PATHOGENETIC FEATURES OF PROTEINURIA

STUDIES ON CONGENITAL NEPHROTIC SYNDROME OF THE FINNISH TYPE

by

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals:

I Haltia A, Solin M-L, Holmberg C, Reivinen J, Miettinen A, Holthöfer H: Morphologic changes suggesting abnormal renal differentiation in congenital nephrotic syndrome. Pediatr Res 43: 410-414, 1998

II Haltia A, Solin M-L, Muramatsu T, Jalanko H, Holmberg C, Miettinen A, Holthöfer H: Expression of nine developmental stage -specific genes in human kidney and cultured renal cells. Exp Nephrol 5: 457-464, 1997

III Haltia A, Solin M-L, Jalanko H, Holmberg C, Miettinen A, Holthöfer H: Mechanisms of proteinuria: Vascular permeability factor in congenital nephrotic syndrome of the Finnish type. Pediatr Res 40: 652-657, 1996

IV Haltia A, Solin M-L, Luimula P, Kretzler M, Holthöfer H: mRNA differential display analysis of nephrotic kidney glomeruli. Exp Nephrol 7: 52-58, 1999

V Holthöfer H, Kretzler M, Haltia A, Solin M-L, Taanman J-W, Schägger H, Kriz W, Kerjaschki D, Schlöndorff D: Altered gene expression and functions of mitochondria in human nephrotic syndrome. FASEB J 13: 523-532, 1999

In addition, some unpublished data is presented.

ABBREVIATIONS

Abbreviations and symbols used:

A	adenine
ACTN	α-actinin gene
ATCC	American type culture collection
ATP	adenosine triphosphate
BN-PAGE	blue native PAGE
bp	basepair
CD2AP	CD2-associated protein
cDNA	complementary DNA
CNF	congenital nephrotic syndrome of the Finnish type
CNS	congenital nephrotic syndromes
COL4	collagen type IV gene
COX	cytochrome-c oxidase
cRNA	complementary RNA
Da, kDa	dalton, kilodalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DDRT-PCR	differential display RT-PCR
DDS	Denys-Drash syndrome
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP)
dT	deoxythymidine
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid disodiumsalt-2 hydrate
EGR-1	early growth response gene 1
FITC	fluorescein isothiocyanate
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
GDNF	glial cell line-derived neurotrophic factor
gp	glycoprotein
HB-GAM	heparin binding growth associated molecule
hox	homeobox
HSPG	heparan sulphate proteoglycans
Ig	immunoglobulin
IGF	insulin like growth factor
IGFBP	IGF binding protein
IL	interleukin
ILK	integrin linked kinase
kb	kilobase
KDR	kinase-insert domain receptor
L-CAM	liver cell adhesion molecule
LPO	lipid peroxidation
MCN	minimal change nephropathy
MDA	malondialdehyde
MGN	membranous glomerulonephritis
MK	midgestation and kidney, midkine molecule
MPGN	membranoproliferative glomerulonephritis
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NAD+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
N-CAM	neural cell adhesion molecule
NGFR	nerve growth factor receptor

NPHS1	CNF, CNF gene
NS	nephrotic syndrome
NTE	NaCl - Tris-HCl - EDTA
PAGE	polyacrylamide gel electrophoresis
PAN	puromycin aminonucleoside
pax	paired box
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
RCA	Ricinus communis agglutinin
RFLP	restriction fragment lenght polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	RNase inhibitor
ROS	reactive oxygen species
rpm	rounds per minute
rRNA	ribosomal RNA
RT	reverse transcription
SCF	stem cell factor
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulphate
SSC	salt sodium citrate (NaCl-NaCitrate)
TGF	transforming growth factor
Tris	Tris[hydroxymethyl]aminomethane
trk-B	tyrosine kinase B gene (high affinity neurotrophin receptor)
tRNA	transfer RNA
UTP	uridine triphosphate
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor
WGA	wheat germ agglutinin
Wnt	wingless gene
WT-1	Wilms´ tumor gene 1
Å	1 Å (ångström) = 0.1 nm

INTRODUCTION

The kidneys have multiple central physiological functions including filtration of metabolic waste products from blood. In a healthy human adult approximately 180 liters of ultrafiltrate is formed each day, resulting finally in 1.0-1.5 liters of urine (Tisher & Madsen 1991). The primary filtration unit of the kidney is the nephron, which consists of the glomerulus and its tubular system. During glomerular filtration, plasma fluid traverses the wall of glomerular capillaries which make up the complex structure of the glomerular filtration barrier. From inside outward, it consists of fenestrated endothelial cells, glomerular basement membrane (GBM), and visceral epithelial cell (podocyte) foot processes with intervening slit diaphragms. The integrity of each of these structural elements in essential for the maintenance of normal ultrafiltration (Kanwar 1984, Kanwar et al. 1991).

The mechanisms regulating plasma ultrafiltration in glomerulus have been an object of great interest and research work during the last decades. The passage of plasma fluid and proteins through the glomerular filtration barrier is regulated by a number of factors including the biochemical and biophysical properties of the glomerular capillary wall and the size, charge and shape of molecules being filtered (Brenner et al. 1978, Kanwar et al. 1991, Remuzzi & Remuzzi 1994). Glomerular ultrafiltrate is normally free of large plasma proteins and cellular elements. However, if the integrity of the filtration machinery is disrupted, the glomerular permselectivity is reduced allowing increased amounts of albumin and other plasma proteins to pass into the urinary space. This leads to proteinuria and nephrotic syndrome, which may, when persisting, lead ultimately to scarring and end-stage kidney requiring dialysis treatment and renal transplantation. Although a common medical finding, the etiology and pathogenesis of proteinuria are still poorly understood.

Nephrotic syndrome represents a series of physiological consequences resulting from the occurence of significant urinary loss of protein. Congenital nephrotic syndrome of the Finnish type (CNF) forms a distinct entity among congenital nephrotic syndromes with its typical clinical picture and an autosomal recessive pattern of inheritance. CNF manifests already at the fetal stage as heavy proteinuria in utero and severe nephrotic syndrome develops within the first weeks of life (Hallman et al. 1967, Seppälä et al. 1976). The proteinuria in CNF is resistant to any treatment and today, renal transplantation preceded with an early nephrectomy and peritoneal dialysis is the only therapeutic option (Holmberg et al. 1995).

The pathology of CNF kidneys has been characterized thoroughly (since its discovery in 1956), but the basic defect leading to proteinuria remained obscure until very recently, when Kestilä et al. (1998) identified a putative CNF gene and its protein product termed nephrin. Extensive morphological, histological and biochemical studies on CNF kidneys have been performed by several investigators, but none of the findings seemed to be causative for the CNF. The normal development of children following kidney transplantation, apparently without any manifestations of disease in other organs (Holmberg et al. 1995) implies that CNF is a kidney specific disease and thus, an ideal human model disease to study the pathogenesis of proteinuria.

The aim of this thesis was to study the molecular mechanisms of proteinuria in the kidneys of CNF patients. At the time of this research, the basic defect of CNF was still unknown, but it had been suggested that it involves a gene essential to establish a functional glomerular filtration barrier, either primarily or secondarily. Several different hypotheses have been proposed to explain the

mechanisms of proteinuria in CNF, including aspects of the development of functional glomeruli and the molecular regulation of filtration. This study was undertaken to examine CNF kidneys in the light of these theories and further, to identify any candidate molecules involved in the pathogenesis of CNF and proteinuria.

1. KIDNEY DEVELOPMENT

1.1. Organogenesis

The permanent mammalian kidney, the metanephros, is created by two tissue components, mesenchymal nephric cord (blastema) and epithelial, originally mesoderm-derived Wolffian duct. In human embryos, development of the permanent kidney starts during the first trimester of gestation with the formation of a Wolffian duct derived ureter bud which invades the metanephric mesenchyme (Spitzer 1985, Saxen 1987). The subsequent inductive interaction initiates a sequence of morphogenetic changes starting with dichotomous branching of the ureteric bud to form the developing collecting duct system, and condensation and polarization of the mesenchymal cells to form renal vesicles around the ampullae of collecting ducts. After the comma-shape and S-shape stages the early nephrons fuse into the collecting duct tree leading ultimately to the formation of mature renal glomeruli with their tubular systems *(Figure 1)* (Horster et al. 1999). During tubule elongation the pretubular cells differentiate to epithelial cell types of proximal and distal tubules and loop of Henle.

The growth of the kidney occurs from the center toward the periphery forming a progressive, centripetal gradient of developing nephrons: as nephrogenesis in the inner cortex proceeds, new nephrons are being formed at the ampullar end of collecting ducts in the subcapsular region of the outer cortex (Saxen 1987). Recent reports have shown that while the growth in the kidney volume is dramatic during development, it is accompanied by a significant amount of apoptosis or programmed cell death (Koseki et al. 1992, Bard et al. 1994, Yasui et al. 1997); there are certain secreted molecules that prevent nephrogenic mesenchyme from undergoing apoptosis, and thus determine the number of available nephron units. The fact that different nephron populations and different nephron segments develop at different times results in a high degree of structural heterogeneity that is reflected to the functional complexity of the kidney at various stages of development (Spitzer 1985).

1.2. Nephron differentiation

During nephrogenesis different cell types are formed as a result of strictly timed and synchronized regulatory mechanisms (Bacallao & Fine 1989, Horster et al. 1999). There are three different cell types in the adult glomerular tuft: visceral epithelial cells (podocytes), mesangial cells and endothelial cells (*Figure 2*). The **podocytes** develop from metanephric mesenchyme. The stimulus which initiates epithelial cell formation from the mesenchyme is provided by the reciprocal interaction of the epithelium of the branching ureter (Horster et al. 1999). **Endothelial cells** comprise all vasculature and are probably originated from external blood vessels which grow into the kidney and migrate to the differentiating glomerulus at the S-shaped body stage (Abrahamson et al. 1998, Risau 1998). Although embryonic kidney is known to produce angiogenic factors, the precise mechanisms which direct growing capillaries into the primitive glomerulus are still unknown. **Glomerular basement membrane** (GBM) separates the endothelial cells from epithelial podocytes and acts as a filtering membrane of the kidney. The GBM forms from the fusion of the epithelial and endothelial derived basement membranes during glomerular capillary loop

development (Sariola et al. 1984b). An important third cell type of the glomerulus, the **mesangial cell**, is closely associated with the endothelial cell. An obvious question is whether this cell type is derived from the metanephric mesenchyme or from the endothelial cell lineage. From the morphological and regulatory point of view mesangial cells resemble vascular pericytes (Michael et al. 1980, Martinez-Hernandez & Amenta 1983), but mesangial cells also have capacity for phagocytosis. The studies of different marker expressions support a common origin of mesangial cells and vascular smooth muscle cells of glomerular arterioles (Lindahl et al. 1998), although direct evidence of this is still missing.

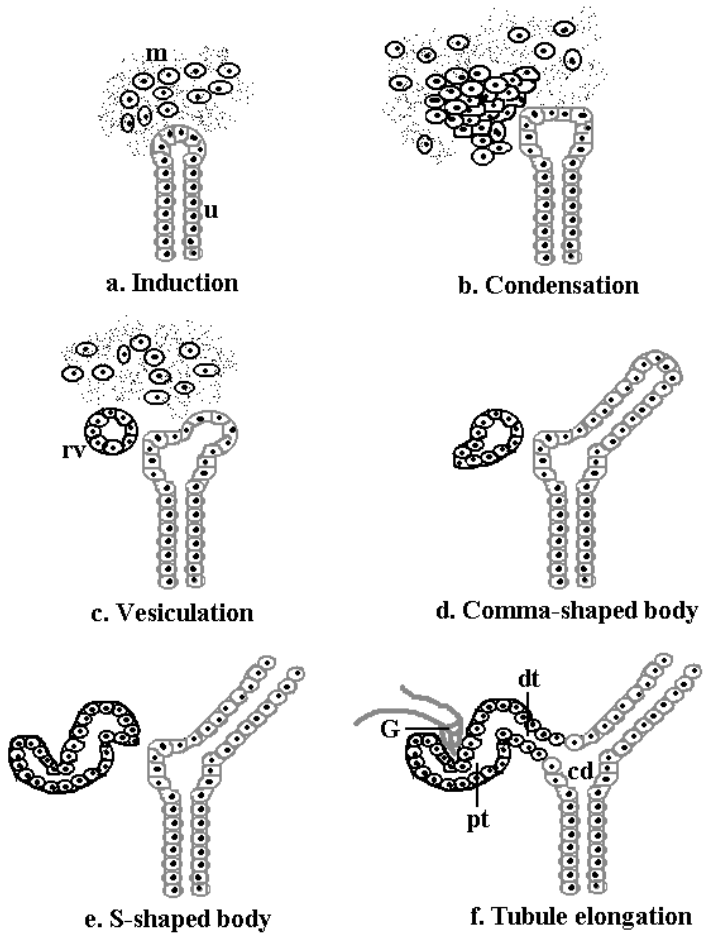

Figure 1. Stages of nephrogenesis. The mesenchymal cells around the tips of the ureteric bud condense and gradually recieve an epithelial character: (a) Induced mesenchymal cells adhere and (b) form condensed aggregates close to the tip of ureteric bud. (c) Condensates give rise to the renal vesicle stage and further diversification leads to the formation of (d) comma-shaped body and (e) S-shaped body with two crevices. (f) The lower crevice expands to form the glomerular cup invaded by capillaries. The middle part develops into the proximal tubulus and the upper part into the distal tubulus, which fuses to the extending collecting duct system. The strictly dichotomous branching of collecting duct tree is not shown here. m, mesenchyme; u, ureteric bud; rv, renal vesicle; G, glomerular cup with capillary tuft; pt, proximal tubule; dt, distal tubule; cd, collecting duct. Adapted from Saxen (1987) and Horster et al. (1999).

In addition to the visceral epithelial podocytes, epithelial components of the kidney comprise the **parietal epithelium** of the Bowman's capsule and the characteristic complex epithelial lining of the renal tubules and collecting duct (Sorokin & Ekblom 1992). During nephron differentiation the condensed cells acquire their polarity with the formation of distinct apical, basal and lateral borders which characterize the **epithelial tubular cells**. Cell interactions and cell-cell adhesion appear to be an important step in the polarization processes (Horster et al. 1999).

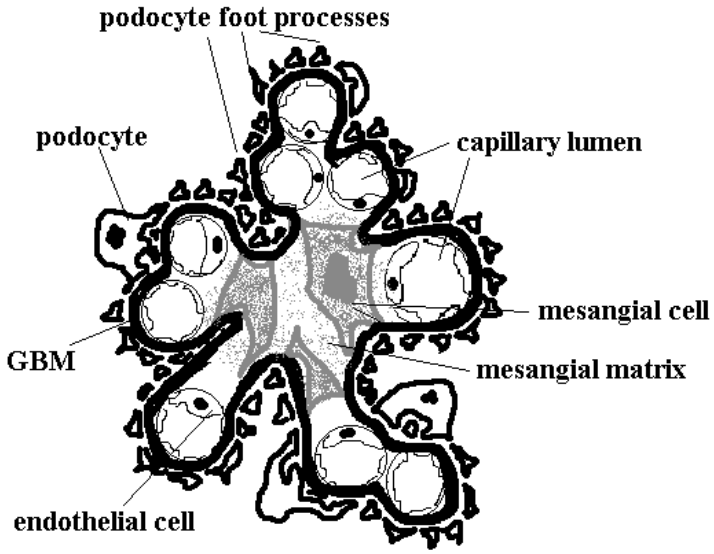

Figure 2. Cross-sectional presentation of the glomerular capillary lobule. The capillary lumens are surrounded by the fenestrated endothelial cells, the glomerular basement membrane (GBM) and podocytes with foot processes. In the middle of the capillary lobule lie mesangial cells and mesangial matrix.

1.3. Molecular expression in the developing kidney

Development is accompanied by characteristic changes in gene expression that precede the morphological changes. Over the last few years there has been considerable progress in determining the cellular and molecular events at the earliest stages of nephrogenesis (Bard & Woolf 1992, Bard et al. 1994, Horster et al. 1999, Kuure et al. 2000). However, looking at the processes of nephrogenesis in the context of the regulatory information now available, it is obvious that only a small proportion of the regulatory genes involved in kidney development have still been identified and the information achieved so far gives only a superficial insight to how nephrogenesis is mediated at the genetic level. It is obvious that these processes are autonomous to the developing organ as much of this development will take place similarly in vitro. Some of the most important developmental factors and their roles in glomerular growth and differentiation are introduced here (see also *Table 1a-d* p.21-23):

1.3.1. Transcription factors

Patterns of gene expression in differentiating cells during tissue morphogenesis are largely regulated by changes in the pool of DNA-binding transcription factors (Kessel & Gruss 1990). As a cascade of sequential transcriptional control has been shown to determine the early *Drosophila* development, mammalian development and organogenesis requires similar cascades of factors with strictly timed and spatially limited expressions specifying positional information and the fate of differentiating cells (Gruss & Walther 1992). In situ hybridization analyses in mice have revealed the expression of many homeobox and paired box containing genes, the Wilms' tumor gene and several other control genes in the condensed mesenchyme. Their expression seems to be linked to the complex morphogenetic program driving kidney organogenesis (Bard & Woolf 1992, Eccles et al. 1995).

Homeobox genes
The homeobox (Hox) sequence encodes a specific protein domain which has a DNA-binding motive. This DNA-binding region allows the homeobox gene products to regulate the transcription of other genes. In the mouse, most **Hox** genes are expressed in the mid-gestation phase (day 9 to 12), a time when organogenesis prevails (Jackson et al. 1985, Kessel & Gruss 1990). The studies with transgenic mice have provided strong direct evidence that homeobox genes constitute critical control elements (Kessel et al. 1990, Chisaka & Capecchi 1991). In transgenic mice, in which the Hox 1.1 gene (HOXA7, see Scott 1993) was expressed in an abnormally wide range of tissues, the embryos developed a malformed face (Kessel et al. 1990). Chisaka and Capecchi (1991) engineered mice with abolished expression of Hox 1.5 gene (HOXA3) and these animals lacked both the thymys and parathyroid glands, a phenotype which resembled the human DiGeorge syndrome. As several of the murine Hox genes are expressed in the developing kidney, the protein products of these Hox genes are implicated in the inductive interactions underlying nephrogenesis (Bard & Woolf 1992). E.g. Hox 2.3 (HOXB7) transcripts were located on the branching ureter tree of the metanephros and a similar pattern was also found in the adult, with transcripts located in the ureter and the collecting ducts, both of which are derived from the bud of Wolffian duct (Kress et al. 1990). The wide expression of homeobox genes in the early kidney may be involved in specifying the fate of the mesodermal domain that will become metanephric mesenchyme (Bard et al. 1994).

Paired box genes
The paired box (Pax) is another DNA-binding domain that is conserved in the evolution of the fly, mouse and man (Burri et al. 1989). Pax-containing genes comprise a family of transcription factors active in specific tissues during embryonic induction and development and have been associated with a variety of known mutations in both the mouse (Balling et al. 1988, Epstain et al. 1991, Hill et al. 1991) and man (Ton et al. 1991, Baldwin et al. 1992, Tassabehji et al. 1992). The expression of **Pax-8** transcripts in the mesenchymal condensations and Pax-2 in the ureteric bud mesenchymo-epithelial cells as well as their transient expression patterns are suggestive of functions in the regulation of early kidney development (Dressler et al. 1990, Poleev et al. 1992, Rothenpieler & Dressler 1993, Eccles et al. 1995). **Pax-2** is one of the earliest markers of induced mesenchyme and is quickly repressed as the mesenchyme derived epithelium matures (Dressler & Douglass 1992, Eccles et al. 1992). Thus, Pax-2 is suggested to initiate a genetic cascade controlling a complex series of events that results in the conversion of kidney mesenchyme to epithelium. Persistent expression of Pax-2 and Pax-8 may be key events during kidney oncogenesis (Maulbecker & Gruss 1993, Tagge et al. 1994). Interestingly, deregulated Pax-2 expression in transgenic mice results in histologically abnormal and dysfunctional renal epithelium with properties similar to congenital

nephrotic syndrome: multifocal microcystic tubular dilation, paucity of podocyte foot processes and proteinuria (Dressler et al. 1993).

Wilms´ tumor gene

The Wilms´ tumor gene **WT-1** is a tumor suppressor gene that encodes a transcription factor with four DNA-binding zinc finger motifs (Buckler et al. 1991, Madden et al. 1991). The role of WT-1 appears crucial for the control of metanephric stem cell differentiation as well as for the whole kidney development; this is based on the results by Kreidberg et al. (1993) showing that the inductive events that lead to formation of metanephric kidney are seriously affected in WT-1 knockout mice. During normal kidney maturation WT-1 activation turns off the expression of molecules involved in embryonal cell proliferation and turns on genes whose expression is required for terminal differentiation (Madden et al. 1991, Drummond et al. 1992, Wang et al. 1993). In specific types of Wilms´ tumors, the gene retains its responsiveness to its upstream regulators in the proliferative response pathway, so that it is transcribed in appropriate cell types, but mutation alters the ability of its product to regulate downstream target genes, thus leading to uncontrolled proliferation and tumors (Pritchard-Jones et al. 1990).

Early growth response genes

Other genes that encode for putative DNA-binding proteins and have restricted expression during development include early growth response genes, such as **EGR-1**. EGR-1 gene is induced by a variety of mitogenic or differentiation-specific stimulation protocols in every mammalian cell type tested including B-lymphocytes, T-lymphocytes, kidney mesangial, glomerular and tubular epithelial cells, hepatocytes and endothelial cells (Sukhatme et al. 1988, Cao et al. 1990). EGR-1 functions as a ubiquitous transcriptional activator, but its interaction with tissue specific factors, e.g. WT-1, gives rise to respective tissue specific effects. EGR-1 shares 65 % amino acid sequence homology with WT-1 in the zinc finger region. WT-1 protein is able to repress transcription through EGR-1 binding site, and also represses transcription of the EGR-1 promoter itself (Rauscher et al. 1990). Thus, EGR-1 and WT-1 are thought to form a binary on/off element in the condensed metanephric mesenchymal cells: EGR-1 is upregulated as a cell receives a mitogenic signal, but as differentiation proceeds, WT-1 levels rise, mitogenic molecules are downregulated and growth comes to a stop (Sukhatme 1992, Rackley et al. 1995).

Myc oncogenes

Proto-oncogens are believed to play key roles in the growth and differentiation of embryonic tissues. The expression of many proto-oncogenes reaches a peak in early development and ceases before or shortly after birth, but failure of repression has a role in preventing the terminal differentiation of cells (Forrester et al. 1992). The myc oncogene family of nuclear DNA-binding proteins consists of three known members, the c-myc, N-myc and L-myc. All myc genes are expressed at high levels in embryonic and fetal tissues with distinct tissue- and stage-specific patterns (Zimmerman et al. 1986). Northern blotting and in situ hybridization have revealed that N-myc expression is highest in brain and kidney (Hirvonen et al. 1989). It has been suggested that **N-myc** is linked to differentiation rather than proliferation, and in the developing kidney, N-myc is not expressed in the most rapidly proliferating cell population (cells of the ureteric bud) but rather in the adjacent mesenchyme. **C-myc** on the other hand, is expressed strongly by the urogenital ridge in the mouse and is suggested to be linked to the mitogenesis of cells. This is supported by the observation that transgenic mice carrying the c-myc gene develop polycystic kidney disease and die of renal failure at an early age (Bacallao & Fine 1989). In these animals the kidneys show evidence of tubular hyperplasia, microadenomas, and hyperplasia of parietal epithelium of the glomerulus.

1.3.2. Growth factors

In addition to transcription factors and other nuclear proteins, various secreted growth factors causing a rapid but strictly organized cell division are involved in the developmental control system. The importance of growth factors as potential morphogens in the control of development has been well documented in lower vertebrates (Green & Smith 1991). In the developing kidney, the requirement for specific growth factors is not yet established, but, for example, the importance of insulin like growth factor system and neural growth factors are well studied.

Insulin like growth factors
Insulin-like growth factors (IGFs) have significant effects on renal function and have been implicated in renal development and glomerular cell differentiation (Chin & Bondy 1992). Many actions of growth hormone on renal size and kidney functions appear to be mediated indirectly through stimulation of the synthesis and release of **IGF-1** (Mathews et al. 1986, Murphy et al. 1987, Hammerman & Miller 1997). All glomerular cell types have also been shown to express IGF-1 receptors (Conti et al. 1988, Conti et al. 1989) which suggests that the glomerulus is a site for the autocrine and paracrine action of IGF-1. Direct actions of IGF-1 include an increase in glomerular filtration rate and renal plasma flow in both human and rat when injected into the renal artery, and chronically elevated circulating IGF-1 levels are associated with renal enlargement and glomerular hypertrophy (Fagin & Melmed 1987). In experimental chronic renal failure of rats, IGF-1 protects the glomerulus from injury by restoring autoregulatory control of renal blood flow (Lin et al 1998). In addition, IGF-1 induced biochemical changes may have a role in the pathogenesis of the initial stages of diabetic nephropathy, perhaps by modulating the metabolism of various glycoproteins of the extracellular matrix (Watanabe et al. 1992).

IGF-2 has been shown to be heavily expressed in fetal kidneys and in embryonal renal tumors and has been attributed an autocrine and paracrine role in nephrogenesis (Scott et al. 1985). The expression of IGF-2 mRNA in fetal kidneys is largely confined to the stromal and blastemal cells and proposedly provides a stimulus for the growth and differentiation of epithelial structures; its expression in Wilms' tumors reflects a failure of complete differentiation of the tumor cells (Hirvonen et al. 1989). In contrast, IGF-2 expression in normal kidney is strictly downregulated at the time of birth.

The biological effects of IGFs are mediated through binding to high affinity receptors on the cell surface. Two types of IGF receptors have been identified, with different relative affinities for IGF-1, IGF-2 and insulin. All known biological actions of IGF-1 and IGF-2 are mediated by type I IGF-receptor (Czech 1989). In addition to cell surface receptors, the multiple biological functions of IGF-1 and IGF-2 are modulated by specific extracellular IGF-binding proteins (IGFBP). **IGFBP**s prolong the half-lives of IGFs, serve as their carriers in the circulation and act as autocrine/paracrine regulators of the biological actions of IGFs by regulating their availability to cell surface receptors (Hsu & Olefsky 1992). Six different human IGFBPs have been identified so far, differing from each other by the sites of synthesis and specific tissue expression patterns. The role of IGFBPs in nephrogenesis is undefined, but most probably, together with IGFs, they participate in regulating the various cellular events of nephrogenesis (Matsell et al. 1994). Matsell et al. (1994) have demontrated a distinct temporal and spatial expression of IGFBP mRNAs in the developing human fetal kidney. The expression of some IGFBP mRNAs has been demonstrated also in adult kidneys. Suikkari et al. (1992) have studied IGFBP-1, and detected its expression in fetal liver and kidney

but not in other fetal tissues. Its transcription in the developing kidney is most remarkable in the epithelial cells of the collecting ducts, in developing glomeruli, especially in most immature glomeruli, and in the subcapsular zone of undifferentiated nephrogenic mesenchyme.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is essential for vasculogenesis and angiogenesis (Breier et al. 1992, Risau 1998). It is also a potent agent inducing and enhancing microvascular permeability as demonstrated experimentally in the guinea pig skin and peritoneal wall permeability assays (Senger et al. 1983). It is a bioactive product of various solid tumors, where **VEGF** proposedly maintains the basal permeability of microvascular endothelia essential for the exchange of nutrients and plasma proteins into the perivascular tissue (Senger et al. 1983, Nagy et al. 1988, Brown et al. 1992b).

The expression of VEGF is high in embryonic brain and kidney when angiogenesis occurs, but in the kidney, VEGF expression remains high in glomerular podocytes even in the adult (Breier et al. 1992, Brown et al. 1992a). VEGF receptors 1 and 2 are endothelial specific receptor tyrosine kinases (Mustonen & Alitalo 1995). Similar to the ligand, expression of these receptors is high during brain and kidney angiogenesis, low in adult brain, but high in adult glomerular endothelium. Because VEGF is also a vascular permeability factor, the expression in the adult correlates with the low permeability of blood-brain barrier endothelium and the high permeability of fenestrated glomerular endothelium, and it has been suggested that VEGF is involved in the regulation of glomerular permeability (Brown et al. 1992a, Simon et al. 1995, Shulman et al. 1996). VEGF receptor-2 is also known as kinase-insert domain receptor KDR in human. The crucial importance of this receptor is underlined by the early lethality resulting from the absence of endothelial cells in mice deficient for this molecule (Shalaby et al. 1995). Later during embryonic development, KDR becomes restricted to endothelial cells consistent with the function of its ligand VEGF as a vascular permeability factor (Kaipainen et al. 1993). It has been speculated that injury to podocytes, the cytologic site of VEGF production, may lead to changes in VEGF synthesis and/or secretion and thereby to abnormal glomerular permeability and proteinuria (Brown et al. 1992a, Shulman et al. 1996).

Transforming growth factor β

The data presented by Lehnert & Akhurst (1988) argue for a major role for transforming growth factor β1 (TGF-β1) in a variety of developmental processes, involving tissues of different lineages and diverse mechanisms of action. **TGF-β1** mRNA has been detected in mouse embryos at 10.5-15.5 days of gestation and of importance, is present throughout the nephrogenic mesenchyme. Many mesenchymal and epithelial cells whose proliferation and differentiation are affected by TGF-β demonstrate increased expression of extracellular molecules in response to it. In developing mouse kidney it may act in a paracrine fashion to affect growth and differentiation of the nephron by promoting extracellular matrix formation (Bacallao & Fine 1989). Furthermore, there is now convincing evidence that the process of matrix deposition in the diseased kidney is regulated by TGF-β (Border et al. 1992, Border & Noble 1993). In tissue cultures, TGF-β1 has been implicated in the regulation of ureteric bud growth and branching (Santos & Nigam 1993, Ritvos et al. 1995). However, mutant mice lacking TGF-β1 (Shull et al. 1992) show no apparent defects in kidney morphogenesis suggesting that it is not necessary to kidney development in vivo.

Neural growth factors

Neural elements including neurons, neurotrophic factors and their receptors seem to be crucially involved in the induction and development of the nephrogenic mesenchyme (Sariola et al. 1991). The effects of neural growth factors are mediated by interaction with specific receptors on target cells. Nerve growth factor receptor (NGFR) serves as the binding site for many of the neurotrophic factors (Meakin & Shooter 1992). In addition to some neuronal cells and glial cells, **NGFR** is expressed in several embryonic tissues, including metanephric cell condensates and S-shaped nephrons (Sariola et al. 1991). Sariola et al. (1991) have shown that inhibition of NGFR expression in rat kidney cultures inhibits kidney morphogenesis: no tubules were formed and only rudimentary branching of the ureter was seen. In addition, the high affinity neurotrophin receptor, protein tyrosine kinase **trk-B** has been found to be differentially expressed during renal development (Durbeej et al. 1993); its expression pattern is mostly restricted to the cortical mesenchyme cells that will differentiate into stroma.

Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic polypeptide distantly related to TGF-β. The importance of **GDNF** in the development of metanephric ureteric epithelium has been demonstrated by several investigators (Pichel et al. 1996, Sánchez et al. 1996, Sainio et al. 1997). GDNF is expressed in the condensing mesenchyme that surround the developing ureter system of kidneys. Sainio et al. (1997) have shown that GDNF can induce primary ureteric bud formation from various segments of Wolffian duct. It binds directly to the tips of the ureteric bud and attract branches towards the source of GDNF. It functions through increasing cell adhesion rather than cell proliferation. One known receptor for GDNF is the **cRet** receptor tyrosine kinase, which is expressed in several tissues adjacent to sites of GDNF synthesis. Transgenic mice deficient for GDNF and deficient for cRet show remarkably similiar phenotypes characterized by a severe defect in intestinal innervation, and renal aplasia or hypodysplasia (Schuchardt et al. 1996).

Heparin binding growth factors

Recently, a new family of heparin binding growth and differentiation factors with regulatory functions in development have been identified (Muramatsu 1993). The initial members of the new family were heparin-binding growth-associated molecule (HB-GAM) and midkine (midgestation and kidney, MK), the product of a retinoic acid responsive gene. The occurence of **HB-GAM** correlates with the perinatal developmental phase of neuronal connections during rat brain, heart and kidney development (Rauvala 1989, Merenmies & Rauvala 1990). Studies on the neurite outgrowth-promoting activity suggest that HB-GAM functions as an extracellular matrix-associated protein that enhances axonal growth during perinatal period (Raulo et al. 1992). **MK** is significantly homologous with the HB-GAM (Tsutsui et al. 1991). The mode of temporally and spatially controlled localization of MK expression during mouse embryogenesis has been studied extensively by several investigators (Kadomatsu et al. 1990, Nakamoto et al. 1992, Mitsiadis et al. 1995). By immunohistochemistry, both MK and HB-GAM expression were localized on the surface of differentiating cells and in basement membranes of organs undergoing epithelial-mesenchymal interactions, including the urogenital system. This observation suggests their signalling roles during morphogenesis of epithelio-mesenchymal organs (Mitsiadis et al. 1995).

Wingless genes

The wingless (Wnt) gene family encodes a group of secreted glycoproteins, which are involved in cell-cell communications. Wnt-signalling is thought to play diverse roles in the development of embryonic tissues (Wilkinson et al. 1987, Gavin et al. 1990, Roelink & Nusse 1991). In particular, the expression of **Wnt-4** gene has been documented in mouse kidney mesenchyme: Stark et al.

(1994) have detected Wnt-4 mRNA in condensed mesenchymal cells, comma-shaped bodies and later in S-shaped bodies in the region where epithelial fusion was occuring with the collecting duct. Mice lacking Wnt-4 activity fail to form pretubular aggregates or more developed tubules, but the mesenchyme remains morphologically undifferentiated. However, the other aspects of mesenchymal and ureteric development are unaffected, suggesting normal induction by the ureter and branching of the ureteric epithelium.

1.3.3. Extracellular matrix

Matrix components
In the course of renal development, both in vivo and in vitro, significant changes in the composition of the extracellular matrix occurs. The GBM originates in development from fusion of subendothelial and subepithelial matrices. The appearance of certain extracellular matrix proteins coincides well with metanephric differentiation and cell polarization (Ekblom 1989, Ekblom et al. 1990). Undifferentiated kidney mesenchyme expresses fibronectin and types I and III of collagen (Ekblom et al. 1981). During epithelialization these extracellular matrix components disappear and are replaced by components typical to epithelial cells, such as collagen type IV, laminin and heparansulfate proteoglycan (HSPG) (Ekblom 1981a and 1981b). The appearance of type IV **collagen** is entirely dependent on the presence of an inducing stimulus (Ekblom et al. 1981). Type IV collagen molecules organize into the basal lamina surrounding the first tubular cell aggregates and ultimately form a tridimensional lattice-like network to which the laminin network with other basement membrane components are suggested to attach. Collagen molecules are heterotrimers; until now six different monomeric chains of type IV collagen have been identified (α1-α6). During GBM assembly and maturation, the α1- and α2-chains of type IV collagen are replaced by α3-, α4- and α5-chains, whereas the α6-chain is present mostly in the Bowman's capsule and parts of the tubular basement membrane (Kuroda et al. 1998, St John et al. 2001).

Laminin, a major glycoprotein in basement membranes, has been shown to accumulate at early developmental stages in the forming basement membranes, and antibodies to certain domains of laminin have been the most effective in inhibiting epithelial cell polarization in vitro (Klein et al. 1988b). Laminins are heterotrimeric proteins which are assembled from a repertoire of five α-, three β- and three γ-chains (Miner 1999). The transition of laminin from a monomeric to a polymerized state is thought to be a crucial step in the development of basement membranes (Colognato et al. 1999). In an organ culture model, it was found that the laminin B (β, γ) chains were constitutively expressed by the mesenchymal cells already before the onset of morphogenesis, but the laminin α-chain could not be detected until the first signs of mesenchymal condensates and epithelial cell formation became evident. The appearance of the α-chain is fundamental for induction of early polarity of tubular epithelial cells (Sorokin et al. 1990, Sorokin & Ekblom 1992, Dearcangelis et al. 1996). The glomerular basement membrane contains restricted isoforms of laminin (as well as collagen IV). The maturation of GBM is marked by the replacement of laminin 1 (consisting of α5-, β2-, and γ1-chains) with laminin 11 (α5β2γ1). GBM is rich in β2-chain of laminin, whereas the renal tubular basal laminae bear no detectable β2 but is rich in the more widely distributed laminin β1-chain (Noakes et al. 1995). The importance of laminin β2-chain to glomerular ultrafiltration was verified, as a null mutation of laminin β2-chain gene in mice leads to nephrotic syndrome resembling human congenital nephrotic syndrome and the minimal change nephropathy (Noakes et al. 1995).

A number of biological activities, especially cell adhesive and cell migratory ones, have been attributed to **fibronectin** expression in embryogenesis. Fibronectin deposition in the developing glomerulus seems to be linked to endothelial cell organization within the Bowman´s capsule to form the glomerular tuft (Sariola et al. 1984a). The functions of alternatively spliced variants of fibronectin are not yet resolved but their temporal and spatial variation during embryogenesis suggests a role in differentiation. **Nidogen** (or entactin) is a glycoprotein found in GBM, mesangial matrix and Bowman´s capsule. The ability of nidogen to bind to various other basement membrane proteins suggests a potential role for nidogen in complex formation of extracellular matrix (Aumailley et al. 1993). **Tenascin** is an extracellular matrix protein which has been implicated in embryonic mesenchymal-epithelial interactions. It is absent in undifferentiated metanephric mesenchyme but begins to appear around condensates of epithelial cells and the S-shaped bodies of early nephrogenesis, and again disappears when tubules achieve their mature size. These findings suggest an important role for tenascin in the process of differentiation, perhaps by mediating the interaction of mesenchyme and the budding ureteric epithelium (Aufderheide et al. 1987).

The major proteoglycan of human GBM is **HSPG**, whereas mesangial proteoglycans contain mainly chondroitin sulphate (Kanwar & Farquhar 1979). In human GBM, **agrin** is a principal HSPG component (Groffen et al. 1998). Agrin can stabilize the matrix structure by binding to laminin and can be involved also in transmembrane signalling of cells (Groffen et al. 1998 and 1999). Its negatively charged structure appears to be of vast importance to glomerular permeability.

Integrins

In general, the receptors for collagen, fibronectin, and laminin belong to the **integrin** supergene family (but receptors do exists that do not belong to this family (Buck & Horwitz 1987)). Integrins are cell surface transmembrane proteins with two noncovalently linked subunits, α and β, and each of the $\alpha\beta$ integrins has specific ligands amongst the extracellular molecules (Müller et al. 1997). In the developing kidney, different $\alpha\beta$ integrins have a distinct spatiotemporal, developmentally regulated expression pattern (Korhonen et al. 1990). In the beginning of condensation of the pretubular mesenchyme $\alpha1$ and $\alpha4$ subunits disappear. During the first stages of epithelial polarization $\alpha6\beta1$ is expressed in a basally polarized manner and during the distal invagination $\alpha3\beta1$ is found in podocytes and in Bowman´s capsule, $\alpha1\beta1$ in mesangial cells and $\alpha2\beta1$ integrin in endothelial cells (Kreidberg & Symons 2000). The importance of $\alpha3\beta1$ integrin has been addressed by the use of the knockout mice (Kreidberg et al. 1996).

Cell adhesion molecules

In the developing nephron, the induced mesenchyme begins to express molecules at the cell surface, which determine cell-cell adhesion, shortly after the elaboration of extracellular matrix proteins. The cell surface adhesion molecules include uvomorulin (or liver cell adhesion molecule L-CAM), P- and N-cadherin and N-CAM (neural cell adhesion molecule). The initial condensation seems to be associated with the production of **syndecan**, a sulphated proteoglycan, which acts as an adhesion molecule between cells in the condensate (Vainio et al. 1989 and 1992). **N-CAM** is an adhesive molecule for determined but yet uninduced mesenchyme and it gradually disappears during conversion to an epithelium (Klein et al. 1988a); as the aggregates form, they lose their mesenchymal character and form epithelia and at the same time the expression of N-CAM ceases, and the production of a new adhesion molecule, **uvomorulin** (L-CAM), starts. Based on the in vitro experiments in mouse embryonic kidney, it can be deduced that production of uvomorulin is related to morphological differentiation and appears only in epithelial cells that form a true epithelial monolayer but not in the epithelial podocytes of the glomerulus (Vestweber et al. 1985). During

development its expression is strongest in cells derived from the ureter and is weaker in mesenchymally derived cells, e.g. proximal and distal tubular cells. However, antibodies to both N-CAM and L-CAM have failed to inhibit tubulogenesis, making their precise contributions to the process of nephrogenesis unclear (Vestweber et al. 1985, Klein et al. 1988a, Bacallao & Fine 1989).
Cadherins are transmembrane adhesion molecules that act as important participants in cell recognition and cell sorting during development. Their appearance and disappearance correlate with major morphogenetic events during tissue differentiation. Many cadherins, including N-, E-, P-, K- and Ksp-cadherins, have been associated with segment-specific morphogenesis of the kidney (Goto et al. 1998). Recently, Reiser et al. (2000) localized P-cadherin into the podocyte slit diaphragms and speculated that it could have an important structural role in establishing glomerular permeability barrier.

TABLE 1. Gene expression during kidney development. Examples of transcription factor, signalling molecule, extracellular matrix, cytoskeleton and cell adhesion molecule expressions in kidney. For references and species specifications see Davies & Brandli (2002) and Bard et al. (1994). +, expressed; -, not expressed; ?, unclear or not determined; MM, metanephric mesenchyme. Adapted from Davies & Brandli (2002) and Bard et al. (1994).

Table 1a. Transcription factors.

GENE	ureter bud	uniduced MM	stem cells	renal vesicle	glomerulus
Hox genes					
Hox A3	+	?	?	?	?
Hox A4	+	?	+	?	?
Hox A5	+	?	+	?	?
Hox A6	+	?	+	?	?
Hox B3	+	?	-	+	+
Hox B5	+	?	+	+	?
Hox B7	+	-	-	+	+
Hox C6	+	?	+	?	?
Hox C8	+	?	+	?	?
Hox C9	-	-	+	+	-
Pax genes					
Pax-2	+	-	+	+	?
Pax-8	-	-	-	+	+
Zinc finger genes					
Kid-1	+	-	-	-	-
WT-1	-	+	+	+	+
Pou genes					
KDN-1	+	-	-	-	-
Other nuclear proteins					
c-myc	-	?	+	+	-
L-myc	+	?	+	-	-
N-myc	-	?	+	+	-
GATA-3	+	?	?	?	+

Table 1b. Signalling molecules and their receptors.

GENE	ureter bud	uniduced MM	stem cells	renal vesicle	glomerulus
Growth factors					
IGF-1	expressed only in nephric tubules				
IGF-2	-	+	+	+	+
TGF-β1	expressed only in stroma				
GDNF	-	+	+	?	?
HBGAM	+	?	?	?	?
MK	+	?	+	+	?
HGF	-	?	+	-	-
PDGF A	+	-	-	+	+
PDGF B	-	?	-	-	+
FGF-2	+	?	?	+	+
BMP-7	+	-	+	+	+
VEGF	?	?	-	-	+
Wnt genes					
Wnt-4	-	-	?	+	-
Wnt-5b	+	+	+	+	+
Wnt 11	+	?	?	?	+
Other messenger molecules					
bcl-2	?	?	-	+	+
Signal receptors					
IGFBP-2	-	?	+	+	+
IGFBP-3	+	-	-	-	-
IGFBP-4	-	?	+	-	+
IGFBP-5	?	?	?	-	+
IGFBP-6	?	?	?	+	-
NGFR	-	?	+	+	+
c-ret	+	-	-	-	-
trk-B	?	?	+	?	?

Table 1c. Extracellular matrix (ECM) components and receptors.

GENE	ureter bud	uniduced MM	stem cells	renal vesicle	glomerulus
ECM					
collagen I	-	+	+	-	-
collagen III	-	+	+	-	-
collagen IV	?	-	-	+	+
fibronectin	-	+	+	-	-
laminin β1	+	-	-	+	+
laminin α1	+	-	-	+	+
laminin γ1	+	-	-	+	+
nidogen	+	+	+	+	+
ECM receptors					
α1 dystroglycan	?	-	-	+	+
α1 integrin	-	+	?	+	+
α2 integrin	+	-	-	-	+
α3 integrin	-	-	-	-	+
α6 integrin	+	-	-	+	+
α8 integrin	-	-	+	+	-
β1 integrin	+	+	+	+	+
β4 integrin	+	-	-	-	-
Proteoglycans					
syndecan 1	+	+	+	+	+
syndecan 2	-	+	+	-	-

Table 1d. Cytoskeleton and cell adhesion molecules.

GENE	ureter bud	uniduced MM	stem cells	renal vesicle	glomerulus
Cytoskeleton					
vimentin	-	+	+	+	+
cytokeratins	+	-	-	+	+
Cell adhesion molecules					
N-CAM	?	+	+	+	-
E-cadherin	+	-	-	+	+
K-cadherin	-	?	-	+	+
P-cadherin	?	?	?	?	+

2. GLOMERULAR FILTRATION

2.1. Filtration function

The two kidneys are a pair of bean-shaped organs in the retroperitoneal space. The kidneys have multiple central functions including filtration of metabolic waste products from blood and regulation of the body fluid and electrolyte balance and blood pressure. The filtration of fluid through the glomerular capillary begins in the human fetus between the 9^{th} and 12^{th} weeks of intrauterine life (Spitzer 1985). In a healthy human adult approximately 180 liters of glomerular ultrafiltrate resulting in 1.0-1.5 liters of urine is formed each day (Kriz & Kaissling 1985, Tisher & Madsen 1991). Mechanisms regulating the plasma ultrafiltration in glomerulus have been an object of great interest and research work during the last decades. The studies have shown that the transcapillary passage of plasma water and proteins is regulated by a number of physical factors (Kanwar 1984). These include glomerular plasma flow rate, the hydrostatic and oncotic pressure in the capillaries, the size, charge and configuration of molecules being filtered, the biochemical and biophysical properties of the glomerular capillary wall, and the hemodynamics within the glomerulus. All of these factors are important in maintaining a delicate balance and glomerular homeostasis and preventing the leakage of plasma proteins into the urinary space.

The primary functional unit of the kidney is the nephron, which consists of the glomerulus and its tubular system. An adult human kidney contains approximately one million nephrons. Each nephron microvascular unit consists of an afferent glomerular arteriole, a glomerular capillary tuft, an efferent glomerular arteriole, and a peritubular capillary bed (Tisher & Madsen 1991, Abrahamson et al. 1998). The glomerulus is composed of 5-7 capillary branches originating from the afferent artery and comprising lobule-like structures in which there are numerous anastomoses (Bohle et al. 1998). The tuft of capillaries is surrounded by the parietal epithelial cells forming Bowman´s capsule. The glomerular ultrafiltrate flows from the glomerular capillaries into the Bowman's space, further to the tubular lumen and is then concentrated by reabsorption by the tubular endothelium.

2.2. Structure of the filtration unit

Mature human glomerulus contains three structural entities: the fenestrated capillary endothelial cells, podocyte (visceral epithelial cell) lining between the glomerular space and capillaries, and mesangial cells in the middle of the capillary lobule (see *Figure 2*). A layered GBM is situated between podocytes and endothelial cells, and the mesangial matrix surrounds the mesangial cells. During glomerular filtration, plasma fluid traverses several cellular and extracellular layers that make up the complex structure of the ultrafiltration unit. From inside outward, it consists of endothelial fenestrae, GBM, and epithelial foot processes with intervening slit diaphragms (*Figure 3*). The integrity of each of these structural elements in essential for the maintenance of normal ultrafiltration (Kanwar 1984).

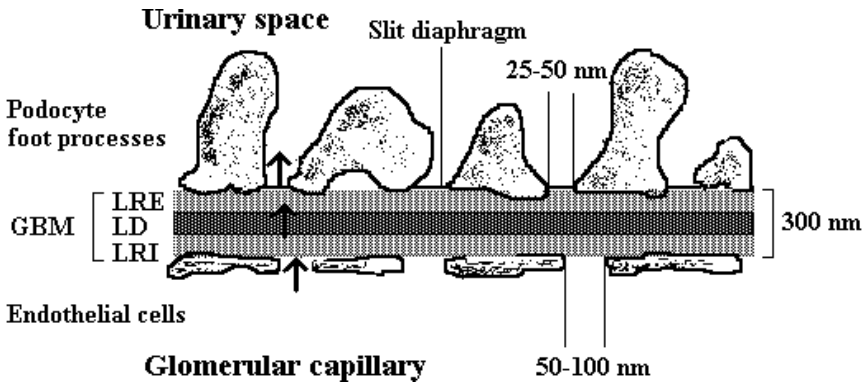

Figure 3. Structure of the glomerular filtration barrier. Glomerular filtration barrier between blood and the urinary space, forms the sieve through which the primary urine is formed (arrows). The barrier has three elements: the fenestrated endothelial cells, the three-layered basement membrane (GBM), and the podocytes. The slit diaphragm is a highly specialized structure between adjacent podocyte foot processes, providing the final barrier to the loss of protein. LRE = lamina rara externa, LD = lamina densa, LRI = lamina rara interna. For details, see text.

2.2.1. Endothelial cells

The glomerular capillary endothelial cells line the GBM on the inner aspect of the capillary wall. They have highly attenuated and fenestrated cytoplasm and a negatively charged plasmalemma rich in sialic acid and also HSPG (Kanwar 1984, Kanwar et al. 1991). The endothelial fenestrae are circular openings with a width of up to 100 nm in cross-section. They are quite large and devoid of diaphragms or limiting membranes and allow thus the passage of large volumes of fluid and solute. The endothelial cells express vasoactive peptides and their receptors and many contractive and relaxant growth factors regulating the hemodynamics of glomerular capillaries (Savage 1994).

2.2.2. Glomerular basement membrane

The GBM is an extracellular matrix interposed between the attenuated endothelium and the epithelial foot processes. Studies have shown that GBM is the main filtration barrier of the glomerulus which restricts the passage of macromolecules according to their size, charge and shape (Bohrer et al. 1978, Brenner et al. 1978, Kanwar 1984, Remuzzi & Remuzzi 1994). In man, it is approximately 300 nm wide and consists of a central electron-dense layer, the lamina densa, which is flanked on either side by electron lucent regions referred to as lamina rara interna and externa (Kanwar 1984, Kanwar et al. 1991). The GBM contains mainly type IV collagen, laminin, nidogen/entactin and HSPG. The specialized isoforms of laminin and type IV collagen in GBM are critical for its structure and filtration function (Miner 1999). The compact networks of laminin and type IV collagen cross-linked and stabilized by nidogen, probably play a vital role in the size selective sieving properties of the ultrafiltration unit.

The GBM is a charge selective barrier which filters cationic molecules more easily than anionic ones. GBM is rich in HSPG, which are highly negatively charged and by electrostatic repulsion,

negatively charged plasma proteins are restricted from penetrating and passing through the glomerular capillary wall. The effect of electrical charge is important in small polyanionic molecules with an Einstein-Stokes radius less than 4.2 nm but more than 2.0 nm (usually 70-150 kDa). Molecules of the size of 150 kDa and larger are retained in the circulation on the basis of their size (Bohrer et al. 1978, Brenner et al. 1978, Kanwar 1984, Remuzzi & Remuzzi 1994).

2.2.3. Epithelial cells and slit diaphragm

The central body of the epithelial cell lies freely in the urinary space. Cytoplasmic extensions arising from these cells anchor to the outside of the GBM as a series of interdigitating foot processes. The podocyte is richly coated with podocalyxin, a negatively charged protein, as well as other charged molecules thought to provide an additional electrostatic barrier to the movement of proteins across the capillary wall (Kerjaschki et al. 1984). The slit diaphragm, which is a modified tight junction between foot processes of adjacent podocytes, provides the final size selective barrier to the loss of protein. The slit diaphragm has a ladder or zipper like structure with pores of 40x140 Å separated by a central filament and cross bridges *(Figure 4)* (Rodewald & Karnovsky 1974, Abrass 1997). According to the hypothesis of Rodewald and Karnovsky (1974), the pore size is slightly smaller than the size of albumin. The molecular nature of the slit diaphragm has hitherto been a mystery, but during last two years, significant advancement has been made in understanding of its structure and function in regulating glomerular permeability (see chapter "5.7. Identification and characterization of the CNF gene") (Tryggvason 1999, Kerjaschki 2001, Khoshnoodi & Tryggvason 2001).

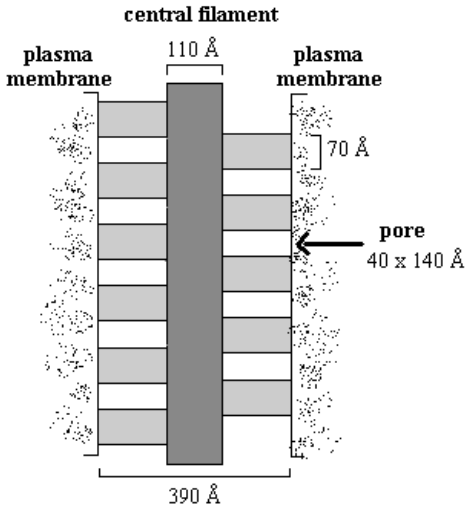

Figure 4. Slit diaphragm. Schematic model of the podocyte slit diaphragm according to Rodewald & Karnovsky (1974). Cross bridges extending from the adjacent foot processes connect to the central filament, leaving filtration pores between them. The average cross section dimensions are indicated.

2.2.4. Mesangial cells

The mesangial cells are centrally located in the intercapillary region of the glomerulus providing structural support for the capillary loops. They are irregular in shape and have number of primary processes extending from the body. Mesangial cells contain contractile elements by which they modulate glomerular filtration and have capacity to phagocytose, synthesise and degrade matrix components and take part in the repair of glomerular injury (Kriz et al. 1990, Latta 1992). Mesangial cells are embedded in the mesangial matrix, which is a highly fibrillar, dense network of microfibrils. The mesangial matrix contains collagens, fibronectin, laminin, nidogen and chondroitin sulfate proteoglycans, and it attaches the mesangial cell to the folding perimesangial GBM and counteracts the distending forces during increased glomerular pressure. It is the failure of the mechanical integrity of this system that leads to the characterized lesion in tuft architecture, such as local expansions of the tuft, seen in human glomerulopathies (Kriz et al. 1990 and 1998).

3. PROTEINURIA

3.1. Pathogenesis of proteinuria

Protein excretion in normal adult urine is usually below 150 mg/day, but even as high as 300 mg/day is considered to be within the normal range. When the integrity of the kidney filtration machinery is disrupted, increased amounts of proteins pass into the urinary space leading to proteinuria. In proteinuria, urinary proteins are comprised of mostly albumin, plasma immunoglobulins, other plasma proteins and different kinds of tissue proteins (Dennis and Robinson 1985). On the basis of its pathogenesis, proteinuria is divided into five classes: glomerular proteinuria, tubular proteinuria, overflow proteinuria, secretory proteinuria and histuria. Of these five types, glomerular proteinuria is the commonest (Dennis & Robinsson 1985); it is an early sign of many renal disorders, and it may in turn promote the progression of kidney diseases. Glomerular proteinuria can present as selective or non-selective (Dennis & Robinsson 1985). The dominant urinary protein is albumin. The dysfunction of tubular reabsorption causes tubular proteinuria. The main markers of tubular proteinuria are β2-microglobulin and globulins (Maack et al. 1985). If the levels of plasma proteins are increased, they can be filtered in excess of the reabsorption capacity of the tubules and then be present in the urine; this is called overflow proteinuria. Proteinuria is referred to as secretory proteinuria or histuria when the urinary proteins originate from surrounding tissues or other organs via excretion and secretion (Dennis & Robinsson 1985).

3.2. Glomerular changes in proteinuria

The histological findings in clinical entities and experimental models of proteinuria include alterations in both the cellular and extracellular components of the glomerular capillary. The same basic considerations apply regardless of the nature of the initiating injury and explain the remarkable uniformity of the lesion seen in several types of progressive injury of the glomerular tuft (Kriz et al. 1998). The available data regarding the mechanisms by which proteinuria is produced are mostly consistent with the idea that GBM, podocytes and their slit-junctions, and the extracellular glycoproteins are sequential barriers to the filtration of protein, and that each of these barriers are essential for normal glomerular function. The common morphologic findings in proteinuric conditions are discussed below and summarized in *Table 2*.

Altered **podocyte** morphology is universal and includes retraction and fusion of foot processes, decrease in the total lenght of slit-pore junction, displacement of the junction away from the GBM, and vacuolization in podocyte cytoplasm, evidence of podocyte activation. Regional denudation of the GBM may occur (Savin 1993). Some investigators have presented evidence for podocyte loss or podocyte ”insufficiency” (Fries et al. 1989, Rennke 1994, Pagtalunan et al. 1997). Due to its inability to replicate effectively postnatally and its unique susceptibility to specific injury, the podocyte is maybe the most vulnerable component of the glomerular tuft and in many cases, it is injury to the podocyte that initiates the definitive pathologic sequence of glomerulosclerosis and eventual glomerular tuft destruction (Kriz et al. 1998).

The **GBM** may appear normal or may be thickened with altered distribution or density of anionic charges due to abnormal regulation of synthesis or degradation. In some cases, serum proteins, antibodies to glomerular components or to other substances, or complement may present within the GBM (Savin 1993). Abnormalities of the **mesangium** may include increased cellularity, protein or antibody deposition, and increased matrix. Mesangial or interstitial cell proliferation with subsequent matrix deposition, leading to glomerular capillary occlusion and to interstitial expansion and tubular atrophy, have been invoked as central mechanisms in glomerular injury (Kashgarian & Sterzel 1992, Couser & Johnson 1994).

Table 2. Glomerular findings in proteinuria (Adapted from Savin 1993)

Podocyte alterations:
Foot process retraction and fusion
Slit diaphragm changes
Cytoplasmic vacuolization
Cell activation: increased organelles and expanded surface area
Podocyte loss (and local denudation of GBM)
Basement membrane alterations:
Charge density decrease
Matrix composition changes
Immune complex and complement deposition
Mesangial alterations:
Mesangial hypercellularity
Mesangial matrix expansion
Abnormal collagen composition
Immune complex and complement deposition

3.3. Mechanisms of glomerular proteinuria

As would be expected from the variation in morphological expression of human glomerular injuries, the underlying mechanisms are also very variable. The ones most often presented are those concerning immunological mechanisms, decreased glomerular charge, and detached glomerular epithelial cells (Kanwar 1984, Savin 1993). The initial events, such as charge neutralization, deposition of antibody-antigen complex, and enzymatic or nonenzymatic biochemical changes may affect primarily the extracellular components (Bruijn et al. 1997, Müller & Brändli 1999). Proteinuria may be initiated by glomerular reactions to growth factors, cytokines, circulating permeability factors, or antibodies to cell membrane antigens, which may lead to enhanced

synthesis or degradation of extracellular matrix or to proliferation of glomerular cells (Savin 1993, Couser 1998, Gibson & More 1998). Interactions between abnormal matrix and podocyte may affect maintenance of the intercellular junctions and matrix homeostasis. If hemodynamic parameters are also altered by matrix changes, there may be direct effects on the podocyte resulting from tension or stress. In other instances, exogenous toxins, inflammatory mediators, or viruses may initiate podocyte injury, altering normal morphology and intercellular junctions (Savin 1993). The importance of alteration in basement membrane, of cellular injury, and of cell activation in response to mediators undoubtly varies in different clinical syndromes and experimental models. Some of the basic theories suggested for the pathogenesis of proteinuria are presented here in more detail.

3.3.1. Charge neutralization

The importance of charged molecules in the glomerular capillary wall for the selective glomerular permeability has received a vast amount of research both in experimental animal models of glomerular disease and human renal diseases. Key studies include the demonstration that the fractional clearance of similar sized dextrans varied dependent upon their charge (Bennett et al. 1976, Chang et al. 1976, Brenner et al. 1977). Negatively charged structures have been found on the surface of podocytes and endothelial cells and throughout the GBM. These sites are provided mostly by HSPG as demonstrated by enzyme incubation and immunoelectron microscopy experiments. Removal of HSPG by enzyme digestion increased the leakage of ferritin and albumin through the GBM and suggests that a decrease in anionic sites could play a role in the pathogenesis of proteinuric states (Kanwar et al. 1980, Rosenzweig & Kanwar 1982). Charge neutralization by polycations, such as protamine sulphate (Kelley & Cavallo 1978), hexadimethrine (Hunsicker et al. 1981) and polyethyleneimine (Barnes et al. 1984), resulted in fusion of podocyte foot processes similar to the electron microscopic appearance in nephrotic syndrome and increased permeability to immunoglobulins and albumin due to alterations in the structural integrity of the capillary wall in charge selective barrier (Goode et al. 1996).

In human disease, the importance of altered basement membrane charges is supported by the finding of decreased sialic acid content and anionic binding sites in many studies (Hirsch et al. 1981, Levin et al. 1985, Quatacker et al. 1987), but also opposite findings exist (Kanwar & Jakubowski 1984, Furness et al. 1986). The glomerular size and charge selectivity in normal and nephrotic humans have been defined by fractional sieving of charged and neutral dextrans (Winetz et al. 1981, Deen et al. 1985). Abnormal permeation of serum proteins has been demonstrated by the presence of protein A-immunogold -stained albumin throughout the GBM in biopsies of humans with proteinuria whereas albumin is confined to the subendothelial space in glomeruli from nonproteinuric patients (Russo & Bendayn 1990).

3.3.2. Immunological mechanisms

It is now becoming increasingly apparent that many immunologically mediated glomerular diseases in man are autoimmune diseases. These lesions result from the glomerular binding of antibodies directed specifically against normal glomerular structures or planted antigens at different locations. Many types of immunological glomerular diseases are inflammatory in nature with glomerular hypercellularity due to infiltration by circulating inflammatory cells and proliferation of intrinsic glomerular cells, particularly mesangial cells. In other types, notably membranous glomerulonephritis, the morphology has a non-inflammatory pattern, with minimal or absent hypercellularity (Couser 1993).

Inflammatory mechanisms

Neutrophils are found infiltrating glomeruli in variable numbers in many types of glomerulonephritis, in response to immune complex deposition and complement activation. Their role in mediating subsequent glomerular injury is established through the release of oxidants and proteases (Johnson et al. 1994). Toxic oxygen metabolites such as H_2O_2 and myeloperoxidase interact to produce substances capable of directly damaging the GBM. Macrophages also localize in inflammatory glomerular lesions (Main et al. 1992). They are attracted by chemotactic factors produced by mesangial cells. Like neutrophils they cause glomerular injury through the release of oxidants (e.g. nitric oxide) and proteases (Main et al. 1992). Through the release of cytokines such as interleukin-1 (IL-1) and TGF-β, macrophages may also stimulate mesangial cells to proliferate and synthesize extracellular matrix proteins. As activated, mesangial cells release many inflammatory mediators, such as platelet-derived growth factor (PDGF) and TGF-β. PDGF may stimulate mesangial proliferation apparently by an autocrine mechanism. In response to TGF-β, mesangial cells increase their production of extracellular matrix components contributing matrix expansion, glomerulosclerosis and irreversible damage (Yoshika et al. 1993, Gibson & More 1998). This characteristic response has been well documented in IgA nephropathy, a disease which primarily affects the mesangium (Gibson & More 1998).

Non-inflammatory mechanisms

In membranous glomerulonephritis (membranous nephropathy), the glomerular lesion is non-inflammatory in pattern. However, there are extensive subepithelial deposits of antibody and complement components including C3 and C5b-9 (Couser 1998). Many aspects of the immunopathogenesis of membranous glomerulonephritis are now understood based on studies of the Heymann nephritis models in rats which closely simulate the human lesion. In Heymann nephritis complement fixing antibodies bind to antigens on the podocyte cell membrane. The best characterized of these antigens is gp330/megalin, which is localized in clathrin-coated pits (Farquhar et al. 1995). Complement activation results in production of C5b-9 membrane attack complex. The resulting podocyte damage causes oxidant production which in turn damages the GBM and the consequent altered permeability leads to the nephrotic syndrome in rats. The nature of the circulating antibody and the pathogenic epitopes of human idiopathic membranous glomerulonephritis remain unknown, but the investigations have revealed that the defect in membranous glomerulonephritis results mainly from a loss of size selectivity of the filtration barrier (Couser 1998).

Antibodies to glomerular antigens

Studies of the effects of the antibodies on the filtration barrier give clues to alterations in cell membrane interactions in for example minimal change nephropathy (MCN) and focal segmental glomerulosclerosis (FSGS). Antibodies to GBM components may act by disrupting the organization of the matrix, by altering the fixed charges of the matrix, or by interacting with podocytes to produce secondary changes in metabolic activities or interactions with the matrix (Savin 1993). However, the majority of the injury appears to depend on the activation of complement and on inflammation, rather than on direct effects of interaction between matrix, antibodies, and cells. Anti-GBM antibody is primarily directed against an antigen on type IV **collagen** of the GBM (Gunwar et al. 1991). Injection of anti-GBM antibody results in acute inflammatory injury produced by complement, platelets, neutrophils and monocytes. Reaction to inflammation may lead to loss of negative charge (Kreisberg et al. 1979, Salant 1987). Antibodies to **heparan sulfate** cause milder and variable glomerular responses. Rabbit anti-heparan sulfate antibodies are only weakly

nephritogenic when administered to rats resulting in antibody binding, complement fixation, monocytic infiltration, endothelial cell injury and basement membrane thickening, but not proteinuria (Makino et al. 1986, Miettinen et al. 1986). Injection of monoclonal antibody against heparan sulfate induced transient, dose-dependent, selective proteinuria immediately after injection in rats (van den Born et al. 1992). The investigators surmised that this was secondary to neutralization of heparan sulphate-associated anionic sites in GBM. Antibodies to **laminin** formed linear deposits along the GBM, but did not induce either inflammation or proteinuria (Feintzeig et al. 1986).

Antibodies to components of the **podocyte** membrane may also result in the induction of proteinuria. In Heymann nephritis, antibody binding to the podocyte membrane induces complement activation and proteinuria (see *"Non-inflammatory mechanisms"*). A monoclonal antibody to a sialoglycoprotein (SGP115/107) of podocytes and proximal tubules induced foot process obliteration, vacuole formation, microvillus transformation and focal retraction from underlying basement membrane (Mendrick & Rennke 1988a and 1988b). Another monoclonal antibody (5-1-6) which binds to slit diaphragms causes massive proteinuria. The investigators speculated that the antigen to which the antibody binds may regulate the permeability of the capillary wall, and that its decrease may lead to proteinuria (Orikasa et al. 1988, Kawachi et al. 1992). Later they have shown that this nephritogenic antibody is directed against nephrin, the CNF gene product (Topham et al. 1999, see "5.7. Identification and characterization of the CNF gene"). A model for renal injury using **mesangial** specific anti-Thy-1 antibody has also been described. Administration of antibody leads to lysis of mesangial cells, subsequent regeneration of mesangium, and later mesangial sclerosis (Bagchus et al. 1986, Johnson et al. 1992).

3.3.3. Circulating factors

In the primary glomerulopathies associated with nephrotic syndrome and glomerular proteinuria without cellular infiltration or immune complex deposition, e.g. in MCN and FSGS, the glomerular damage may partially be mediated by circulating permeability factors (Savin 1993). In these diseases, the podocyte is morphologically damaged with swelling, alterations in charge density, extensive foot process effacement and sometimes detachment from the GBM; these changes are associated with changes in the functional integrity of the slit diaphragm. Possible injury mechanisms include stimulation of release of materials which directly damage underlying GBM such as oxidants and proteases, or interference with molecules such as intergrins which regulate cell-matrix interaction leading to cell detachment from GBM (Couser 1998, Gibson & More 1998). Putative circulating permeability factors are probably not immunoglobulins but are thought to be most likely T-lymphocyte derived lymphokines, circulating cationic proteins or other permeability factors such as vascular permeability factor (VPF), human focal sclerosis factor or vasoactive serum factor, which neutralize anionic sites in glomerular capillaries or otherwise increase glomerular permeability to proteins (Bakker & van Luijk 1989, Savin 1993).

Several lines of evidence implicate circulating factors in the pathogenesis of MCN and FSGS. The decrease in proteinuria following plasma exchange in FSGS has been used as evidence in favor of the presence of a permeability factor, and there are reports of transfer of proteinuric activity by injecting serum into experimental animals (Zimmerman 1984, Wilkinson et al. 1986) or in isolated glomeruli (Savin 1993). The finding of the specific serum factors can lead to therapeutic interventions such as removal of the causative factor by plasmapheresis (Koyama et al. 1991) or by plasma protein absorption (Ingulli & Tejani 1991). The proteinuria in MCN is thought to result

mainly from a loss of charge selectivity of the glomerular filtration barrier. Much has been learned recently regarding the molecular mechanisms which regulate the adherence of glomerular cells to GBM, and disorders of these processes may also be important in the pathogenesis of MCN (Cosio & Orosz 1993).

3.3.4. Toxic injury

Administration of puromycin aminonucleoside (PAN) or doxorubicin has been used to induce injury to podocytes (Caulfield et al. 1976, Messina et al. 1987, Whiteside et al. 1989). These treatments resulted in proteinuria, renal insuffuciency and eventual mesangial sclerosis. Structural changes in the podocytes and their slit-junctions occur and include formation of occluding junctions and epithelial cell detachment. Micropuncture studies have defined both size- and charge-selective abnormalities, as well as decrease in hydraulic permeability (Bohrer et al. 1977). The primary effects of these agents appear to be on the podocyte, as suggested by the finding of direct toxicity to these cells in culture (Fishman & Karnovsky 1985). Podocyte toxicity may result in alterations in the composition of the charge barrier of the glomerular capillary. Mahan et al. (1986) showed that a decrease in anionic sites in the GBM preceded foot process widening or proteinuria in PAN nephritis. Some alterations in metabolism of matrix have been shown (Abrahamson et al. 1983, Nakamura et al. 1991), but interestingly, no decrease in heparan sulphate could be detected (Lelongt et al. 1987).

4. NEPHROTIC SYNDROME

4.1. Manifestation of nephrotic syndrome

Reduced glomerular permselectivity leads to proteinuria and ultimately to nephrotic syndrome (NS). Clinically NS is characterized by the presence of proteinuria, lipiduria, hypoalbuminemia, hypercholesterolemia, and variable tendency for edema (Abrass 1997, Eddy & Schnaper 1998). Classically, NS is thought to be initiated when integrity of the glomerular capillary wall is disrupted and increased amounts of albumin and other plasma proteins pass into the urinary space. Primarily, intermediate size (60-200 kDa) plasma proteins are lost, which leads to a marked change in plasma protein composition. Plasma oncotic pressure falls and plasma viscosity rises because of the relative increase in higher molecular weight proteins. The changes in plasma protein composition and oncotic pressure determine most of the secondary consequences of NS (Abrass 1997). In the milder forms of NS, plasma albumin levels are reasonably preserved (>25 g/l) and the plasma volume is expanded. Severe NS is characterized by marked hypoalbuminemia (< 20 g/l and can fall below 10 g/l), severe edema and occasionally hypovolemia that is reflected by normal or low blood pressure (Abrass 1997).

4.2. Classification of nephrotic syndromes

NS is not a disease per se, but rather a manifestation of various diseases that cause proteinuria. It can be cathegorized as **primary** and **secondary** NS; the former is due to primary or idiopathic glomerular disease, while the latter is associated with specific etiologic events or a complication of other diseases, certain **systemic** diseases or a part of general malformation syndromes (**syndromic** NS). On the basis of their etiology glomerular injurys can be divided into **acquired** diseases caused by e.g. metabolic or environmental (toxins, infection) factors and **genetic** diseases which include

hereditary and familial kidney diseases. It appears, that these categories may have significant overlap with each other and in some cases, the cause of the disease is less apparent. Furthermore, NS with onset in the first year of life is conventionally divided into **congenital** and **infantile** types, the arbitrary age limit being 3 months of age (Rapola 1987). This division is, however, of quite limited diagnostic value since the onset of clinical symptoms in congenital NS can vary significantly between different forms of the disease. The use of renal biopsies has been accompanied by considerable improvement in the classification of glomerular disease, but still the clinical and histological tools available in diagnosis sometimes lack accuracy (Peten & Striker 1994). Recently, the classification has been aided by applying molecular genetics to identify genes involved in the pathogenesis of NS. Some examples of primary and secondary glomerular kidney diseases are introduced here, mostly in respect of their pathology and basic defects (if known).

4.3. Idiopathic glomerular diseases

According to histologic lesions, primary NS can be categorized as several classes including minimal change nephropathy, focal segmental glomerulosclerosis, mesangial proliferative glomerulonephritis, IgA nephropathy, membranous glomerulonephritis and membranoproliferative glomerulonephritis (Abrass 1997).

Minimal change nephropathy

Minimal change nephropathy (MCN) is the most frequent cause of NS in children, with an incidence peak from 3 to 5 years accounting for almost 80 % of pediatric NS cases (Savin 1993, Eddy & Schnaper 1998). Patients classically present with edema, selective proteinuria and normal glomerular filtration rate (Savin 1993). The disease is also called minimal change lesion or minimal change disease, to stress the relative paucity of glomerular findings under light microscopy. The glomeruli appear largely normal, with mild increase of cellularity in the mesangial area and enlargement of epithelial cells. Proximal tubules may contain fine lipid droplets (Glassock et al. 1991a). The glomeruli lack of deposits of immunoglobulins and complement. In electron microscopy, effacement and retraction of the epithelial podocytes has been shown. The obliteration of the slit pore membrane complex is also noted in most glomeruli and glomerular capillaries (Glassock et al. 1991a). Several factors have been considered to explain the dysfunction of the glomerular permselectivity in MCN. Proposed etiologic factors include (1) charge neutralization of GBM-related molecules by circulating cationic proteins, (2) glomerular permeability factors, (3) lymphokines, (4) allergens, and (5) overproduction of oxygen radicals (Kanwar & Farquhar 1979, Savin 1993, Couser 1998). These findings have been mimiced in a rat **PAN model**, which is widely utilized to explore the pathogenesis of MCN.

Focal segmental glomerulosclerosis

Focal segmental glomerulosclerosis (FSGS), like MCN, presents with insidious onset of NS. It occurs both in children and adults, accounting for 7-15 % and 15-20 % of the primary NS, respectively. Pathological findings on light microscopy show focal and segmental sclerotic lesions affecting a variable minority of the glomeruli, usually those in the deeper, juxtamedullary cortex due to the higher perfusion pressure (Glassock et al. 1991a). Electron microscopy examination presents, in most cases, the characteristics of the effacement of podocytes and the detachment of foot processes from the GBM, especially when heavy proteinuria is observed (Glassock et al. 1991a). In addition, there exists IgM and C3 deposition in the glomeruli. Patients with FSGS have variable responses to corticosteroid therapy. FSGS may recur after renal transplantation, but treatment with plasma exchange has resulted in a decrease or disapperance of proteinuria. This has

strongly suggested that a circulating substance may be responsible for glomerular injury in some patients with this entity (Savin 1993). In some certain types of FSGS, genetic factors in the pathogenesis of this disorder has been noticed (Eddy & Schnaper 1998, see chapter "4.4. Genetic defects").

Mesangial proliferative glomerulonephritis

According to the components of deposits in the mesangium, mesangial proliferative glomerulonephritis can be divided to non-IgA glomerulonephritis and IgA nephropathy (Glassock et al. 1991a). **Non-IgA glomerulonephritis** is a relatively uncommon type of NS. It is characterized by hematuria and nonselective proteinuria. Typical findings in light microscopy are accumulation of mesangial matrix and increased cellularity of the mesangium (Glassock et al. 1991a). In addition, sedimentation of IgM and C3 are frequently found in the mesangium. The pathogenesis of non-IgA glomerulonephritis is not fully known, but based on the observation of IgM and C3 deposits as well as circulating immune complexes, it appears to be an immune complex disease. An **anti-Thy-1.1** (anti thymocyte serum, ATS) rat **model** shows similar mesangial changes in the glomeruli (Jefferson & Johnson 1999). In this model, the injection of antibody to the Thy-1.1 antigen on the mesangial cell membrane induces an initial phase of mesangiolysis followed by platelet infiltrate and mesangial cell proliferation (Couser & Johnson 1994). A variety of factors, such as IL-1, TGF-β, and PDGF, might participate in the mediation of pathologic mesangial cell proliferation (Jefferson & Johnson 1999).

IgA nephropathy is definied as prominent and diffuse granular deposits of IgA in the glomerular mesangium (Nolin & Courteau 1999). In many cases, deposits of IgG are concomitantly seen in the mesangium. Other changes in glomeruli may be variable, but mesangial hypercellularity and matrix accumulation are typical (Glassock et al. 1991a). Several lines of evidence indicate that IgA nephropathy is some kind of immune complex glomerulonephritis. The effects of reactive oxygen species, IL-6, viral infection and genetic factors have also been reported (Glassock et al. 1991a).

Membranous glomerulonephritis

Membranous glomerulonephritis (MGN) is the leading cause of primary NS in adults (Shankland 2000). MGN refers to a specific entity of NS associated with diffuse deposits of immune complexes under the epithelium. The typical feature is diffuse thickening of the GBM and increase in mesangial matrix, usually without any significant proliferation of endothelial, mesangial, or epithelial cells (Ambrus & Sridhar 1997). The findings on electron microscope reveal subepithelial and in advanced cases intramembranous electron-dense deposits (Ambrus & Sridhar 1997). Immunofluorescence reveals, in almost all cases, that IgG and C3 are present in a uniform granular distribution in capillary loops (Glassock et al. 1991a). The pathogenesis of human MGN is not clearly known. A rat model of **Heymann nephritis** has been widely used for the exploration of the mechanism, owing to its similarity with the features of human MGN (Miettinen et al. 1980). Several pathogenetic possibilities have been proposed, such as circulating or in situ formed immune complexes (Shankland 2000).

Membranoproliferative glomerulonephritis

Membranoproliferative glomerulonephritis (MPGN) is mainly found in children and adolescents rather than in the elderly. The clinical features are variable, but proteinuria often with NS, is usually present (Ambrus & Sridhar 1997). Based on ultrastructural and immunofluorescence patterns, idiopathic MPGN can be further categorized as three subtypes (Glassock et al. 1991a). In type I, subendothelial and mesangial electron-dense deposits are noted. Immune complexes can be IgG,

C3, C1q or C4. This type accounts for 65-75 % of all MPGN patients. In type II, electron-dense deposits can be found along the GBM ("dense deposit disease"). C3 is predominant in immune complex deposits, IgG is not usually found. This type accounts for 20-30 % of all cases. Type III is very rare; the electron dense deposits are mainly found under endothelium, epithelium, and in the mesangium. C3 is predominant, accompanied by IgG. MPGN is thought to be an immune complex disease and also genetically related (Ambrus & Sridhar 1997). Lack of successful animal models has restricted the delineation of the mechanisms. Heterogenity in pathogenesis is suggested due to diversity characteristics in pathology.

4.4. Genetic defects

The pathomechanisms of kidney disease manifested by proteinuria and NS have been a mystery for decades. The underlying causes of these diseases often lie in changes of the glomerular filtration barrier, but the molecular properties of these alterations have been poorly understood. Recent molecular genetic research has, however, identified the causes of many rare familial kidney diseases, such as steroid-resistant NS, FSGS, and congenital nephrotic syndrome of the Finnish type. The identification of podocyte-specific proteins affected in these diseases, has increased our knowledge of the kidney ultrafilter, especially of podocytes and the slit diaphragm. Similarly, the studies on Alport syndrome have improved our knowledge of the glomerular basement membrane. In addition to these known genetic defects, there are a number of glomerular diseases which are thought to have a genetic, yet unidentified, component based on family clustering. Diseases in this category include IgA nephropathy, diabetic nephropathy, and human immunodeficiency virus (HIV) nephropathy (Striker et al. 1994).

Steroid-resistant nephrotic syndrome
Steroid-resistant NS (SRN or NPHS2) is an inherited autosomal recessive disease, which is mostly observed in children between three months and five years of age. The patients do not respond to steroid treatment and, after onset of proteinuria, progression towards end-stage renal disease is rapid. The causative gene NPHS2 was mapped to chromosome 1q25-q32 by Fuchshuber et al. (1995) and has also been isolated recently (Boute et al. 2000). The gene product, named podocin, is a putative 42 kDa integral membrane protein with a short extracellular domain, followed by a transmembrane spanning region and a long carboxy-terminal cytoplasmic end. Expression of the gene is restricted to glomerular podocytes, and it has been speculated that podocin may be involved in organizing the cytoskeleton of podocyte foot processes at the cytoplasmic site of the slit diaphragm (Khoshnoodi & Tryggvason 2001).

Focal segmental glomeruloclerosis
Another genetic cause of NS has been clarified recently by positional cloning of a gene from patients with adult onset of FSGS. This disease represents a heterogenous group of autosomal dominant kidney disorders that are manifested by proteinuria and slow progression of segmental glomerular sclerosis, and finally end-stage renal disease in adulthood (Khoshnoodi & Tryggvason 2001). Two genes have been located to chromosomes 19q13 (FSGS1, Mathis et al. 1998) and 11q21-22 (FSGS2, Winn et al. 1999). The gene for FSGS1 has been cloned recently by Kaplan et al. (2000) and shown to encode α-actinin-4; the gene for FSGS2 has not been identified yet. Actinins are proteins that crosslink actin filaments in cytoskeleton, and their expression patterns vary throughout the body (Honda et al. 1998). Four α-actinin coding ACTN genes are known in man (ACTN1-ACTN4). ACTN4 is widely expressed in different tissues, and it has been shown to be highly expressed in glomerular podocytes (Kaplan et al. 2000). Analysis of mutations in affected

individuals showed non-conservative amino acid substitutions in the actin-binding region of α-actinin-4. On the basis of co-sedimenation studies, the investigators (Kaplan et al. 2000) have proposed that the dominant mutations in the ACTN4 gene might lead to an increased affinity for actin filaments. This finding implicates the importance of proper organization of the cytoskeleton for normal function of podocyte foot processes (Khoshnoodi & Tryggvason 2001).

Denys-Drash syndrome

Denys-Drash syndrome (DDS) is characterized as a congenital or early onset NS associated with severe urogenital aberrations, pseudohermaphroditism and Wilms´ tumor (Pelletier et al. 1991). Wilms´ tumor, or nephroblastoma, is the most common pediatric kidney cancer resulting in the formation of tumors and developmental abnormalities (Mrowka & Schedl 2000). The characteristic glomerular lesion - diffuse mesangial sclerosis and nephropathy- is usually discovered at birth or within the first months of life. DDS arises from mutations in WT-1 gene, encoding a transcription factor known as the Wilms´ tumor suppressor (Pelletier et al. 1991). The gene, located at chromosome 11p13, has a critical role in kidney and gonad development, as demonstrated in WT-1 knockout mice lacking both kidneys and gonads (Pritchard-Jones et al. 1990). WT-1 is highly expressed during the embryonic development, but in mature kidney its expression persist only in podocytes and epithelial cells of the Bowman´s capsule (Kreidberg et al. 1993). The protein has transcriptional regulatory region and four zinc-finger DNA-binding domains. The mutations in WT-1 found in DDS patients seem to act as a dominant trait, and nearly all are heterozygous missense mutations affecting zinc-finger regions of the protein (Pelletier et al. 1991). The WT-1 gene product has a down-regulatory effect on large number of genes, such as transcription factors Pax-2, Pax-8, novH and the WT-1 gene itself, and some growth hormones and their receptors, such as IGF-2, IGF receptor, PDGF-A, TGF-β and epidermal growth factor receptor (Mrowka & Schedl 2000). The alterations in the binding capacity of the mutated protein and the loss of suppression may explain the glomerular lesions found in DDS patients.

Disorders of GBM

Advances in our knowledge of the biology of the GBM and the molecular genetics of specific genes, have contributed the understanding of glomerular diseases of GBM components. The biochemistry of the GBM involves a complex interaction of collagen IV with laminin and other crosslinking proteins and proteoglycans. The first defect described in molecular terms was **Alport syndrome**. Alport syndrome is a hereditary nephritis presenting as hematuria, proteinuria and end-stage renal disease (Barker et al. 1990). It shows wide genetic heterogeneity with X-linked, autosomal dominant and autosomal recessive forms (Goodyer & Kashtan 1998). It has been unequivocally identified as the phenotypic expression of mutations affecting type IV collagen; to date, variable mutations in genes COL4A5, COL4A6, COL4A3 or COL4A4 have been documented (Tryggvason et al. 1993, Goodyer & Kashtan 1998). During the past few years, several other matrix-associated glomerular diseases have been described, such as **collagen type III glomerulopathy**, **fibronectin glomerulopathy** and **lipoprotein glomerulopathy**. These are all slowly progressive familial diseases and usually present with proteinuria or NS (Abt & Cohen 1996, Gibson & More 1998).

Congenital nephrotic syndrome of the Finnish type

Congenital nephrotic syndromes (CNS) are defined as severe proteinuria leading to the clinical symptoms of NS already the first months of life (Holmberg et al, 1996). Congenital nephrotic syndrome of the Finnish type (CNF) is an inherited type of CNS with massive proteinuria; the

nature of CNF and the specific genetic defect are introduced thoroughly in the next chapter (Chapter 5).

4.5. Systemic or syndromic nephrotic syndromes

Systemic diseases that may lead to nephrotic proteinuria include diabetes mellitus, amyloidosis and systemic lupus erythematosis (Glassock et al. 1991b, Abrass 1997). Diabetes mellitus is the most prevalent single metabolic disease assosiated with progressive glomerulosclerosis, and **diabetic nephropathy** is the most common cause of nephrotic proteinuria in adults (Ritz & Stefanski 1996). In diabetes, the size selectivity of the GBM can become altered by disease-associated chemical modifications. Nonenzymatic glycation of the GBM may alter the pore size creating channels that allow proteins larger than albumin to reach the subepithelial space more easily. However, recent data have shown that the risk for renal disease in diabetics is familial indicating the existence of a genetic component too (Seaquist et al. 1989, Borch-Johnsen et al. 1992). NS may also be a part of general malformation syndromes, such as central nervous system malformations, Nail-Patella syndrome and DDS (Rapola 1987, for DDS see chapter "4.4. Genetic defects").

4.6. Acquired types of nephrotic syndrome

The most common types of acquired NS are those associated with bacterial or viral infections, drug reactions and toxins (Glassock et al. 1991b). For example, **human immunodeficiency virus nephropathy** typically causes nephrotic proteinuria and renal insufficiency, occasionally as the first clinical manifestations of the acquired immunodefiency syndrome. Infection-associated glomerulonephritis may be caused also by hepatitis B or C or malaria viruses and some bacteria (Eddy & Schnaper 1998, Orth & Ritz 1998). The patients with **Goodpasture syndrome**, an anti-GBM antibody disease, develop rapidly progressive nephropathy during adulthood, but presumably have normal kidneys prior to the onset of specific antibody response (Striker et al. 1994). The Goodpasture antigen has been identified as collagen IV, more specifically the NC1 domains of α3(IV) and α4(IV) chains (Hudson et al. 1993). It has been suggested that a primary event such as infection or toxin exposure triggers the unmasking of these epitopes, causing the initial production of anti-GBM antibodies (Hudson et al. 1993, Gibson & More 1998). A role for many environmental factors, such as heavy metal intoxication, in the initiation or progression of some glomerular diseases have also been implicated, but their nephrotoxicity in the general population is not yet clear (Striker et al. 1994).

5. CONGENITAL NEPHROTIC SYNDROME OF THE FINNISH TYPE (CNF)

5.1. Basics of the disease

CNS are kidney diseases which are manifested by the leakage of normal circulating proteins into the urine and which are present at or soon after birth (Rapola 1987). CNS are a heterogeneous group of diseases, and the molecular pathomechanisms of leaky glomerular filtration barrier in most CNS are still obscure. CNF was first described in 1956 by Hallman et al. (1956). CNF forms a distinct entity among CNS with its typical clinical picture and an autosomal recessive pattern of inheritance (Norio 1966, Hallman et al. 1973, Rapola 1987). It is much more common in Finland than anywhere else, but a few cases have been reported throughout the world (Rapola et al. 1992, Habib 1993, Savage et al. 1999, Aya et al. 2000). In recent years, 2-8 CNF children have been born in Finland each year.

The incidence has been reported to be 14.2 in 100 000 newborns during years 1975-1988, including births and aborted CNF pregnancies (Laakso et al. 1992).

5.2. Clinical features

The clinical picture of CNF is quite uniform. The disease is manifested already at the fetal stage as heavy proteinuria in utero, which can be diagnosed by elevated concentrations of alpha-fetoprotein in the amniotic fluid and maternal serum (Seppälä et al. 1976, Aula et al. 1978). The typical clinical signs are prematurity ranging from 35th to 38th gestation weeks, low birth weight, and large placenta equivalent to over 25 % of child´s birth weight (Kouvalainen et al. 1962, Huttunen 1976). Severe NS with massive urinary loss of proteins, serum hypoproteinemia, edema and secondary manifestations is usually detected immediately or shortly after birth (Huttunen 1976, Holmberg et al. 1995). CNF infants suffer from different kinds of infections, thromboembolism and seizure (Huttunen 1976, Laine et al. 1993). Developmental delay and bony deformities are common (Hallman et al. 1973).

5.3. Pathology of the kidney

The pathology of CNF kidneys has been characterized thoroughly, but the basic defect leading to proteinuria remained obscure until very recently. Extensive morphological, histological and biochemical studies on CNF kidneys have been performed by several investigators from the fetal period to infancy, but none of the findings seemed to be causative for the CNF. The weights of the kidneys of CNF children have been reported to be 2-3 times larger compared to normal kidneys (Huttunen et al. 1980). The number, density and size of kidney glomeruli are increased in infants with CNF (Tryggvason & Kouvalainen 1975, Tryggvason 1978), althought this was not evident in 16-to-22-week CNF fetuses (Autio-Harmainen & Rapola 1981). It has been suggested that in CNF the glomerular formation does not cease normally but continues beyond the 35th gestational week resulting in about 70 % higher number of glomeruli (Autio-Harmainen & Rapola 1981). Furthermore, glomerular maturation is suggested to be enhanced in CNF, because a proportional increase in mature glomeruli and decrease in glomerular visceral epithelial cells in fetal CNF kidneys could be detected by light microscopy (Huttunen et al. 1980, Autio-Harmainen & Rapola 1981).

Typical histological findings of CNF kidneys are the dilation of proximal tubular lumina and expansion of the mesangial area. The fetal tubular dilations are sparse and found mostly at the medullary portion of the cortex, but the dilations increase and extend during the patient´s postnatal life (Huttunen et al. 1980, Autio-Harmainen & Rapola 1981). The epithelium of the dilated tubules is first swollen and filled with eosinophilic colloid-like material, but later becomes flattened and atrophic (Huttunen et al. 1980, Rapola et al. 1984). Mesangial hyperplasia can be detected both in fetal and postnatal kidneys (Huttunen et al. 1980, Autio-Harmainen & Rapola 1981). In infants, progressive accumulation of mesangial matrix is seen; sclerosis and fibrosis of the glomeruli can be detected in patients approaching one year of age (Huttunen et al. 1980). The late changes include also interstitial fibrosis, lymphocytic inflammation (Hallman et al. 1973, Huttunen et al. 1980) and thickening of parietal Bowman’s capsule (Huttunen et al. 1980, Autio-Harmainen et al. 1985). Electron microscopy reveals the near total loss of podocyte foot processes in both fetal and infant CNF glomeruli (Autio-Harmainen 1981, Rapola et al. 1984 and 1992).

The molecular composition of the GBM in CNF has been characterized by immunofluoresence microscopy and biochemical analysis. The basic defect leading to proteinuria in CNF has most often

been suggested to lie in the structure of GBM. Several changes have been found in the amino acid composition of the GBM in CNF kidneys. A decrease in 3- and 4-hydroxyproline, hydroxylysine and glycine, amino acids characteristic for type IV collagen, has been reported by Tryggvason (1977). Similar changes are however seen in other kidney diseases as well and the immunohistochemical staining for laminin, type IV collagen and fibronectin have not revealed any changes in CNF compared to age matched controls (Rapola et al. 1984). HSPG is considered to be mainly responsible for the charge selectivity in the GBM, and a decrease in HSPG concentration has been thought to cause the increased GBM permeability (Vermylen et al. 1989). However, in CNF children, the total glycosaminoglycan content of the GBM, the number of anionic sites, and the urinary excretion of heparan sulphate were all found to be normal (van den Heuvel et al. 1992, Ljungberg 1994, Ljungberg et al. 1995).

5.4. Diagnosis

Traditionally, the prenatal diagnosis of CNF is based on high alpha-fetoprotein concentrations in the amniotic fluid or maternal serum in the second trimester of pregnancy (Seppälä et al. 1976, Aula et al. 1978, Holmberg et al. 1996). This is not specific for CNF, as elevated α-fetoprotein levels can also been found in other fetal disorders, mostly structural anomalies such as neural tube defects and abdominal wall defects. In addition, the α-fetoprotein screening method may lead to false-positive results and subsequent abortions of healthy fetuses (Männikkö et al. 1995). However, CNF is, especially in Finland, a likely cause for very high amniotic fluid α-fetoprotein in pregnancies where ultrasound shows no fetal abnormalities.

Clinically, the postnatal diagnosis of CNF, suspected prenatally on the basis of an increased α-fetoprotein level, can be made using the following criteria: (1) positive family history, (2) severe proteinuria (>20g/l when serum albumin has been corrected to >15 g/l) of intrauterine onset, (3) a large placenta, (4) exclusion of other types of CNS, and (5) normal glomerular filtration rate during the first six months of life (Holmberg et al. 1996). The diagnosis can be further strengthened by a typical renal histology after the first three to six months of life.

The localization of the CNF gene to chromosome 19q13.1 (Kestilä et al. 1994a, Männikkö et al. 1995) and the strong linkage equilibrium of the genetic markers in this region enabled prenatal diagnosis based on haplotype analysis (Männikkö et al. 1997). Haplotype analysis can be made as early as in the 12th to 13th gestational weeks and with up to 95% accuracy. The recent identification of the CNF gene and disease-causing mutations (Kestilä et al. 1998) has now made the exact DNA-based diagnosis and reliable carrier screening possible. Mutation analysis can be done by PCR-restriction fragment lenght polymorphism (RFLP) and by a dual-color oligonucleotide ligation assay (Romppanen & Mononen 2000).

5.5. Treatment and prognosis

Until the beginning of 1980´s, CNF children died, most of them as infants, but at present the prognosis is good. Treatment with aggressive protein supplementation, bilateral nephrectomy and dialysis, and subsequent renal transplantation have been successful and have made normal growth and development of CNF patients possible (Holmberg et al. 1991 and 1995). These treatments were started in Finland in 1985 (Holmberg et al. 1990), and no extrarenal manifestations have developed in patients during the over 10 years follow-up. Some complicated problems, such as lipid abnormalities (Antikainen et al. 1994), anemia (Siimes et al. 1992) and infections (Jalanko et al.

1989) have been, however met. Severe lipid abnormalities, such as hypertriglyceridaemia and abnormal cholesterol levels, indicate increased risk of arteriosclerosis in CNF. Renal transplantation substantially improves lipid abnormalities, but significant disturbances still persist (Antikainen et al. 1994). Furthermore, six patients during years 1987-1992, representing seven out of 29 transplantations performed, developed post-transplantation nephrosis (Laine et al. 1993). The reason for this recurrence has been unknown, but very recently, after identification of the CNF gene, Wang et al. (2001) and Patrakka et al. (2002) reported that recurrence of NS in CNF transplantation patients is associated with preceding high levels of specific antibodies to the CNF gene product, nephrin, possibly explaining the recurrence mechanism.

5.6. Candidate genes for CNF

CNF is thought to be a highly kidney specific disease, and it has been proposed that it is caused by a mutation in a gene coding for either a structural basement membrane component or a component otherwise crucial for the filtration process. Testing the hypotheses, Kestilä et al. (1994b) analyzed eight genes coding for basement membrane components using intragenic polymorphisms and linkage analysis. They excluded the genes for α1-4(IV)chains of type IV collagen, β1, γ1 and γ2 chains of laminin and HSPG as causative for CNF on the grounds of recombination between the CNF and intragenic RFLP markers. At least two unrelated transgenic mice models have been described for nephrotic syndrome. Weiher et al. (1990) described a recessive lethal mouse strain generated by inactivation of the Mpv17 gene coding for a ubiquitous peroxisomal protein. Affected Mpv17 mice developed NS with proteinuria, hypoalbuminemia, and hyperlipidemia, and died from renal failure. Kidney injury was found to be at the glomerular level. However, the human Mpv17 gene localizing to chromosome 2 p21-p23 has been excluded as a disease gene for CNF (Karasawa et al. 1993, Zwacka et al. 1994). Later, Binder et al. (1999) have shown that glomerular damage in this murine model is due to overproduction of oxygen radicals and accumulation of lipid peroxidation adducts causing podocyte foot process flattening and proteinuria. A report by Dressler et al. (1993) demonstrated that deregulation of Pax-2 transcription factor gene led to CNS in transgenic mice resembling that of human CNF. Pax-2 is normally expressed in induced kidney mesenchyme, in early epithelial structures derived from the mesenchyme and in ureter epithelium, and in Wilms´ tumor tissue. Overexpression of the Pax-2 in mice kidneys resulted in development of microcysts, loss of podocyte foot processes and proteinuria, and it was suggested that these mice represent an animal model for congenital nephrotic syndrome. The Pax-2 gene lies in the chromosomal region 10q22-q25 but this location has also been excluded as a mutated CNF locus (Kestilä et al. 1994c). Recently, NS has also been shown in knock-out mice lacking CD2AP, an actin-binding protein that is expressed in many cell types (Shih et al. 1999). CD2AP was first characterized as a cytoplasmic ligand of the CD2 receptor protein in T-cells and natural killer cells (Dustin et al. 1998). CD2AP-deficient mice had certain immune function compromises, but surprisingly they died from NS at the age of 6-7 weeks. Electron microscopy examination of kidneys showed a loss of podocyte foot process integrity accompanied by mesangial cell hyperplasia and extracellular matrix deposition (Shih et al. 1999). Interestingly, by co-precipitation studies, Shih et al. (1999) demonstrate a direct interaction between CD2AP and the CNF gene product (characterized below), and suggest their role in slit diaphragm function.

5.7. Identification and characterization of the CNF gene

Recently, Kestilä et al. (1998) reported a positional cloning of putative CNF gene (NPHS1) and characterization of its predicted protein product, which they termed **nephrin**. The NPHS1 gene is 26 kilobases in size and comprises 29 exons. It encodes a transcript of about 4.3 kb, which was shown to be strongly expressed in human embryonic and adult kidneys, but not in any other human tissues studied. In situ hybridization revealed intense expression signals in the periphery of mature and developing glomeruli, apparently in epithelial podocytes (Kestilä et al. 1998). Further histochemical studies (Holthöfer et al. 1999) with nephrin-specific antipeptide antibodies confirmed the exclusively glomerular localization of nephrin in the kidney, with a preferentially epithelial-like staining pattern. By immunoelectron microscopy Holthöfer et al. (1999), Ruotsalainen et al. (1999) and Holzman et al. (1999) demonstrated nephrin localization in the podocyte foot processes prominently at the level of filtration slits, as well as occasionally in the plasma membrane of podocytes (Holthöfer et al. 1999).

The cDNA-predicted amino acid sequence of nephrin is comprised of 1241 residues, with a calculated molecular mass of 134,742 Da without posttranslational modifications (Kestilä et al. 1998). Because of posttranslational glycosylation, the mature nephrin migrates as a 180 kDa protein when analyzed by Western blot analysis (Ruotsalainen et al. 1999). Based on similarity comparisons and protein structure prediction programs, nephrin was considered as a transmembrane protein of the immunoglobulin superfamily, with a 22-residue amino-terminal signal peptide, an extracellular domain containing eight immunoglobulin-like modules followed by a fibronectin type III domain, a single short transmembrane region and a cytosolic carboxy-terminal end (*Figure 5)* (Kestilä et al. 1998). Several mRNA splicing variants that mainly lack the transmembrane region have also been reported (Holthöfer et al. 1999).

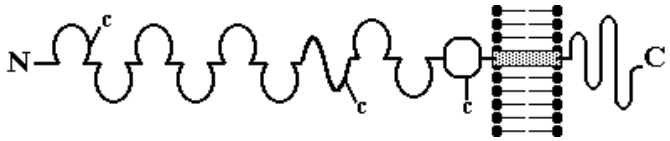

Figure 5. Predicted domain structure of nephrin. Nephrin is composed of eight extracellular Ig-like modules (partial circles), one fibronectin-like motif (octagon), a transmembrane region (rectangle in a membrane lipid bilayer), and a short intracellular domain. The locations of three free cysteine residues are indicated by c. N, amino terminal end; C, carboxy terminal end. Adapted from Kestilä et al. (1998).

With CNF patients, exon sequencing analysis of the NPHS1 gene revealed the presence of two major mutations representing over 90% of the Finnish CNF chromosomes: (1) a 2 bp deletion in exon 2 causing a frameshift and a premature 90-residue long protein, and (2) a nonsense mutation in exon 26 leading to an early stop codon in the cytoplasmic domain of the protein (Kestilä et al. 1998). These two mutations were much more rare in non-Finnish populations, which showed a large number of missense mutations, small deletions and insertions, nonsense, splice-site and promoter mutations, making the total number of known mutations to about 50 (Lenkkeri et al. 1999, Aya et al.

2000, Beltcheva et al. 2001). CNF kidney samples (except one of the 28 studied) failed to show nephrin reactivity with either the extra- or intracellular nephrin antibodies in immunohistochemical studies (Holthöfer et al. 1999). However, some nephrin-specific signals were obtained with immunoelectron microscopy in some of the CNF samples, preferentially at the flattened apical surface of podocytes.

The role of nephrin remains unknown, but it is likely to be a cell adhesion molecule and a signalling protein, as its domain structure resembles that of the many cell adhesion receptors participating in cell-cell and cell-matrix interactions. Kestilä et al. (1998) discussed the possible importance of nephrin for the integrity or anchorage of the podocyte foot processes, and the development and function of the glomerular filtration barrier. The results of its key localization in glomerulus, in podocyte slit membranes (Holthöfer et al. 1999), support this crucial role of nephrin in glomerular filtration and thus, proteinuria in CNF. It has been hypothesized that nephrin may form an isoporous zipper-like membrane structure that functions as the ultrafilter of the glomerular filtration barrier *(Figure 6)* (Ruotsalainen et al. 1999, Tryggvason 1999, Khoshnoodi & Tryggvason 2001). The central role of nephrin in the formation and function of the slit membrane is also demonstrated by the generation of NPHS1 knock-out mice (Putaala et al. 2001). The NPHS1 null mice completely lack a slit diaphragm and develop massive non-selective proteinuria at birth and die within 24 hours. In addition, changes in nephrin expression have been suggested in other pediatric kidney diseases (Furness et al. 1999, Doublier et al. 2001) and experimental animal models (Ahola et al. 1999, Kawachi et al. 2000, Luimula et al. 2000), but not unequivocally (Patrakka et al. 2001).

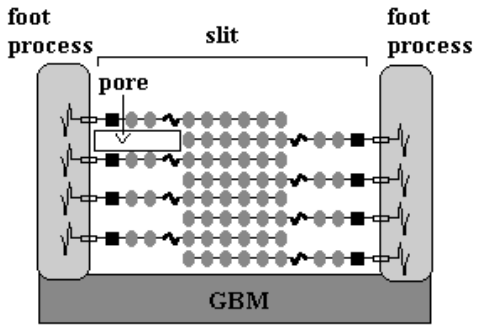

Figure 6. Nephrin - a key component of the slit diaphragm. Hypothetical assembly of nephrin molecules to form an isoporous filter of podocyte slit diaphragm. Ig-like repeats (grey circles) 1-6 of a nephrin molecule associate in an interdigitating fashion with Ig-like repeats 1-6 in a neighboring molecule. Molecules from opposite foot processes are predicted to interact with each other in the center of the slit through disulfide bridges. According to Ruotsalainen et al. (1999) and Tryggvason (1999).

AIMS OF THE PRESENT STUDY

The outline of the present study was to learn more about the molecular mechanisms of proteinuria by using congenital nephrotic syndrome of the Finnish type (CNF) as a model disease. CNF appears an ideal human model disease to study pathogenesis and mechanisms associated with proteinuria, which have remained thus far poorly understood. CNF is a kidney specific, one-gene defect, and results in massive, treatment resistant proteinuria already in utero. At the time of this research, the basic defect of CNF was still unknown, but it had been suggested that it involves a gene essential to establish a functional glomerular filtration barrier, either primarily or secondarily. Several different hypotheses have been proposed to explain the mechanisms of proteinuria in CNF, including the aspects of the **development** of functional glomeruli and the molecular **regulation** of the filtration. The first part of this study (**I, II, III**) was undertaken to test these hypotheses. The second series of experiments (**IV, V**) was planned to find **new candidate molecules** involved in the pathogenesis of CNF.

The specific aims were:

- to characterize morphological and developmental abnormalities of CNF kidneys and glomeruli in more detail using cell-type and differentiation stage-specific markers (**I**),
- to test systematically the expression of kidney development and maturation associated genes in CNF kidneys and cultured glomerular cells thereof (**II**),
- to explore the putative involvement of vascular permeability factor (VPF) in the pathogenesis of proteinuria (**III**),
- to proceed to screening for the differentially expressed gene products in CNF glomeruli compared with normal human glomeruli (**IV**),
- to further characterize the gene products identified by differential display analysis and examine their association with the pathogenesis of proteinuria in CNF (**IV, V**).

MATERIALS AND METHODS

1. TISSUE SAMPLES

1.1. CNF patients and kidney samples

Kidney samples of patients with congenital nephrotic syndrome of the Finnish type (CNF, n=8) were obtained at nephrectomies performed according to an established treatment protocol (Children's Hospital, University of Helsinki, Helsinki, Finland). Prior to nephrectomy, treatment of the patients included daily albumin infusions and aggressive nutrition and, as the weight of 8-9 kg was achieved (at an age of 9-18 months), nephrectomy of both kidneys was performed (Holmberg et al. 1995). Diagnosis of CNF was based on the typical clinical picture (large placenta, massive proteinuria at birth) and exclusion of other types of congenital nephroses, and later by the typical pathology at nephrectomy (Rapola 1987). Some kidneys at nephrectomy were immediately perfused with Ringer's buffer solution (Orion Pharmaceuticals, Espoo, Finland) via the renal arteries.

1.2. Control kidney samples

As control human kidneys we used cadaver kidneys (n=7; ages 3, 12, 18, 22, 25, 37 and 48 years) unsuitable for renal transplantation due to vascular anatomic reasons (Division of Transplantation Surgery, University of Helsinki), or macroscopically normal parts of the kidneys obtained at nephrectomy due to renal carcinoma (n=3; adults; Division of Urology, University of Helsinki) or Wilms' tumor (n=2; ages 3 and 5 years; Division of Urology, University of Helsinki). The fetal kidney sample was from a legal, prostaglandin induced abortion at the 16th week of gestation due to a severe maternal illness (Department of Obstetrics and Gynecology, University of Helsinki).

1.3. Other kidney samples (II, V)

The Wilms´ tumor tissue was obtained from a clinical nephrectomy (n=1; age 5 years; sporadic case; Division of Urology, University of Helsinki) and diagnosed at the Pathology Unit of the Department of Pediatrics (University of Helsinki) based on routine diagnostic procedures combining clinical and histological data. Kidney biopsies from patients with membranous glomerulonephritis (n=3; ages >18 years) and adult minimal change disease (n=3; ages >18 years) were obtained from routine diagnostic biopsies (University of Helsinki).

1.4. Other tissues (V)

Other human tissues used included samples from extrarenal arteries from CNF patients and control humans (Department of Surgery, University of Helsinki).

All procedures used in this study were approved by the Ethical Committees of the respective departments of the University Central Hospital, Helsinki.

2. PREPARATION OF TISSUE SAMPLES

After removal, CNF kidneys were cut into two halves along the longitudinal axis across the renal pelvis to reveal the gross anatomic appearance of the corticomedullary relationship. Then immediately, the cortical tissues were mechanically separated from medulla and dissected into cubes of about 0.5-1 cm^3. Samples were (a) frozen in liquid nitrogen for RNA extractions, (b) prepared for isolation of glomeruli, (c) snap frozen in isopentane cooled with liquid nitrogen for

histochemistry and immunohistochemistry, or (d) fixed in 3.5% paraformaldehyde and frozen in isopentane cooled with liquid nitrogen for in situ hybridization. For electron microscopy, the samples were fixed and processed as described earlier by Neale et al. (1994) and Shirato et al. (1996). Control kidney samples were processed as CNF samples, usually in 18-48 hours after removal (stored in appropriate conditions for transplantation). For later experiments, all samples were stored at -70°C or liquid nitrogen. In addition, kidney biopsy samples and arterial samples were snap frozen and stored in liquid nitrogen until analyzed.

3. ISOLATION OF GLOMERULI

Glomeruli were isolated from fresh kidney samples as described previously (Striker & Striker 1985, Holthöfer et al. 1991). Aseptically isolated cortical tissue was placed into ice cold PBS, minced to macroscopic homogeneity, and pushed sequentially through sieves of 250 µm and 150 µm pore sizes. The glomeruli were collected by rinsing from a third sieve of 106 µm pore size. The preparation was examined under light microscopy for purity and usually 94-96 % pure glomeruli were obtained. Isolated glomeruli were then used immediately for the establishment of glomerular cell culture or isolation of glomerular RNA.

4. CELL CULTURES (II, III)

4.1. Establisment of glomerular cell culture

For culture, the isolated glomeruli were placed in 25 cm^2 plastic tissue culture bottles (Greiner Labortechnik, Frickenhausen, Germany) in RPMI-1640 medium (Gibco Biocult, Paisley, Scotland) supplemented with penicillin (100 E/ml; NordCell, Skärholmen, Sweden), streptomycin (100 µg/ml; NordCell), glutamine (2 mM; NordCell) and 10 % fetal calf serum (Collaborative Research, Cambridge, MA, USA). The medium was changed twice a week after the proliferating cells appeared typically by day five of culture. For fetal cell culture, the fetal kidney sample was throughly homogenized with a razor blade and placed in culture bottles in medium as described above.

4.2. Identification of glomerular cell types

The outgrowing glomerular cell types were identified by their morphology and the expression of cell-type specific epitopes studied by immunofluorescence microscopy as described earlier (Dekan et al. 1990, Holthöfer et al. 1990 and 1991, Miettinen et al. 1990). For mesangial cells these included antibodies against Thy-1.1 (clone OX-7; Serotec, Oxford, UK), alpha smooth muscle actin (Progen Biotechnik GmbH, Heidelberg, Germany), fibronectin (Locus Genex, Helsinki, Finland), integrin alpha-1 (Becton-Dickinson, San Jose, CA, USA) and alpha-5 (Telios Pharmaceuticals, San Diego, CA, USA), and *Ricinus communis* agglutinin (RCA I; Vector Laboratories Inc., Burlingame, CA, USA). For epithelial markers we used anti-PHM5 (Dakopatts, Glostrup, Denmark) and anti-gp44 (kindly provided by Dr. Peter Mundel, University of Heidelberg, Heidelberg, Germany) antibodies, and wheat germ agglutinin (WGA; Vector Laboratories Inc.). The cells used in the present study (passages 3 to 5) contained both epithelial and mesangial cells, with a predominance of mesangials, ranging from 66 to 78 % of the total cell counts.

4.3. Other cell lines

Adenovirus-transformed human embryonic kidney epithelial cell line A293 (cell repository line (CRL) 1573; American Type Culture Collection (ATCC), Rockville, MD, USA) was cultured in the same conditions as the glomerular cells.

5. HISTOLOGICAL STAININGS

5.1. Histochemistry (V)

Frozen sections 3-4 µm thick were cut with a cryostat from the cortical kidney samples. Fresh cryostat sections were incubated with specific substrate solutions, producing coloured insoluble granular deposits at sites of enzymatic activity. Histochemical methods for respiratory chain complex II (succinate dehydrogenase, SDH) and IV (cytochrome-c oxidase, COX) were used in this study (Seligman et al. 1968, Bancroft 1975).

5.2. Immunohistochemistry

Fozen tissue sections (as for histochemistry) were fixed for 3 min in acetone at -20°C, washed in phosphate buffered saline (PBS, pH 7.4), incubated with the appropriate dilutions of the primary antibodies overnight at 4°C, washed with PBS, then incubated for 1-2 hours with the secondary antibodies conjugated with fluorescein isothiocyanate (FITC) and finally mounted in a nonfading mounting medium (Mowiol; Calbiochem Corp., La Jolla, CA, USA). The immunofluorescence microscopy and photography were performed with an Olympus Ox50 microscope (Olympus Co., Japan) with a filter system for FITC fluorescence, as previously described (Dekan et al. 1990, Miettinen et al. 1990) .

5.3. Antibodies and lectins used

The following primary antibodies were used (**I**): rabbit (R) anti-fibronectin (Serotec), R anti-laminin (Bethesda Research Laboratories, Gaithersburg, MD, USA), mouse (M) anti-cytokeratin (Pkk1; Labsystems, Helsinki, Finland), M anti-human monocytes/macrophages (M 718; Dakopatts), M anti-human leukocyte common antigen (M701; Dakopatts) M anti-stem cell factor (SCF; R&D Systems, Minneapolis, MN, USA), M anti-Bcl-2 (Upstate Biotechnology, Lake Placid, NY, USA), R anti-Wilms' tumor gene protein product (WT-1, clone HC17; kindly provided by Dr. Frank Rauscher, The Wistar Institute, Philadelphia, PA, USA; described by Amin et al. 1995), M anti-proliferating cell nuclear antigen (PCNA; Boehringer Mannheim Biochemica, Mannheim, Germany), R anti-heparin binding growth associated molecule (HB-GAM; kindly provided by Dr. Heikki Rauvala, Institute of Biotechnology, University of Helsinki), and R anti-midgestation and kidney (MK; kindly provided by Dr. Takashi Muramatsu, University of Nagoya, Nagoya, Japan; (Muramatsu & Muramatsu 1991, Tsutsui et al. 1991)); (**III**): R anti-vascular permeability factor (VPF, VEGF-A20; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); (**V**): M anti-cytochrome-c oxidase subunit I (COX I, clone 1D6-E1-A8, kindly provided by Dr. Jan-Willem Taanman, Department of Clinical Neurosciences, University of London, London, UK), M anti-COX IV (clone 10G8-D12-C12, from J.-W. Taanman) (Taanman et al. 1993, 1996 and 1997), R anti-malonyldialdehyde (kindly provided by Dr. Thomas Montine, Department of Pathology, Duke University Medical Center, Durham, NC, USA (Montine et al. 1996)). The secondary antibodies used were FITC-conjugated anti-rabbit (R) or anti-mouse (M) IgG antibodies (Jackson Laboratories, West Growe, PA, USA (**I, V**) or Zymed, San Francisco, CA, USA (**III**)). In addition, FITC-*Triticum vulgaris*-lectin (WGA; Vector Laboratories Inc.) and, to detect apoptosis (method described by Abrams et al. (1993)), acridine orange (Sigma Chemical Co., St. Louis, MO, USA) were used for histological stainings (**I**).

6. IN SITU HYBRIDIZATION (III, IV)

6.1. Hybridization experiments

Tissue sections 3-4 µm thick were cut from the froxen kidney samples on glass slides pretreated with 3'-triethoxysilylpropylamine (TESPA; Sigma Chemical Co.). Prehybridization and hybridization were performed according to Wilcox's (1993) procedure for frozen sections. Posthybridization washes were as follows: (1.) 5xSSC (salt sodium citrate; 1xSSC: 150 mM NaCl, 15 mM NaCitrate) and 10 mM dithiothreitol (DTT; Calbiochem Corp.) for 30 min at 50°C, (2.) 50 % formamide, 2xSSC, and 30 mM DTT for 30 min at 65°C, (3.-5.) 0,5 M NaCl, 10 mM Tris-HCl pH 8, and 5 mM EDTA (NTE) for 3x 10 min at 37°C, (6.) 20 mg/ml RNase A (Sigma Chemical Co.) in NTE for 30 min at 37°C, (7.) NTE for 15 min at 37°C, (8.) same as (2.), (9.) 2xSSC for 15 min at 65°C, and (10.) 0,1xSSC for 15 min at 65°C. After dehydration with increasing concentrations of ethanol and air drying the sections were covered with NTB-2 photoemulsion (Eastman Kodak Co., Rochester, NY, USA), exposed at 4°C, developed by the protocol of Kodak and counterstained with hematoxylin (Shandon Inc., Pittsburgh, PA, USA).

6.2. Preparation of probes

III: VPF complementary RNA (cRNA) probes were synthesized from Hind III (antisense) or Eco RI (sense) restriction enzyme (Promega Corp., Madison, WI, USA) linearized pGem3Zf(+) plasmid containing nucleotides 57-638 of the VPF_{165} coding region (kindly provided by Dr. Daniel Connolly, Monsato Co., St. Louis, MO, USA; Leung et al. 1989), using T7 and SP6 polymerases (Promega Corp.) and ^{35}S-UTP (Amersham Corp., Amersham, UK) (Sambrook et al. 1989). For VPF kinase-insert domain receptor KDR probes, the fragment covering base pairs 6-715 of KDR cloned into pBluescript KS (kindly provided by Dr. Bruce Terman, Lederle Laboratories, Pearl River, NY, USA; Terman et al. 1992) was linearized with Eco RI (antisense) or Hind III (sense) enzyme and transcripted correspondingly. **IV**: cRNA probes were synthesized from Sac I and Apa I (Promega Corp.) linearized PCR-clones (see "9. Differential display RT-PCR") and transcripted as described above.

7. PREPARATION OF RNA

Total glomerular RNA was extracted by guanidine isothiocyanate (Fluka BioChemica, Buchs, Switzerland) lysis and subsequent purification by CsCl-gradient (Serva Feinbiochemika, Heidelberg, Germany) centrifugation (Chirgwin et al. 1979), either from tissue samples of 300-500 mg, from 50-100 µl of freshly isolated glomeruli, or from about 10^7 cells harvested from subconfluent cultures. If required, poly(A)$^+$-RNA was prepared by oligo(dT) cellulose chromatography (Boehringer Mannheim Biochemica). Isolated RNA was then used for Northern blotting experiments, for differential display reverse transcription-polymerase chain reaction (DDRT-PCR) analysis or for semiquantitative RT-PCR.

8. NORTHERN BLOTTING

8.1. Hybridization

30 µg of total-RNA or 3 µg of poly(A)$^+$-RNA per lane were electrophoresed in 0,8-1 % agarose gels containing 2,2 M formaldehyde (Riedel de Haén, Seelze, Germany), and transferred to nylon membranes (Pall Biodyne, New York, NY, USA (**II, III**) or Hypond-N, Amersham Corp. (**IV, V**)) (Sambrook et al. 1989). Membranes were cross-linked according to manufacturers´ suggestions, and

prehybridized in 5x SSC (see ”in situ hybridization"), 50% formamide, 5x Denhardt's reagent, 0,1% sodium dodecyl sulphate (SDS), 50 mM Na-phosphate buffer pH 6,5 and 250 µg/ml salmon sperm DNA (all reagents from Sigma Chemical Co.) at 42°C. The probes were labelled with (α-^{32}P)dCTP (>3000 Ci/mmol; Amersham Corp. (**II, III**) or DuPont NEN, Boston, MA, USA (**IV, V**) by random priming method (Cunningham et al. 1990) using Random Primer DNA Labelling Kit (Boehringer Mannheim Biochemica) and hybridized to blots in prehybridization solution for 16 h. Posthybridization washes varied from the low-stringency (1x SSC, 0,1% SDS at 42°C) to very high-stringency (0,1x SSC, 0,1% SDS at 65°C) conditions depending on the probe used. To control the total RNA content and lack of degradation in the analyzed preparations, the blots were rehybridized with a human β-actin probe. For autoradiography, the filters were exposed on medical X-ray film (Fuji Photo Film Co., Japan) at -70°C with intensifying screens (**II, III, IV**) or on Fuji Bas IIIS Imaging Plates (**V**), and the expression was quantified using a phosphorimager and accompanying MacBAS software (Fuji Photo Film Co.).

8.2. Probes used

The cDNA probes used here were for (**II**): human WT-1 (clone WT-33; kindly provided by Dr. Melissa Little, Western General Hospital, Edingburgh, UK; Call et al. 1990), insulin like growth factor I (IGF-1, clone phigf1; ATCC; Bell et. al. 1984, Tricoli et al. 1984), IGF-2 (clone phigf2, ATCC; Bell et al. 1984, Dull et al. 1984), IGF binding protein 1 (IGFBP-1, clone PP12; kindly provided by Dr. Riitta Koistinen, Department of Obstetrics and Gynecology, University of Helsinki; Julkunen et al. 1988), tyrosine kinase trk-B (prepared by our own; see below) and MK genes (clone hMK-1; kindly provided by Dr. Takashi Muramatsu, University of Nagoya; Tsutsui et al. 1991), and for mouse WT-1 (clone pKS/2; kindly provided by Dr. Jane Armstrong, Western General Hospital, Edingburgh; Buckler et al. 1991), early growth response 1 (EGR-1, clone zif/268, ATCC; Christy et al. 1988) and paired box 2 (Pax-2, clone c31A; kindly provided by Dr. Greg Dressler, National Institutes of Health, Bethesda, MD, USA; Dressler et al. 1990) genes, and for rat HB-GAM gene (clone P18/6 kindly provided by Dr. Heikki Rauvala, Institute of Biotechnology, University of Helsinki; Merenmies & Rauvala 1990); (**III**): human VPF and its receptor KDR (both clones as in ”in situ hybridization”); (**V**): COX I and COX II (mitochondrial DNA clone pCOX2 containing the complete gene for COX II and 714 bp of the gene for COX I; kindly provided by Dr. Jan-Willem Taanman, Department of Clinical Neurosciences, University of London), COX IV (clone pCOX4 containing 682 bp COX IV cDNA fragment; kindly provided by Dr. M. Lomax, Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, MI, USA) and COX VIb (clone pCOX6 containing a 450 bp COX IVb cDNA fragment; provided by J.-W. Taanman).

8.3. Probe preparation for trk-B (II)

First strand (anti-sense) cDNA was synthesized from 2 µg of total RNA from human kidney cortex using random hexanucleotide primers (Promega Corp.) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega Corp.), as described by Kawasaki & Wang (1989). The 702 bp fragment from the coding sequence of trk-b gene was amplified by PCR in a thermal cycler (PTC-100, MJ Research Inc., Watertown, MA, USA) using ampli-Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) and 1mM synthetic oligonucleotide primers (Department of Biochemistry, University of Helsinki). The amplified DNA fragment was cloned and sequenced, and used for Northern hybridization.

9. DIFFERENTIAL DISPLAY RT-PCR (IV, V)

9.1. cDNA synthesis

Contaminating genomic DNA was removed from glomerular total RNA preparations with RNase-free DNase I (Boehringer Mannheim Biochemica) incubation in a presence of human placental RNase inhibitor (Promega Corp.). After purification by phenol-chloroform extraction and ethanol precipitation, RNA samples were split into identical aliquots and reverse transcribed using "anchored" oligonucleotide primers (Anchor Kit Version 1, Operon Technologies Inc., Alameda, CA, USA or GenHunter, Boston, MA, USA) following manufacturers´ instructions. All reactions were done in duplicate.

9.2. PCR amplification

PCR reactions were performed as previously described (Liang & Pardee 1992, Bauer et al. 1993) with some minor modifications using anchored cDNA-products, PCR-buffer, dNTPs, specific random decamer oligonucleotides (OP-DDRT 26x10-mer Primer Set, Operon Technologies Inc. or GenHunter), anchored primers (as above), ^{35}S-dATP (Amersham Corp.) and ampli-Taq DNA polymerase (Perkin Elmer Cetus). The amplification conditions consisted of initial denaturation at 94°C for 3 min followed by 40 cycles of amplification with denaturation at 94°C for 30 sec, annealing at 40°C for 2 min, extension at 72°C for 30 sec, and a final extension at 72°C for 15 min. The PCR products were then denatured at 80°C for 5 min in the presence of formamide loading buffer, and separeted side by side on a 6 % denaturing polyacrylamide gel. After electrophoresis gels were dried and exposed to a medical X-ray film (Fuji Photo Film Co.) for 2-4 days.

9.3. Cloning

Reproducibly differentially expressed bands were excised from the dried polyacrylamide gels, extracted and re-amplified using the same primers and conditions as for the initial PCR. Products were run on 2 % agarose gel, purified with Micropure Separators (Amicon Inc., Beverly, MA, USA) and subcloned using the TA-cloning system (Promega Corp. or Invitrogen, San Diego, CA, USA; Sambrook 1989). For each PCR product, 3-8 colonies from a single transformation were prepared (Kretzler et al. 1996) for sequencing. Sequencing was done with T7 and SP6 primers (Promega Corp.) by the modified dideoxy chain termination method (Hsiao 1991) using DNA Sequencing Kit (version 2.0; USB, Cleveland, OH, USA) and (α-^{35}S)dATP (>1000 Ci/mmol; Amersham Corp.) or by automated DNA sequencer (ABIPrism model version 2.1.1, Perkin Elmer Cetus). Sequences were screened for homologies with database sequences using the BLAST search algorithm via Internet at the National Center for Biotechnology (Washington D.C., USA). Reconfirmations of differential expression of the candidate DDRT-PCR clones were done by Northern blotting analysis.

10. SEMIQUANTITATIVE RT-PCR (V)

Sequence-specific oligonucleotide primers were designed and produced (in Department of Biochemistry, University of Helsinki) for mitochondrially encoded COX I and cytochrome b as well as for nuclearly encoded COX VIIa. Semiquantitation of their expression was done from glomerular total RNA fractions as described by Kretzler et al. (1996), basing on the serial dilutions of control plasmid DNA and sample cDNAs in the linear range of amplification and on the amount of house-keeping (β-actin) amplification product.

11. BLUE NATIVE GEL ELECTROPHORESIS (V)

The steady-state levels of the mitochondrial respiratory complexes I to V were analyzed from kidney tissues of CNF patients (n=3) and control humans (n=3). 10 mg of each kidney sample was solubilized and separated in the native state by blue native polyacrylamide gel electrophoresis (BN-PAGE) and then processed by SDS-PAGE in a second dimension, and quantified by densitometry as described previously (Schägger & von Jagov 1991, Schägger 1995). The staining intensities of individual complexes were normalized to the intensity of complex III.

12. ELECTRON MICROSCOPY (V)

Electron microscopy studies were done as previously described by Neale et al. (1994) and Shirato et al. (1996).

13. STATISTICS (V)

For mitochondrial quantitations, statistics were performed with unpaired two-tailed t-tests. P-values less than 0.05 were considered significant. Data were obtained by direct counting from the electron micrographs at 4500x magnification. Multiple sections were analyzed from each sample.

RESULTS AND DISCUSSION

1. MORPHOLOGY AND HISTOLOGY OF CNF KIDNEYS (I)

1.1. Renal tubules and interstitium

At macroscopic examination, kidneys of patients with congenital nephrotic syndrome of the Finnish type (CNF) regularly revealed a decreased cortical thickness to an average of only 3-6 mm. Areas of poor demarcation between the cortical and medullary compartments were found and sometimes the cortical tissue appeared to ectopically extend to the medullar region. In early studies, Tryggvason and Kouvalainen (1975) have described CNF kidney cortex as thick and medullary tissue as scanty. These partly incompatible findings may be explained by the poor demarcation of areas and also by morphological variability between patients. In histological analyses, we found normal appearing tubular profiles with morphologically distinguishable epithelial cells of the proximal tubules (cells with distinct brush border), loops of Henle (smaller caliber tubular profiles with more flat epithelium), distal tubules (number of tubular profiles within cortex, no brush border) and collecting ducts (heterogeneity in epithelial cell size and morphology, bifurcation of ducts). However, characteristic areas of tubular dilatations as described for CNF also earlier (Hallman et al. 1967) were seen. In all CNF kidneys studied some tubules were focally surrounded by areas of poorly organized cells. When studied by immunohistochemistry, these areas were faintly positive for anti-fibronectin, anti-stem cell factor (SCF) and anti-cytokeratin antibodies, whereas no laminin-specific staining could be detected. Staining for human lymphocytes and monocyte/macrophages colocalized only partially with these areas, and only occasional cells staining with the anti-Bcl-2 (anti-apoptotic factor) and anti-proliferating cell nuclear antigen (PCNA) antibodies or cells with acridine orange reactive compacted nuclei (typical for apoptosis) were found. These results indicate no aberrations in the apoptotic (Bcl-2, acridine orange) or cell proliferation (PCNA) pathways, the mechanisms normally controlling the ultimate cell number of the mature kidney (Herzlinger et al. 1992). However, these peritubular areas expressing markers of early differentiation (fibronectin, stem cell factor, cytokeratin) suggest retarded differentiation in CNF kidneys in this compartment.

1.2. Glomeruli

The arrangement of CNF glomeruli in most samples studied differed characteristically from that of the control kidneys: densely clustered glomeruli separated by narrow Bowman's capsules were often seen especially at the corticomedullary demarcation zone resulting in an abnormally high density of glomeruli in these areas. This observation is in agreement with the early results of Tryggvason & Kouvalainen (1975), who reported an increase in the total number of glomeruli in CNF kidneys. The finding of clustered glomeruli with abnormally high numbers may suggest a failure in the coordinated mesenchymo-epithelial interaction during nephrogenesis in CNF. Furthermore, we could occasionally find large glomeruli suggesting fusion of two to three individual glomeruli. These "fused" glomeruli were typically surrounded by a continuous Bowman's capsule, as demonstrated by laminin staining. The cellular composition within these glomeruli appeared balanced, with no prominence of podocyte, mesangial or endothelial elements, based on the morphology and reactivity for podocyte specific Wilms´ tumor protein (WT-1) antibodies and wheat germ agglutinin (WGA) lectin. No signs of continued cell proliferation (anti-PCNA staining) nor cells with aberrations in the apoptotic pathway (anti-Bcl-2 and acridine orange stainings) could

be seen within the CNF samples as compared to control kidneys. In addition, the functionally important anionic charge seemed to appear normally within the epithelial cells and glomerular basement membranes of the "fused" CNF glomeruli both in intensity and distribution as shown by WGA lectin staining.

The possibility that abnormal early developmental events may lead to a distinct functional abnormality of glomeruli is intriguing and supported by the findings in studies with mice. The early developmental gene Pax-2 deregulation in mice results in proteinuria and morphologic changes closely resembling those in congenital nephrotic syndrome (Dressler et al. 1993). Mice deficient for laminin B2 gene, encoding the mature type of laminin β chain of GBM, similarly produce a phenotype closely resembling that of human congenital nephroses (Noakes et al. 1995). In early studies with CNF, (Tryggvason & Kouvalainen 1975), some CNF glomeruli were found to be immature, but also opposite findings exist (Huttunen et al.1980). Sharif et al. (1998) have shown changes in glomerular epithelial protein 1 (GLEPP1) expression in CNF and considered that as a consequence of failure to complete normal podocyte development. They also stated a dedifferentiation hypothesis for the nephrotic syndrome. In addition, there is evidence that e.g. in the postischemia model of tubular injury and in animal models of liver injury, reverse differentiation or dedifferentiation may take place (Safirstein 1994, Sell & Pierce 1994). Furthermore, Holthöfer et al. (1992, 1994) have earlier shown that the cell type specific glomerular ganglioside, O-acetyl GD3, found only transiently during early stages of glomerulogenesis remain atypically expressed in the CNF kidney glomeruli. Our findings of morphologic abnormalities in CNF kidneys and glomeruli, and the local areas showing lack of appropriate differentiation suggest the possibility of developmental failure or arrest in CNF kidneys. However, the markers used here did not appear optimal to characterize the functional disturbance of CNF kidneys and the resulting proteinuria, which could be consequences of such a proposed developmental arrest.

2. DEVELOPMENTAL STAGE SPECIFIC GENE EXPRESSION IN CNF KIDNEYS (II)

Several genes transiently expressed during the maturation of the metanephrogenic mesenchyme have been reported in recent years. On the basis of our histological results introduced above, as well as suggestions of other investigators (Tryggvason & Kouvalainen 1975, Dressler et al. 1993, Kestilä et al. 1994c, Sharif et al. 1998), it is possible, that the CNF kidneys never reach the stage with normally functioning glomeruli. If so, one would expect to see a developmental arrest with the expression of genes transiently expressed during normal kidney maturation. We studied the expression of nine such candidate marker genes, including transcription factors, growth factors and other signalling molecules associated with renal maturation, by Northern blotting analysis. We compared the expression pattern in CNF kidneys, in renal Wilms' tumor tissue and in normal human kidneys of various ages. Subsequently, we studied cultured glomerular cells for the corresponding expression pattern.

2.1. Kidney tissue samples

WT-1 and paired box gene Pax-2 are transcription factors expressed early during nephrogenesis. In this study, Pax-2 and WT-1 gene expressions were preferentially found in fetal kidney (gestational age of 16 weeks) and in Wilms' tumor tissue, as expected. The CNF kidneys resembled the normal adult and child kidney cortex in maintaining a low, basal level expression of these mRNAs. Thus, both Pax-2 and WT-1 revealed considerable changes in expression levels between different

developmental and functional stages of the kidney, but we could not find specific changes in their expression levels in CNF. Direct association of Pax-2 defects to proteinuria has been suggested by Dressler et al. (1993) who demonstrated that deregulated Pax-2 expression in transgenic mice results in histologically abnormal and dysfunctional renal epithelium with properties similar to congenital nephrotic syndrome: multifocal microcystic tubular dilation, paucity of podocyte foot processes and proteinuria. However, Kestilä et al. (1994c) have previously excluded a defect in the Pax-2 gene locus in CNF kidneys. Kreidberg et al. (1993) have shown that in WT-1 knockout mice the inductive events that lead to formation of metanephric kidney are seriously affected. During normal kidney maturation WT-1 activation turns off the expression of molecules involved in embryonal cell proliferation and turns on genes whose expression is required for terminal differentiation. The third transcription factor analyzed here was the early growth response gene EGR-1, a ubiquitous transcriptional activator. The expression pattern of EGR-1 did not show any considerable developmental stage-specific regulation at the transcriptional level, but a basal level expression was seen in every sample. In the metanephric kidney EGR-1 is normally upregulated as mesenchymal cells receive a mitogenic signal, but as differentiation proceeds and e.g. WT-1 levels rise, mitogenic molecules icluding EGR-1 are downregulated (Sukhatme 1992).

Insulin like growth factor IGF-2 was found to be heavily expressed in fetal kidney and in embryonal Wilms' tumor tissue as well, but it was totally downregulated and not detectable in postnatal kidney samples, not in CNF nor in control kidneys. The expression of IGF-2 mRNA in fetal kidneys has been shown to be largely confined to the stromal and blastemal cells providing a stimulus for the growth and differentiation of epithelial structures. Thus, the continuous expression of IGF-2 in Wilms' tumor proposedly reflects a failure of complete differentiation of the tumor cells (Hirvonen et al. 1989). The expression of IGF-1 only in renal cortex of the 12 year-old child, but not in any other tissue sample tested, reflects most likely the growth hormone induced expression of IGF-1, as reported earlier (Murphy et al. 1987). IGF binding protein IGFBP-1 mRNA was detected in control adult and CNF samples only after prolonged exposure of poly(A)$^+$RNA fractions indicating very low but comparable expression levels.

Neural elements including neurons, neurotrophic factors and their receptors seem to be crucially involved in the induction and development of the nephrogenic mesenchyme (Sariola et al. 1991). The high affinity neurotrophin receptor trk-B has previously been found to be differentially expressed during renal development with a highly restricted expression pattern in the cortical mesenchyme cells that will differentiate into stroma (Durbeej et al. 1993). In this study, however, trk-B showed a low, but equal level of expression in every kidney sample tested without any considerable maturation stage specific regulation. In addition, heparin binding growth and differentiation associated molecule HB-GAM appeared to be expressed at a constant level in all kidney samples, reducing its usefulness as a marker for kidney maturation level. The midkine (midgestation and kidney) MK mRNA was readily detected in the 5-year-old child and CNF kidneys, and at a much elevated level in the fetal kidney tissue. This is well in agreement with the results with mouse kidney showing the highest expression during the fetal life and to be still expressed after birth (Kadomatsu et al. 1990, Nakamoto et al. 1992). Taken together these results, on the basis of the gene expression profiles studied we could not find any conclusive evidence of developmental abnormality in the CNF kidneys.

Recent identification of nephrin as a first and key component of the podocyte filtration slit has opened new possibilities for studying the development of the glomerular filtration barrier. According to Ruotsalainen et al. (2000), nephrin is first expressed in late S-shaped bodies during

human glomerulogenesis. Nephrin localizes in the ladder-like structures between differentiating podocytes before the formation of foot processes and mature slit diaphragms. They also found that the slit diaphragms were completely missing in CNF kidneys lacking the nephrin molecule (Patrakka et al. 2000, Ruotsalainen et al. 2000). In addition, the results of nephrin knock-out mice showed early lethality within 24 hours after birth due to nephrotic syndrome and the histological findings of dilated renal tubules, foot process effacement and mesangial hypercellularity were very similar to those observed in CNF patients (Putaala et al. 2001). These results favor the idea that nephrin is essential for the normal maturation of podocytes and formation of the slit diaphragm and its disorder is causative for the massive proteinuria in CNF. However, other functional properties of nephrin e.g. in podocyte signaling, as well as its potential role in other morphological changes observed in CNF kidneys (e.g. increased number of glomeruli, abnormal glomerular morphology) and in renal pathology in general remain to be determined.

2.2. Cultured glomerular cells

Glomerular cell culture allows a specific approach to study the role of individual glomerular cell types in renal physiology and pathology. Also, several inflammatory, metabolic and genetic diseases that affect the kidney have been studied using culture of kidney glomeruli (Oberley et al. 1981, Lovett & Sterzel 1986, Floege et al. 1992). However, when working with cells isolated from glomeruli and when attempting to extrapolate to the in vivo situation, several issues need to be addressed, including morphological characteristics, expression profiles, synthetic activities and response patterns of the cultured cells compared to those observed in vivo (Floege et al. 1994). There are many reports on glomerular cell morphology and cell-type specific gene expressions in glomerular cultures (e.g. Striker & Striker 1985, Dekan et al. 1990, Holthöfer et al. 1990 and 1991, Miettinen et al. 1990). Many of these cell-type specific markers were also tested by us when establishing cell cultures from CNF glomeruli (see Material and methods: "4. Cell cultures"). To further characterize these cultured glomerular cells and to explore their usefulness in CNF studies we tested the cells for the same developmental expression pattern as for kidney tissues.

Cultured cells from the adult and CNF kidney glomeruli expressed Pax-2 mRNA, but the message was not seen in cultured fetal kidney cells or in the fetal kidney cell line A293. Thus, there seemed to be distinct difference in Pax-2 expression between tissues and cultured cells, as tissues that can be considered less mature (fetal kidney and Wilms' tumor) readily expressed Pax-2, whereas the cultured cells from fetal kidney failed to do so. Instead, the expression of WT-1 and EGR-1 transcripts appeared somewhat elevated in the fetal cells compared to other cultured cells.

In cell cultures both adult and CNF glomerular cells produced IGF-1, IGF-2 and IGFBP-1 transcripts as comparable levels, but in fetal kidney cells no expression was observed. For IGF-2 signal, only the 6.0 kb mRNA was seen in cultured cells even in isolated poly(A)$^+$ fractions, in contrast to three approximately 6.0 kb, 4.9 kb and 1.9 kb transcripts detected in tissue samples. This may be due to differencies in splicing of the precursor RNA in different cell types. In addition, all cultured kidney cells tested expressed MK and HB-GAM. With MK, fetal kidney cell line A293 showed the strongest expression. Cultured cells did not show any trk-B expression.

The information obtained here from glomerular cell cultures appeared very restricted. The results showed remarkable inconsistency between respective tissue samples and cultured cells thereof e.g. for Pax-2, MK and IGFs expressions. In vitro culture conditions may themselves lead to the changed expression pattern, because of the uncoupling of cultured cells from local factors regulating

the normal cell proliferation and gene expression in intact tissue. Alternatively, the cells may in fact represent cells clonally selected in culture due to some secondary properties irrelevant to their differentiation stage.

3. VASCULAR PERMEABILITY FACTOR IN CNF KIDNEYS (III)

Vascular permeability factor (VPF), or vascular endothelial growth factor (VEGF), is a potent microvascular permeability-enhancing mediator and a selective mitogen for vascular endothelium. In the kidney, it is expressed preferentially in the glomerular visceral epithelial cells, the podocytes. It has been speculated that injury to podocytes may lead changes in VPF synthesis or secretion and to abnormal glomerular permeability (Brown et al. 1992a, Shulman et al. 1996). Thus, VPF has been among the candidate molecules proposed to be involved in the pathogenesis of proteinuria. We considered it highly interesting and informative to clarify the proposed role of VPF in increased glomerular permeability in CNF. For that, we studied normal human, fetal and CNF kidney tissues and the corresbonding cultured glomerular cells for VPF and its receptor expression.

In Northern blotting analysis, an abundant expression of VPF mRNA was observed in all kidney tissues and in cultured kidney cells studied. Fetal kidney seemed to express the highest level of VPF mRNA. The major VPF transcript was detected at the ~3,8 kb, but additional faint bands of approximately 1,9 kb, 4,5 kb and 6,5 kb were seen on longer exposures with polyA^+-RNA samples. In in situ hybridization experiments glomeruli labeled strongly for VPF mRNA in a fashion suggesting labeling of podocytes, in accordance with previous reports (Brown et al. 1992a). No significant differences were observed in the amount or localization of VPF mRNA between normal and CNF kidney samples. Rare tubular profiles, identified as collecting ducts based on their morphology, were also labeled. Immunohistochemical staining confirmed collecting duct and the visceral epithelial localization of VPF in the normal and CNF kidneys. In some sections intense juxtaglomerular staining of the protein product could also be seen. In fetal kidney, both the epithelium of the invading ureter and collecting duct as well as the developing glomeruli showed VPF protein expression.

The expression of the VPF kinase-insert domain receptor KDR was also studied. The KDR probe detected a single mRNA of 7,0 kb in Northern blots. The most abundant KDR expression was seen in the samples of normal adult and CNF kidneys and a slightly weaker expression in fetal kidney. Cultured kidney cells showed no KDR-specific signal. In situ hybridization revealed a distinct pattern of KDR expression centrally in the glomerular tuft resembling the pattern of glomerular endothelial reactivity. No KDR-specific signal was seen in the peritubular capillaries. The KDR labeling pattern in the CNF kidneys was comparable to that of the normal kidney. Thus, these findings failed to show any significant differences in the expression of VPF or KDR in CNF kidneys. Other pathogenetic mechanisms involving VPF or members of the growing VPF receptor family cannot, however, be excluded.

In contrast to its transient expression reported in many fetal tissues during development and maturation VPF expression persists in adult kidney, but is reduced into the glomerular visceral epithelium. Interestingly, a constitutive VPF expression has also been reported in the epithelium of the choroid plexus, where the endothelium is similarly highly fenestrated and involved in filtration of the cerebrospinal fluid (Breier et al. 1992). These findings suggest that VPF may be important for the differentiation and maintenance of organotypic endothelial cells with specific filtration

functions. The action of VPF (as VEGF) in inducing endothelial proliferation may not be its function in glomeruli, since proliferation of glomerular endothelial cells is not usually seen in adult kidney. Our observation of VPF expression in a previously unrecognized site, i.e. the juxtaglomerular area of the nephron, is interesting, but a role for VPF in juxtaglomerular regulation of fluid homeostasis is only speculative.

4. NEW CANDIDATE GENES FOR THE PATHOGENESIS OF CNF AND PROTEINURIA (IV, V)

4.1. Differential display analysis

The changes in glomerular gene expression in CNF were studied by a newly developed method called mRNA differential display or differential display RT-PCR (DDRT-PCR, Liang & Pardee 1992). DDRT-PCR analysis combines both the power of PCR amplification and the high resolution of denaturing polyacrylamide gel electrophoresis. The basic principle is to reverse transcribe and systematically amplify the 3' termini of mRNAs with a set of anchored oligo(dT) primers and arbitrary decamers. By changing primer combinations, most of the RNA species in a tissue can be represented. Side-by-side comparison of radioactively labeled PCR products from different RNA samples in a polyacrylamide gel allows the identification of differentially expressed genes. Corresponding DNA fragments are then recovered, cloned, sequenced, and used as probes for hybridization and library screening experiments. Since its presentation, many modifications and improvements to the original technique have been reported (e.g. Bauer et al. 1993, Liang et al. 1993 and 1994, Callard et al. 1994, Hadman et al. 1994, Li et al. 1994, Mou et al. 1994, Sompayrac et al. 1995, Zhao et al. 1995, Vogeli-Lange et al. 1996). The major advantages to other, more classical comparative gene expression screen methods, like subtractive or differential hybridization techniques, are its speed and sensitivity. In addition, only a modest amount of starting material is required making the study with also rare human material possible.

DDRT-PCR technique has been successfully applied in studies on mouse renal organogenesis (Kretzler et al. 1996), human liver carcinoma (Begum et al. 1995), hyperglycemia and diabetes (Aiello et al. 1994, Nishio et al. 1994 and 1995), mouse preimplantation development (Zimmermann & Schultz 1994), graft vasculopathy (Chen et al. 1996), TGF-β (Subramaniam et al. 1995) and fibroblast growth factor-1 (Hsu et al. 1993, Donohue et al. 1994) regulated gene expression, metastatic capacity of human melanoma cells (van Groningen et al. 1995), nerve injury (Kiryu et al. 1995), pathogenesis of myelodysplastic syndrome (Kroef et al. 1996), and stomach cancer (Salesiotis et al. 1995) among many other studies. Since all the evidence suggests that the pathology of the CNF defect is restricted to the kidney glomeruli, we considered the application of DDRT-PCR to isolated glomeruli as an effective possibility to identify the proteinuria-associated genes in CNF.

In applying this method to screening of glomerular genes as presented here, the only assumption is that the selected genes are differentially expressed in glomeruli of CNF kidneys compared with normal glomeruli. Thus, the method enables not only the diagnosis of changes in gene expression pattern involved in the pathogenesis of CNF and proteinuria but also the characterization of novel glomerular genes. By using isolated glomeruli from CNF patients and normal human donors and 58 different primer pair combinations a total of approximately 3800 reproducibly displayed amplification products were identified. Of these, 37 PCR products showed systematic differences

between CNF and control glomeruli: 8 (22%) were either only seen in CNF or greatly increased in intensity, 11 (30%) showed moderate increase in CNF, whereas 12 and 6 (32% and 16%, respectively) of the bands showed moderate or greatly reduced intensity in CNF. We succeeded in cloning 27 of the differentially expressed PCR fragments, sizes between 100-600 bp, but more than half of these were undetectable by Northern analysis, probably because of low expression level or presence of contaminating sequences, or gave several mRNA signals indicating unspecificity of the probe. Finally, 12 PCR products showing distinct changes in expression levels were chosen for further characterization including reconfirmation of expression differences, sequencing and screening of genetic databases for homologies.

4.2. Identification of new candidate genes

Sequence comparisons and databank searches for the differentially expressed PCR products from CNF glomeruli could reveal some genes not previously associated to proteinuria. Most prominently, one of the products showed a 98 % similarity with mitochondrially encoded cytochrome-c oxidase subunit I (COX I), a gene centrally involved in the oxidative phosphorylation and cellular energy metabolism (Suomalainen 1997). The other homologies identified included the genes for integrin-linked kinase (ILK), eotaxin and insulin-like growth factor 2 receptor (IGF-2R) and also fragments resembling anchyrin and cadherin adhesion molecule family like consensus sequences. All these molecules are of interest in respect to glomerular filtration function.

The finding of **ILK**-like gene as one of the differentially expressed genes in CNF glomeruli is intriguing. Integrins are expressed in podocyte foot processes regulating their firm attachment to the underlying glomerular basement membrane (Adler 1992). The classic morphologic sign of proteinuria is fusion and retraction of the podocyte foot processes. Thus, recognition of a novel signalling molecule involved in cytoskeleton-cell matrix interaction as downregulated in CNF glomeruli is interesting, and should be studied further. Recently, the role of ILK in regulating podocyte phenotype and permeability changes has been demonstrated by Kretzler et al. (2001). **IGF-2R** gene (also known as mannose-6-phosphate receptor) encodes a receptor which targets IGF-2 to the lysosomes for degradation (Zhang et al. 1997) and, therefore, inhibits the mitogenic function of IGF-2. In kidney, changes in the expression of IGF-2 and IGF-2R are associated with many pathological situations including Wilms' tumor and clear cell sarcoma (Reeve et al. 1985, Yun 1993). Overexpression of a receptor for IGF-2 in CNF kidneys might indicate defective regulation of kidney growth and development. This would be well in line with our results of abnormal glomerular morphology in CNF (**I**). The finding of overexpressed PCR product with marked homology to **eotaxin** could also be functionally appropriate in proteinuria. Eotaxin is among recently identified chemokines important in attracting eosinophils to tissues (Hein et al. 1997). Among many other tissues, kidney shows significant constitutive eotaxin expression (Hein et al. 1997). Upon activation eosinophils release histamine with its known effects in cellular permeability. Our earlier results have also shown the markedly increased levels of histamine as measured both immunohistochemically and by mass spectrometry in CNF cortical kidney (Holthöfer et al. unpublished data), but the meaning of this finding has remained unclear.

Among the other DDRT-PCR identified expression differencies we also found clones showing limited homologies to **cadherin** and **anchyrin** molecules. The cadherin superfamily is a large and diverse group of calcium-dependent, membrane associated glycoproteins, which play a prominent role in the mediation of intercellular interactions, cell recognition and adhesion. Kidney-specific cadherins have been implicated in tissue morphogenesis and cell polarization during development

(Xiang et al. 1994, Thompson et al. 1995). If any of our clones represents new family members of the extensive cadherin and anchyrin superfamilies remains to be explored until the corresponding full length cDNAs have been cloned and characterized.

The observation that one of the DDRT-PCR products (clone CNF B36) showed 98% similarity over 363 bp to the mRNA of mitochondrial cytochrome-c oxidase subunit I (COX I) is striking. In Northern blotting analysis with this clone, a mRNA of the expected size for COX I could be detected and the intensity of the signal was 70% lower in CNF than in controls. The decreased expression of COX I in CNF kidneys has since been verified with several independent methods, as described below, indicating defects in energy metabolism of CNF glomeruli.

5. CHARACTERIZATION OF MITOCHONDRIAL INVOLVEMENT IN CNF KIDNEYS (V)

5.1. Normal and abnormal mitochondrial function

Mitochondria are the cytoplasmic organelles responsible for cellular energy production. The basis for energy production is oxidative phosphorylation system, an enzymatic cascade located in the inner mitochondrial membrane (Singh et al. 1996). Functionally the oxidative phosphorylation system consists of five distinct multimeric protein assemblies: (1) NADH-dehydrogenase (complex I), (2) succinate dehydrogenase (complex II), (3) cytochrome b-c1 (complex III), (4) cytochrome-c oxidase (complex IV), and (5) ATP synthase (complex V) (Hatefi 1985). The first four complexes, with ubiquinone and cytochrome-c, comprise the mitochondrial respiratory chain, also called the electron transport chain *(Figure 7)*. The operation of the respiratory chain is characterized by two distinct, but linked processes, electron transport and proton pumping. Because of the resulting gradient, the protons flow back from the intermembrane space to the mitochondrial matrix through complex V, ATP synthase, and the released energy is captured in the form of ATP (Hatefi 1985).

The majority of mitochondrial proteins are nuclear encoded and imported into the mitochondria from the cytoplasm. In addition, mitochondria possess their own unique genome, which demonstrates maternal inheritance in humans carried by the ovum, because the sperm mitochondria do not survive after fertilization. Human mitochondria usually contain from two to ten copies of mitochondrial genome (mtDNA). MtDNA is a double-stranded circular molecule which contains 16 569 base pair *(Figure 8)*. It is a compact piece of genetic information with little intervening, non-coding sequences with the exception of its short regulatory region termed displacement- or D-loop. MtDNA encodes two ribosomal RNAs (12S and 16S rRNAs), 22 transfer RNAs (tRNAs) and 13 messenger RNAs (mRNAs). The protein coding mRNAs are transcripted and translated in the mitochondria using the mitochondrial rRNAs and tRNAs, independently of nuclear DNA (Clayton 1992, Johns 1995). These mitochondrially encoded polypeptides are all components of the oxidative phosphorylation system. MtDNA has a high mutation rate, about ten times that of nuclear DNA, probably because the mtDNA polymerase lack the proofreading ability and because there are no histone proteins protecting the mitochondrial genome. Moreover, the high oxygen consumption in mitochondria produces toxic reactive oxygen species (ROS), which may result in DNA damage (Singh et al. 1996).

Mitochondrial diseases are a diverse group of disorders that result from the structural, biochemical, or genetic derangement of mitochondria. The molecular defects that underlie these disorders may

arise from mutations of either the mitochondrial or the nuclear genomes or both. The variable tissue involvement observed in many patients is generally ascribed to the tissue-specific differencies in their dependence on energy production and the degree of heteroplasmy (Taanman et al. 1997). Heteroplasmy is a condition, where both normal and mutant mtDNA coexist within the same cell. The proportion of mutant mtDNA required for the occurrence of a deleterious phenotype and clinical symptoms, known as the threshold effect, varies among persons, among organs systems, and within a given tissue. The threshold effect depends on the delicate balance between oxidative supply and demand (Johns 1995). Furthermore, tissues with a high mitotic index are able to select against heteroplasmic cells, whereas in tissues with little mitotic activity the ratio of mutant to normal mitochondria rather increases (Larsson et al. 1990).

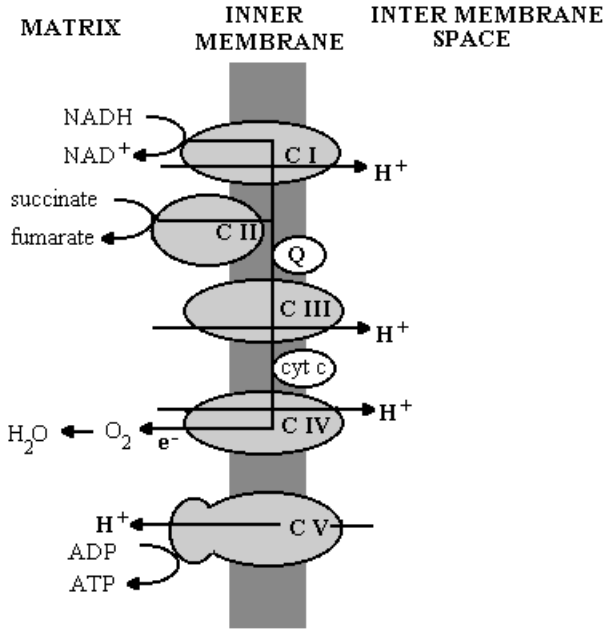

Figure 7. Mitochondrial respiratory chain and ATP-synthase. Complexes I-IV form the electron transfer chain: first, NADH is oxidized by complex I and succinate by complex II, followed by electron transfer to ubiquinone, and then further to complex III, to cytochrome-c, to complex IV and to the final electron acceptor, oxygen. The electron transport is coupled to proton pumping from the matrix to the intermembrane space, producing an electrochemical gradient. The gradient is used by complex V to make ATP. C I-V = complexes I-V, Q = ubiquinone, cyt = cytochrome. Direction of electron (e-) and proton (H+) flow is indicated by arrows.

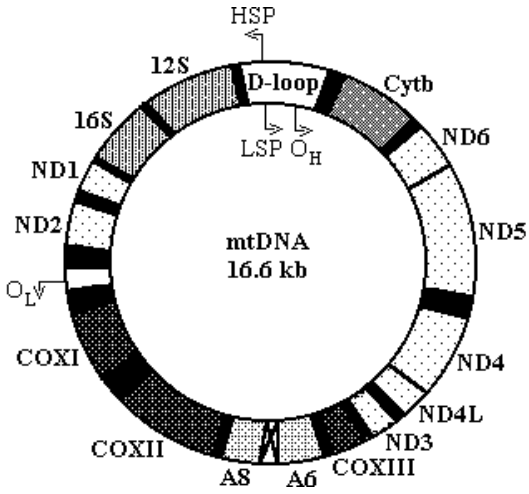

Figure 8. Mitochondrial DNA. The duoble-stranded circular mitochondrial genome consists of an outer H-strand (heavy) and an inner L-strand (light). MtDNA encodes two rRNAs (12S, 16S) and 22 tRNAs (black areas) for its own protein synthesis, and 13 mRNAs for protein subunits of the respiratory chain complexes: ND1-6 for complex I, Cyt b for complex III, COX I-III for complex IV, and A6 and A8 for complex V. D-loop, displacement loop; O_H and O_L, replication origins of H- and L-strands; HSP and LSP, promoters for transcription from H- and L-template strands.

In adult patients, abnormalities of oxidative phosphorylation are often associated with rearrangements or point mutations affecting a subpopulation of mitochondrial genomes, i.e. these mutations are usually heteroplasmic (Schon et al. 1994, Taanman et al. 1997). An important cause of mitochondrial dysfunction in neonates and infants is a depletion of mtDNA (Moraes et al. 1991, Taanman et al. 1997). These patients present soon after birth with muscle weakness, hepatic failure or renal tubulopathy associated with defective oxidative phosphorylation and a severe depletion of mtDNA (88-99 %) in affected tissues. The infants usually die before 9 months of age. Patients often show a deficiency of complex IV (COX) and other enzyme complexes containing mtDNA encoded subunits (Moraes et al. 1991, Taanman et al. 1997). The molecular defect responsible for mtDNA depletion syndrome has not yet been identified. Although the basic mechanism of mtDNA replication has been elucidated, knowledge of the regulatory factors is still limited and only a few protein factors have been purified. Furthermore, knowledge concerning maintenance of mtDNA in the cell is scarce, making it difficult to suggest candidate genes that could be defective. Autosomal recessive inheritance of mtDNA depletion syndrome suggests a mutation in nuclear DNA as the primary cause of the defect (Moraes et al. 1991). In the study by Bodnar et al. (1993), mtDNA levels could be restored by complementation with nuclear DNA from a control cell line, providing further evidence for the involvement of nuclear-encoded controlling factor. Taanman et al. (1997) have speculated that the replication is arrested due to the nuclear-encoded factor, which is tissue-

specifically and developmentally regulated reflecting tissue-specific and neonatal expression of symptoms. This factor could be involved in the fine tuning of mtDNA levels to the demands of the cell and tissue.

Mitochondrial diseases often show changes in the kidney as a part of multiorgan involvement, more frequently in children (Szabolcs et al. 1994, Hsieh et al. 1996, Singh et al. 1996, van Biervliet et al. 1977, Jansen et al. 1997, Niaudet & Rötig 1997). The most frequent renal manifestations are proximal tubular damage, glomerular disease with nephrotic syndrome and tubulointerstitial nephropathy. Renal filtration is highly dependent on mitochondrial energy production (Singh et al. 1996) and the kidney shows strong COX activity in histochemical staining earlier than most other tissues at the second trimester of pregnancy (Moggio et al. 1989), coinciding with the onset of glomerular filtration. It is noteworthy, that the vicinity of the nephrin gene NPHS1 locus in the long arm of chromosome 19 contains some genes involved in energy metabolism or mitochondrial function, including genes for COX VIb (Taanman et al. 1991), ATPase ATP4A (Song et al. 1992), and phospholipid hydroperoxide glutathione peroxidase (PHGPx) (Kelner & Montoya 1998). In this respect, it is interesting that Männikkö et al. (1995) have reported an apparent mitochondrial disease in one of their CNF families. Whether this genomic region, or the nephrin in general, has any connections to our mitochondrial findings remains to be studied.

5.2. Cytochrome-c oxidase subunit (COX) gene expression

Cytochrome-c oxidase (COX) is the terminal enzyme in the respiratory chain. The mammalian enzyme is composed of 13 subunits (Capaldi et al. 1995). The three largest subunits (I, II, and III) are encoded on mtDNA and are synthesized inside the mitochondrion. The remaining subunits (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII; nomenclature of Kadenbach et al. 1983) are encoded in the nucleus, synthesized in the cytosol, and imported into mitochondria. The catalytic site of the COX enzyme complex is composed mainly of mitochondrially synthesized subunits (Capaldi et al. 1995, Liu & Wong-Riley 1995). The occurrence of tissue-specific isoforms of some nuclear encoded subunits (VIa, VIIa, and VIII) suggests that they confer a regulatory role by adjusting the enzyme activity to the metabolic demands of various tissues (Liu & Wong-Riley 1995). Enhanced interest in cytochrome-c oxidase has come from the realization that deficiences of this enzyme are associated with a number of human diseases, including Leigh syndrome, chronic progressive ophthalmoplegia, Kears-Sayre syndrome, and fatal and benign infantile mitochondrial myopathy (Larsson et al. 1990, Moraes et al. 1991, Schon et al. 1994, Capaldi et al. 1995, Johns 1995, Singh et al. 1996).

Because of our findings with COX I in CNF, we continued analyzing the expressions of also other components of the cytochrome-c oxidase complex. Mitochondrially encoded subunit COX II as well as two nuclearly encoded subunits COX IV and VIb were studied by Northern blotting analysis. Similarly to COX I, COX II mRNA showed consistent underexpression, about 30 % of normal, in CNF kidney tissues. In contrast, the levels of the nuclearly encoded mRNAs for subunits COX IV and VIb did not differ from the levels of controls. To localize the mRNA expressions we performed in situ hybridization experiments with our DDRT-PCR clone CNF B36, COX I, COX II, COX IV, and COX VIb. Decreased expressions of CNF B36, COX I and COX II mRNAs were observed especially in the proximal and distal tubuli in CNF cortical kidneys as compared to the controls, but again, the nuclearly encoded COX IV and VIb mRNAs failed to show any difference in signal intensity between CNF and control kidney samples. Due to the overwhelming number of mitochondria in tubular cells, only a very faint reactivity of COX mRNAs could be seen in

glomeruli. Thus, we analyzed the transcript levels directly from isolated glomeruli by semi-quantitative RT-PCR. We found the same downregulation of COX I but not the nuclearly encoded subunits also in CNF glomeruli. In order to extend the analysis to other complexes of the respiratory chain, we also performed RT-PCR for glomerular cytochrome b expression, a respiratory chain complex III component encoded by the mitochondrial genome. This showed an underexpression comparable to that of COX I in CNF glomeruli.

To study the mitochondrial defect in CNF at the protein level, we first used immunohistochemical analysis. A monoclonal antibody recognizing the mitochondrially encoded COX I showed an intense, finely granular pattern of protein expression in the proximal and distal tubular cells, whereas glomerular reactivity was diffuse and faint. Again, a consistent decrease in staining was seen in tubuli of CNF kidneys as compared to controls. Immunohistochemical staining for the nuclear encoded COX IV showed intense reactivity in tubular cells, but no difference was apparent between CNF and control tissues.

COX biosythesis is a very complex process not only because it is involved in the expression of both nuclear and mitochondrial genomes, but also because the regulatory factors required for gene expression differ between the two genomes. Moreover, modulations of transcriptional and translational controls may be via tissue and cell type-specific factors, and the same subunit may be regulated differently among tissues or cell types. COX subunits may also be regulated independently from one another. The maintenance of an optimal mitochondrial function requires finely tuned participation of tens of nuclear and mitochondrial encoded, tissue-, cell type- and developmental stage specific factors which must be correctly targeted, recognized, processed and assembled into the mitochondria in stoichiometric amounts (Voos et al. 1994). It has been reported that, at steady state, nuclear and mitochondrial genomes are proportionally regualted for COX subunit mRNAs. This is evident from studies where the ratio of COX III mRNA to COX VIc mRNA is equal in each rat tissue analysed on slot blots (Hood 1990, Gagnon et al. 1991) and a similar pattern of distribution of subunits I, IV and VIII mRNA in in situ hybridization (Hevner & Wong-Riley 1993). However, little is known about the regulation of gene products from the two genomes in response to the altered cellular activity. Hevner & Wong-Riley (1993) and Liu & Wong-Riley (1995) have shown that nuclear and mitochondrial genomes are disproportionately regulated at COX subunit mRNA and protein levels by altered neuronal activity. In addition, the disproportionate regulation of the two genomes is found to be true in skeletal muscles of patients with partial COX deficiencies (Mita et al. 1989). Our findings of a constant underexpression of mitochondrially encoded mRNAs and proteins indicate a disproportionate expression of the two genomes in CNF kidneys. This may result from a defective nuclear-mitochondrial interaction or from a primary mitochondrial defect (Nagley 1991, Scarpulla 1997). We could, however, exclude the mitochondrial defect at the posttrancriptional level, because the levels of mitochondrial mRNAs were comparable to protein levels.

5.3. Other respiratory chain complexes

To further study the respiratory chain impairment in CNF kidneys, we performed histochemical analysis for the activity of respiratory complex II (succinate dehydrogenase, SDH) and complex IV (cytochrome-c oxidase, COX). Similar to the in situ hybridization and immunohistochemistry, the histochemical reaction products in the glomeruli were weak in both control and CNF kidneys precluding reliable comparison. However, the tubular reactivity of both SDH and COX were remarkably decreased in CNF in comparison to control kidney cortex. The steady state levels of also

other mitochondrial respiratory chain complexes were determined with the blue native gel electrophoresis technique (BN-PAGE). Here, CNF patient kidneys showed a consistent decrease of all respiratory chain complexes from 10% (complex I) to 30% (complexes II-V) of controls. These findings indicate that the enzymatic activities and the amounts of complexes show a good correlation not only to each other but also to mitochondrial expression of subunit protein and mRNA levels. Thus, it appears as the levels of mitochondrial subunits are the limiting factor in the formation of functional enzyme complexes in CNF kidneys, and in this respect, the nuclear subunits are overexpressed. Interestingly, also complex II, which is completely nuclearly encoded and thus apparently trancripted in normal levels in CNF, shows decreased activity.

The normal ratios of the complexes II-V in BN-PAGE excluded a specific deficiency in any of these complexes in CNF. Rather, these findings suggest an impaired mitochondrial transcription machinery or defective replication of mtDNA leading to mtDNA depletion. Indeed, Solin et al. (2000) have later continued these studies and found a general decrease of mtDNA level in CNF kidney tissues in Southern blotting analysis, suggesting a mtDNA depletion in CNF. This could well explain our findings of the underexpression of mitochondrial mRNA and protein subunit levels.

Interestingly, Bentlage et al. (1995) have described neuron specific symptoms in a disease with a similar remnant mitochondrial activity. This was explained by the fact that terminally differentiated neurons can not compensate functional over non-functional mitochondria due to lack of cell (and interrelated mitochondrial) proliferation (Johns 1995). This could also apply to glomerular podocytes which in general do not proliferate (Pabst & Sterzel 1983). Furthermore, Mandel et al. (1993) have shown that a decline in cellular ATP levels leads to increased epithelial permeability in MDCK (cockerspaniel kidney epithelial) cells; such a decrease of ATP content specifically disrupts the architecture of actin and intermediate filaments (Gabai & Kabakov 1993) which are considered especially important for the maintenance of the complex structure of podocytes (Shirato et al. 1996). These findings could explain the common morphologic changes including flattening and retraction of podocyte foot processes seen in CNF.

5.4. Mitochondrial morphology

The size, shape and quantity of mitochondria vary between tissues and even in different locations within the same tissue (Yaffe 1999). A common feature of all mitochondria is the presence of a double membrane. By electron microscopy, we wanted to study the amount and morphology of mitochondria in CNF and control kidney cortex. No statistically significant differences were found in the amount or density of mitochondria in CNF samples as compared to controls (51.7 ± 6.6 / study area in CNF, 61.5 ± 2.1 in controls, p-value not significant). As a morphological sign of possible mitochondrial involvement in CNF, we found characteristic concentric intramitochondrial bodies consisting mostly of double membranes within otherwise normal appearing mitochondria. In the CNF samples studied, these morphologic changes were present in 13.0 to 44.4% of all mitochondria (mean 26.9%); in controls, only 0 to 2% of such abnormal mitochondria (mean 0.7%, $p<0.001$) were found. Thus, the morphological abnormality in CNF is apparent, but as the amount of abnormal mitochondria does not directly correlate to the decreased expression levels, the meaning remains unclear. However, it is known that many mitochondrial diseases are defined by morphologically abnormal mitochondria in addition to defects in energy metabolism (Moraes et al. 1991, Szabolcs et al. 1994, Hsieh et al. 1996, Niaudet & Rötig 1997).

5.5. Reactive oxygen species (ROS) and lipid peroxidation (LPO)

Mitochondria consume about 90 % of the body´s oxygen and are particularly rich source of reactive oxygen species (ROS) (Chance et al. 1979). Molecular oxygen that is needed for energy production is itself toxic and its further reactions produce additional ROS, such as superoxide and hydroxyl radicals, singlet oxygens, and hydrogen peroxide (Halliwell 1991). ROS are constantly generated in cells like granulocytes and are benefical in many situations. However, an imbalance between generation and the defence mechanisms against ROS can result in damage. ROS can cause DNA damage, protein oxidation, and lipid peroxidation (LPO) affecting critical enzyme systems and destroying membranes (Halliwell 1991). ROS have been connected with several disease states, such as neurological damage, diabetic cataract, Down´s syndrome and immunological conditions. Furthermore, in tissue injury due to another cause, free radicals may exacerbate destruction. There is also plenty of recent evidence that ROS and LPO are distinctly associated with various types of experimental glomerulonephritis (Thakur et al. 1988, Diamond 1992, Johnson et al. 1994, Neale et al. 1994, Binder et al. 1999).

It has been postulated that mitochondrial respiratory chain-associated diseases also may involve toxicity caused by ROS and recently, a deficiency of mitochondrial complex I has been shown to result in excessive production of superoxide radicals, which in turn causes excessive formation of hydroxyl radicals and aldehydic lipid peroxidation (Pitkänen & Robinson 1996, Luo et al. 1997). Whether free radicals are a major cause of cell injury in mitochondrial disease or whether they arise as secondary products in deteriorating tissue has not been fully established. As a secondary indication of mitochondrial dysfunction in CNF, we wanted to analyze local lipid peroxidation within CNF glomeruli. LPO is an autocatalytic process that damages lipid-containing structures, such as membranes and intracellular lipids, and yields reactive by-products, such as malondialdehyde (MDA) (Montine et al. 1996). In immunohistochemistry with anti-MDA antibodies, we found finely granular glomerular staining within podocytes and along the glomerular basement membrane in CNF, whereas neglible reactivity was seen in the control samples. This suggests enhanced glomerular LPO in CNF kidneys. This is an intriguing finding as glomerular lipid peroxidation has been implicated as an important factor in the generation of proteinuria. A detailed analysis of the pathogenesis of proteinuria in the Heymann nephritis rat model has revealed a chain of events that include intraglomerular overproduction of ROS, generation of LPO adducts in the podocytes and GBM, and crosslinking of type IV collagen via its NC-1 domains (Neale et al. 1994, Kerjaschki & Neale 1996). Furthermore, in rats with PAN nephritis overproduction of ROS and local accumulation of LPO adducts were identified as prime causes for glomerular damage (Johnson et al. 1994). Interestingly, we have found (Holthöfer et al., unpublished data) that in puromycin induced glomerular damage mitochondrial dysfunction develops prior to the onset of proteinuria suggesting of a causal relationship. Overproduction of ROS and intraglomerular LPO are also clearly involved in the glomerular disease of Mpv 17-inactivated mice in knock out mice model. Mpv 17 gene product is a peroxisomal membrane protein which probably plays a major role in the peroxisomal metabolism of ROS (Zwacka et al. 1994).

Together these data suggest a pathogenic role for ROS and LPO in proteinuria, also in CNF, but the exact mechanisms involved are yet to be defined. In the kidney, antioxidant enzyme expression has been shown to be particularly high compared with several other organs (Marklund 1984, Gwinner et al. 1998). However, despite this high antioxidant expression, the glomerulus seems to be a target for oxygen radical attack in many glomerular diseases (Gwinner et al. 1998). In CNF, we have later

confirmed a distict increase of local LPO products 4-hydroxynonenal (4-HNE) and MDA especially in the glomerular epithelial cells, and also shown that the local antioxidant defence against LPO provided by the enzyme phospholipid hydroperoxide glutathione peroxidase (PHGPx) was decreased (Solin et al. 2001). This could make CNF glomeruli even more susceptible to oxygen stress and damage. Based on these considerations and on our results of decreased mitochondrial activity and enhanced LPO in CNF kidneys the following hypothesis can be stated: in CNF, an unknown damage leads to mtDNA depletion which results in a decrease in local energy production and modification of ROS generation. Together with defective antioxidant defense, as shown in CNF glomeruli, this may result in severe functional consequences, such as proteinuria.

SUMMARY AND CONCLUSIONS

The pathomechanisms of kidney disease manifested by proteinuria and nephrotic syndrome have been a mystery for decades, and the molecular properties of the underlying causes have remained poorly understood. Congenital nephrotic syndrome of the Finnish type (CNF) is a clinically well-characterized disorder of the Finnish disease inheritage, with severe primary proteinuria. CNF is now known to be caused by a mutation in the recently described nephrin gene. At the time of this study, the basic defect of CNF was still unknown, although several hypotheses on its pathogenesis and mechanisms of proteinuria had been presented. The first part of this thesis was undertaken to test the prevailing theories on failed kidney maturation (**I, II)** and abnormal regulation of filtration **(III)** in CNF. The second series of experiments (**IV, V**) was planned to find new candidate molecules possibly involved in the pathogenesis of CNF and proteinuria.

In morphological examination (**I**), CNF kidneys revealed a decreased cortical thickness and areas of poor demarcation between cortical and medullary compartments. Peritubular areas of poorly organized cells expressing markers of early differentiation (fibronectin, stem cell factor, cytokeratin) were found in CNF, which suggests retarded differentiation in this compartment; however, we could not show aberrations in the apoptotic (Bcl-2, acridine orange) or cell proliferation pathways (PCNA) by immunohistochemical stainings. In CNF, densely clustered glomeruli were often seen at the corticomedullary demarcation zone resulting in abnormally high density of glomeruli in these areas. This may reflect abnormal mesenchymo-epithelial interactions during renal development in CNF. Furthermore, we could occasionally find large glomeruli suggesting fusion of two to three individual glomeruli. Taken together, these findings of morphologic abnormalities and areas of irregular tissue organization in CNF kidneys and glomeruli suggest the possibility of developmental failure or arrest in CNF kidneys.

In the second study (**II**) of this thesis, we systematically studied the expression of nine genes associated with kidney maturation from samples of normal human fetal, juvenile and adult kidneys and cultured glomerular cells using Northern blotting analysis. Subsequently, kidneys from patients with CNF were studied for evidence of dedifferentiation or persistence of a fetal expression pattern. The candidate marker genes for kidney maturation level analyzed here included transcription factors Pax-2, WT-1 and EGR-1, growth factors IGF-1, IGF-2, MK and HB-GAM and receptors IGFBP-1 and trk-B. However, on the basis of these gene expression profiles, we could not find any conclusive evidence of developmental abnormality in the CNF kidneys. The cultured glomerular cells showed remarkable inconsistency to respective tissue samples reducing their usefulness in developmental studies.

Vascular permeability factor (VPF) has been among the candidate molecules proposed to be involved in the pathogenesis of proteinuria. The third study (**III**) was designed to clarify the role of VPF in increased glomerular permeability in CNF kidneys. For that, we studied normal human, fetal and CNF kidney tissues and the corresponding cultured glomerular cells for VPF and its receptor KDR expression using Northern and in situ hybridization techniques and immunohistologic stainings. Our findings failed to show any significant differences in their expressions in CNF kidneys and thus, propose that VPF system is not directly involved in the pathogenesis of proteinuria in CNF. However, we observed VPF expression in a previously unrecognized site, in the juxtaglomerular area of the nephron.

Differential display RT-PCR is a new powerful technique for identification and characterization of altered gene expression. Using DDRT-PCR approach and isolated glomeruli, we compared the gene expression patterns of normal human and CNF kidney glomeruli (**IV**). Differential expression of candidate genes was verified by Northern blotting, and the corresbonding PCR fragments were sequenced and compared to known sequences in databanks. We found several genes and sequence tags with altered expression in CNF glomeruli including fragments with close homologies to cytochrome-c oxidase subunit I, integrin-linked kinase, insulin-like growth factor 2 receptor and eotaxin, and also fragments resembling anchyrin and cadherin family-like consensus sequences. Thus, the method enabled us not only to diagnose changes in gene expression pattern involved in the pathogenesis of proteinuria but also to reveal potentially novel glomerular members of known gene families.

Especially, the observation that one of the DDRT-PCR products showed 98% similarity to the subunit I of the mitochondrial respiratory chain enzyme cytochrome-c oxidase (COX I) is striking: in Northern blotting analysis with the DDRT-PCR clone, a mRNA of the expected size for COX I was detected and the intensity of the signal appeared 70% lower in CNF than in controls. Renal filtration is highly dependent on mitochondrial energy production and the kidney shows strong COX activity earlier than most other tissues during fetal life, coinciding with the onset of glomerular filtration. Furthermore, a decrease of ATP content specifically disrupts the architecture of cytoskeletal filaments which are considered especially important for the maintenance of the complex structure of the podocytes.

In the fifth study of this thesis (**V**), we proceeded to further characterize the mitochondrial involvement in CNF kidneys. We found a constant underexpression, about 30 % of normal, of all the mitochondrially encoded respiratory chain complex subunit mRNAs and proteins tested, but the nuclearly encoded subunits did not differ from the levels of controls, as examined by Northern and in situ hybridization, by immunohistochemical staining and by semiquantitative PCR of isolated glomeruli. This indicates a disproportionate expression of the nuclear and mitochondrial genomes in CNF kidneys resulting probably from a defective nuclear-mitochondrial interaction or from a primary mitochondrial defect.

In histochemical analysis, the activities of respiratory chain enzyme complexes were remarkably decreased in CNF in comparison to control kidney cortex. By blue native polyacrylamide gel electrophoresis (BN-PAGE) technique, CNF patient kidneys showed a consistent decrease of all enzyme complexes from 10% to 30% of controls. These findings indicate that the enzymatic activities and the amounts of complexes show a good correlation to each other and also to mitochondrial expression of subunit protein and mRNA levels. Thus, it appears that the levels of mitochondrial subunits are the limiting factor in the formation of functional enzyme complexes in CNF kidneys. The normal ratios of the complexes in CNF excluded a specific deficiency in any of these complexes, but suggest an impaired mitochondrial transcription machinery or defective replication of mtDNA leading to mtDNA depletion. Solin et al. (2000) have later studied these possibilities and found a general decrease of mtDNA level in CNF kidney tissues in Southern blotting analysis, suggesting a mtDNA depletion in CNF. This could well explain our findings of the underexpression of mitochondrial mRNA and protein subunit levels.

By electron microscopy, no statistically significant differences were found in the amount or density of mitochondria in CNF samples as compared to controls. As a morphological sign of possible

mitochondrial involvement in CNF, we found characteristic concentric intramitochondrial bodies within otherwise normal appearing mitochondria. In the CNF samples studied, these morphologic changes were present in 26.9% of all mitochondria; in controls, only 0.7% of such abnormal mitochondria were found. Thus, the morphological abnormality in CNF is apparent. As a secondary indication of mitochondrial dysfunction in CNF, we analysed a local lipid peroxidation (LPO) product, malondialdehyde (MDA) within CNF glomeruli. In immunohistochemistry with anti-MDA antibodies, we found glomerular staining within podocytes and along the glomerular basement membrane in CNF, whereas neglible reactivity was seen in the control samples. This suggests also enhanced glomerular LPO in CNF kidneys. This is an intriguing finding as reactive oxygen species (ROS) and glomerular LPO have been implicated as important factors in the generation of proteinuria in experimental types of glomerulonephritis.

In conclusion, based on all these considerations and on our results of decreased mitochondrial activity and enhanced LPO in CNF kidneys the following hypothesis can be stated: in CNF, an unknown damage leads to mtDNA depletion and subsequently to a decrease in local energy production and modification of ROS generation. Together with defective antioxidant defense, as shown in CNF glomeruli, this may result in severe functional consequences, such as proteinuria. If this chain of events could somehow be triggered by a change in nephrin gene remains to be studied.

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