD – minimal change disease; HSA – human serum albumin; BSA – bovine serum albumin.

was observed at the tubular brush border and in glomerular podocytes (Figure 2C). The protein expression of dynamin in rat glomeruli was slightly but not significantly elevated in 4-week-old Dahl SS rats compared to SHR. At 6 weeks, we could not find a difference in glomerular dynamin expression between the two strains (figure 2B).

As in rat kidneys, tubular epithelial brush borders were dynamin-positive in human renal tissue. Endothelial cells of larger vessels showed expression of dynamin as well. In human glomeruli, dynamin was present in podocytes and occasionally in parietal epithelial cells. Glomerular endothelial cells were not labeled (figure 3A). We observed an increase in dynamin protein expression in proteinuric kidney diseases, with minimal change disease and lupus nephritis showing a statistically significant increase ($P < 0.01$). Results of the digital image analysis measurements of glomerular dynamin expression are depicted in figure 3B.

Recent studies suggest that proteolytic cleavage of dynamin by cathepsin L may underlie the development of proteinuria (24). We therefore evaluated the mRNA expression of cathepsin L in proteinuric kidney diseases. In most renal diseases studied, we found a downregulation of cathepsin L at the mRNA level that was inversely correlated to the extent of proteinuria ($r = -0.39$, $P = 0.018$; figure 3C).

Proteinuria-induced glomerular gene expression patterns

Increased albumin trafficking through the glomerular filtration barrier within the context of proteinuria may have toxic effects on podocytes. We performed an immunostaining to visualize albumin within the glomerulus. As expected, albumin droplets were more often seen in Dahl SS rat glomeruli than in those of SHR rats. Co-staining of albumin and the podocyte stress marker desmin in Dahl SS tissues revealed that desmin accumulated in a podocyte-pattern in glomeruli or segments of glomeruli that showed the most extensive albumin accumulation (Figure 4).

These observations may support the hypothesis that increased albumin passage through the glomerular filtration barrier in Dahl SS rats is associated with podocyte stress. The intraglomerular albumin may consequently exert alterations in glomerular gene expression. From the literature we compiled a list of 13 genes of which the expression was found to be changed in proximal tubular epithelial cells upon proteinuria (Table 4). Using the global test, we found that the expression pattern of this group of genes was also significantly different in glomeruli of Dahl SS compared to SHR rats ($P < 0.03$ and $P < 0.008$ if time was taken as a covariate, Figure 5). If many genes are differentially expressed between groups of interest, a random group of genes may also be expected to be significantly associated with the difference between the two groups. We therefore compared this group of 13 'proteinuria-related' genes to 1,000 randomly selected groups of genes of the same size. We found that the group of proteinuria-related genes performed better than ~80 percent of the randomly selected groups. This indicates that the association between the expression pattern of the group of proteinuria-related genes with the difference between Dahl SS and SHR rats is specific.

Discussion

We used microarray technique to obtain more insight in gene expression differences during the spontaneous development of proteinuria in the Dahl SS rat. We report the following findings: i) we found and validated several genes that are differentially expressed and located within genomic regions previously identified to be associated with proteinuria; ii) pathway analysis showed that differences between the proteinuric and non-proteinuric rat strains are for a large part dependent on cytoskeletal genes; iii) from all genes involved in the regulation of the cytoskeleton, we further investigated the upregulation of dynamin expression in glomeruli of the Dahl SS rat and in human kidney diseases. iv) genes known to be differentially expressed upon proteinuria in tubular epithelial cells are differentially regulated in Dahl SS compared to SHR rats.

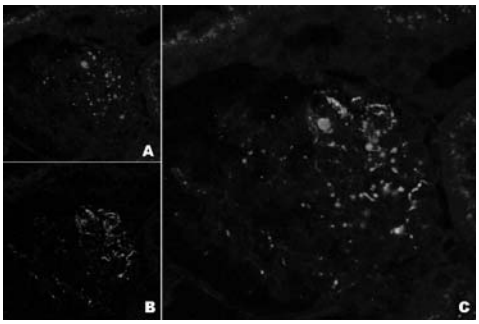


Figure 4. Co-immunostaining of desmin (green) and albumin (red) in a section of an 8-week-old proteinuric Dahl SS rat. Increased desmin expression is seen mostly in areas with extensive albumin accumulation. Desmin (A), albumin (B), merge (C).

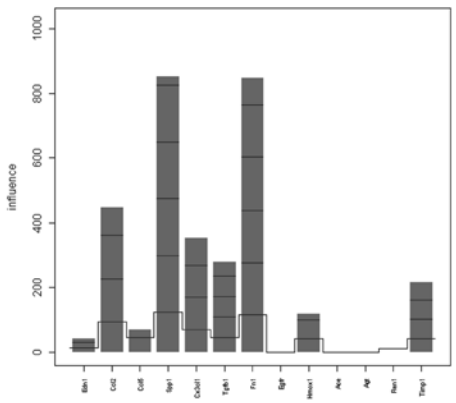


Figure 5. Proteinuria-induced gene expression patterns. We selected a group of genes of which expression was previously found to be induced in the tubulointerstitial compartment upon proteinuria. We used the global test to evaluate whether the glomerular expression of these genes was altered in proteinuric Dahl SS rats. The glomerular expression of the group of genes significantly differed between proteinuric Dahl SS rats and SHR ($P < 0.03$). In the graph, the contribution of the individual genes on the test result can be seen. On the x-axis, the individual genes are listed. The reference line marks the expected expression of the gene if it is not differently regulated (null-hypothesis); the height of the bar indicates the relative contribution of the individual gene to the test result; the marks indicate the number of standard deviations that the bar exceeds the expected (null-hypothesis) height. The graph shows a relatively large contribution of ssp1 (osteopontin) and fibronectin to the overall test result.

To find genes of interest for the development of proteinuria in the Dahl SS rat, we selected differentially expressed genes that are located on genomic regions previously identified in studies by Kreutz and Garrett et al (5-8). In these studies, a QTL on rat chromosome 19 was shown to be of importance for the development of proteinuria in the Dahl SS rat (5,6,9). We found that the mRNA expression of hedgehog-interacting protein (Hhip) and polyamine modulated factor binding protein 1 (Pmfbp1, also named outer dense fiber 3, ODF3), located within this genomic region, were significantly up- and downregulated, respectively. Although we confirmed the differential regulation of the mRNA transcripts in a time course analysis of individual rats, we have not been able to study the expression and distribution of the respective proteins.

Hedgehogs (Hh) are signaling molecules that play a role in tissue morphogenesis through influence on differentiation and proliferation of cells. Hh signaling is initiated by binding of Hh to the membrane receptor Patched, which via an elaborate pathway results in the transcriptional activation of target genes by Gli proteins (25). Hhip is a transcriptional target of Hh signaling and is expressed at the plasma membrane of Hh responsive cells where it is able to bind hedgehogs, thus preventing binding of hedgehogs to Patched (26). In the embryonic kidney, sonic Hh is thought to control the expression of various genes that play a role in the branching morphogenesis (27). Little is known about the activity of the Hedgehog signaling pathway in the adult kidney. Humphreys et al showed that in mice sonic Hh expression is limited to the renal medulla; Indian Hh is expressed in proximal

tubular epithelial cells, but its presence is dispensable for normal renal development and function (Humphreys et al, J Am Soc Nephrol 2006, 665A). Thus, it remains unknown whether increased glomerular Hhip expression has an effect on glomerular hedgehog signaling.

Pmfbp1, also termed outer dense fiber 3 (ODF3), is a coiled-coil protein that was originally identified in rat spermatozoa. Petersen et al (28) found that pmfbp1 is also expressed in the brain, and suggested that the protein is a component of the cytoskeleton. With regard to the organization of their cytoskeleton, podocytes show important similarities to neurons (29). It would be of interest to see whether the glomerular mRNA expression of pmfbp1 is indeed of podocyte origin.

Pathway analysis revealed significant overrepresentation of cytoskeletal genes in the group of genes with a differential expression between the Dahl SS and SHR. This is not surprising regarding the fact that proteinuria is mostly accompanied by profound alterations of the podocyte cytoskeleton. Widening and shortening of foot processes leads to loss of the complex cellular architecture (30,31). The organization of the actin cytoskeleton changes, and an electron dense band of filaments is formed at the base of the flattened cells (31). In a previous study, we found that the onset of proteinuria at five weeks of age precedes the first signs of foot process effacement in Dahl SS rats (15). However, cytoskeleton gene expression differences were already present at week 4, and persisted through week 6. We studied several cytoskeletal genes at the mRNA level, and found a consistent upregulation of genes involved in actin filament, intermediate filament, and microtubule based cytoskeletal networks in Dahl SS compared to SHR rats.

One of the proteins that has recently been implicated in the podocyte actin cytoskeleton rearrangement in proteinuria is the GTPase dynamin (24). We found an upregulation of dynamin mRNA in glomeruli of 4- and 6-week-old Dahl SS rats compared to SHR rats of the same age, although this difference was not clearly present at the protein level. In patients with acquired proteinuric kidney diseases, we did observe an upregulation of dynamin at the protein level. Reiser et al suggested that the cysteine protease cathepsin L is able to cleave and thereby inactivate dynamin (24). We found a downregulation of cathepsin L mRNA levels in patients with proteinuric diseases that was correlated to proteinuria. Taken together, these results may suggest that the absence of cathepsin L leads to the increased levels of dynamin protein. However, this pattern – downregulation of cathepsin L in combination with upregulation of dynamin – contrasts the previous findings by Reiser et al that cathepsin L activity is increased in LPS and polyamine nucleoside-induced nephrotic syndromes in rodents (32). The time frame in which proteinuria and foot process effacement develops is much shorter in these experimental models (days), in comparison to the spontaneous development of proteinuria in Dahl SS rats (weeks). The differences in gene and protein regulation in the current study and that of Reiser et al may be a reflection of this temporal regulation. For example, upregulation of dynamin may be a compensatory reaction to proteinuria, similar to our previous observation that certain critical podocyte proteins

are upregulated in acquired proteinuric diseases (23). We cannot exclude the possibility that the changes in cathepsin L and dynamin mRNA exression are separately influenced by another factor.

We observed that there was little differential regulation over time in the Dahl SS rats in comparison to the SHR rats. Pathway analysis of SHR rats indicated that cell-cycle and cytokinesis promoting genes were upregulated in the SHR rats compared to Dahl SS rats. In a previous study, we found that the glomerular volume per podocyte is increased in Dahl SS rats compared to SHR rats of the same age, i.e. there is a relative paucity of podocytes in Dahl SS glomeruli (15). Several studies have suggested that a shortage of podocytes may increase the susceptibility of the glomerulus to damage. Observational studies in human renal disease have already suggested a link between proteinuria and glomerular podocyte number (33). Macconi et al (34) found a similar relationship between relative "podocytopenia" (35) and proteinuria in Munich Wistar Fromter rats. In an experimental setting, Wharram et al (36) have studied the effects of podocyte depletion in rats: they developed a transgenic rat in which podocytes were depleted in a dose dependent fashion. A small percentage of podocyte depletion led to transient proteinuria, whereas removal of over twenty percent of podocytes resulted in persistent proteinuria, with increasing extent of glomerular damage and podocyte loss. We suggest that the lack of cell-proliferation response in Dahl SS rats that we observed using microarray analysis may relate to the development of proteinuria.

Furthermore, the observation that the Dahl SS rat showed little differential expression between weeks 4 and 6 may indicate that an impaired gene expression response underlies the development of proteinuria in the Dahl SS rats. Alternatively, these findings may indicate that the detection of protein in the urine lags behind the development of proteinuria-related gene expression changes in the gomerulus. We wanted to test whether the differential glomerular expression of genes between Dahl SS and SHR rats could be related to the increased protein trafficking through the glomerular filtration barrier. Gene expression changes upon protein loading are known to occur in tubular epithelial cells. Because tubular and glomerular epithelial cells share a common embryologic origin, we hypothesized that a similar pattern of gene expression might be present in the glomerulus. Our findings using the global test indicate that this may indeed be the case. This has interesting, be it speculative, implications. It is increasingly thought that proteinuria confers a toxic effect to the tubules, a phenomenon that has been regarded as one of the possible explanations of the link between glomerular damage and tubulo-interstitial injury (11). The similarity of proteinuria-induced gene expression pattern in tubules and glomerulus suggests that proteinuria has a comparable toxic effect on these tissue compartments. This could suggest that the increased passage of protein through the glomerular filtration barrier is harmful for podocytes. In support of this hypothesis is the observation that desmin protein expression in podocytes, which indicates podocyte stress, co-localized with albumin reabsorption droplets in the glomerulus.

Morigi et al previously found that exposure of cultured mouse podocytes to human serum albumin or IgG causes upregulation of endothelin 1 via activation of NF-κB and Ap1 (37). Also in the 5/6 nephrectomy model in rats, protein accumulation in podocytes was seen in conjunction with de novo podocyte desmin expression and an increased glomerular TGF-beta mRNA expression (12). In line with suggestions by these and other authors, the increased accumulation of proteins such as IgG and albumin in podocytes may initiate a self perpetuating process of podocyte damage and further proteinuria (38). In a recent study, Nagase et al (39) suggested that the proteinuria seen in Dahl SS rats might be caused by podocyte damage. Although we cannot disprove this hypothesis, our results would also be compatible with a pathogenetic pathway in which podocyte injury is a phenomenon secondary to proteinuria.

In conclusion, we have studied the expression of glomerular genes during the development of proteinuria in the Dahl rat. Our results warrant further investigation of the role of Hhip and Pmfbp1. We found an upregulation of dynamin mRNA and protein expression in rat and human proteinuric glomeruli, underscoring the importance of actin-regulating proteins in proteinuric diseases. Furthermore, our findings substantiate the hypothesis that the increased passage of proteins through the glomerular filtration barrier has a direct toxic effect on the glomerulus.

Acknowledgements

We would like to thank Paul Eilers and Jan Oosting for their help with the statistical analysis of the microarray data, and Jochen Reiser for his help with the dynamin experiment.

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