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# Cell-cycle regulatory proteins in podocyte cell in idiopathic nephrotic syndrome of childhood

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## Cell-cycle regulatory proteins in podocyte cell in idiopathic nephrotic syndrome of childhood.

**Background.** The podocyte cell is believed to play an important role in idiopathic nephrotic syndrome (INS) of childhood. In adults with cellular and collapsing focal segmental glomerulosclerosis (FSGS), the expression of cell-cycle regulatory proteins such as p27, p57, and cyclin D is decreased and expression of cyclin A, Ki-67, and p21 is observed in podocyte cells suggestive of a dysregulated podocyte phenotype. We investigated for alterations in the expression of cyclin kinase inhibitors, p27, p57, p21, and cyclins D and A in the podocyte cell of children with INS.

**Methods.** Forty-two kidney biopsies were investigated; 14 with minimal-change disease (MCD), seven with diffuse mesangial hypercellularity (DMH), 12 with FSGS, four with Alport syndrome (AS), and five normal biopsies. The sections were examined by immunohistochemistry using dual staining method. Podocyte cells were first identified by Wilm's tumor-1 staining after which expressions of cell-cycle regulatory proteins were analyzed. A quantitative analysis was performed for the proportion of podocyte cells that expressed each cell cycle regulatory protein.

**Results.** On light microscopy, all podocyte cells expressed p27, while p57 and p21 expression was seen in a portion of podocyte cells in normal kidney biopsies. Cyclin D was expressed in a small percent of podocyte cells though the expression was more marked in mesangial and endothelial cells. Cyclin A expression was not seen in normal biopsies. The mean expression of p27 decreased significantly in order from normal (100%), MCD (45.9%), DMH (22.4%), and FSGS (16.7%), and the difference between MCD and FSGS was significant. p21 was significantly and equally reduced in MCD (2.3%), DMH (0%), and FSGS (0.7%) compared to normal (66.6%). There was no significant difference in expression of p57, cyclin D and cyclin A in the podocyte cells between normal and children with INS. Children with AS showed a significant decrease in p27 and p21 expression, while the expression of p57, cyclin D and cyclin A were unchanged from normal, thus demonstrating a pattern similar to INS.

**Key words:** cyclin A, cyclin D, p21, p27, p57, idiopathic nephrotic syndrome, immunohistochemistry, children.

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**Conclusion.** The podocyte cell in children with INS down-regulates expression of cyclin kinase inhibitors such as p21 and p27, but not p57, but does not up-regulate cyclin D and cyclin A that are needed to overcome the G<sub>1</sub>/S transition and move the cell forward in the cell cycle process. Thus, the podocyte cell remains trapped in the G<sub>1</sub> arrest phase. In children with INS or AS, the dysregulated podocyte phenotype is different than the one described in adults with cellular or collapsing FSGS.

Idiopathic nephrotic syndrome of childhood (INS) is a common renal disease in children. On biopsy, the common histologies of INS include minimal-change disease (MCD), diffuse mesangial hypercellularity (DMH), and focal segmental glomerulosclerosis (FSGS), which together constitute ~90% of childhood INS [1]. MCD is characterized by minimal changes in the glomerulus, responds well to steroid treatment, and rarely progresses to renal failure. On the other end of the spectrum, FSGS is characterized by segmental glomerular hyalinosis and sclerosis, responds poorly to steroid treatment, and frequently progresses to renal failure [2]. The clinical course of DMH lies between MCD and FSGS. The visceral glomerular epithelial cell or podocyte cell plays an important role in glomerular ultrafiltration, glomerular basement membrane turnover, support for the glomerular capillary tuft, and formation of urine [3]. The podocyte cell demonstrates significant alteration, such as effacement of foot processes, apical displacement of slit diaphragms, and detachment of the podocyte from the glomerular basement membrane in INS [4–6]. In experimental animals, Laurens et al [7, 8] have reproduced INS by isolated injury to the podocyte. We found synaptopodin, a podocyte protein, to decline with increasing severity of INS, manifested by a decrease in synaptopodin expression from MCD to DMH to FSGS [9]. We also showed other podocyte proteins, such as GLEPP-1 and nephrin, to be decreased in INS [10]. It thus seems that in the event of podocyte dysfunction/ injury, it clinically manifests as proteinuria, and at the molecular level podocyte proteins such as synaptopodin, GLEPP-1, and nephrin become disorganized.

The cell-cycle process is a highly organized process that allows the cell to proliferate under both physiologic and pathologic conditions. The process ensures that DNA replication occurs only once and that the DNA replication is completed before mitosis occurs in each cycle [11–15]. This highly organized cell-cycle process is regulated by a number of cell-cycle regulatory proteins: (1) positive cell-cycle regulatory proteins, such as “cyclins” and “cyclin-dependent kinase (CDK),” which aid in the progression and completion of the cell cycle; and (2) negative cell-cycle regulatory proteins, “cyclin kinase inhibitors (CKI),” which inhibit the cell-cycle process. Cells can also exit from the cell cycle at any phase by undergoing apoptosis (death of cell), senescence (permanently growth-arrested cells), terminally committed specialized cells (cells can proliferate under appropriate stimuli), or be uncontrolled as in neoplasia [13]. Mature podocyte cells in the glomerulus are regarded as growth-arrested cells that normally express negative cell-cycle regulatory proteins p27 and p57 and positive cell-cycle regulatory proteins cyclin D, but not cyclin A, cyclin B1, or Ki-67 [16]. In adult subjects with cellular and collapsing variant of FSGS and HIV-associated nephropathy (HIVAN), Shankland et al [17] found decreased p27 and p57 expression and de novo expression for p21 in podocyte cells. Barisoni et al [18] reported a decrease in p27, p57, and cyclin D1 expression with expression for cyclin A and Ki-67 in podocyte cells in collapsing glomerulopathies. On the other hand, Nagata et al [19] showed that the proliferating epithelial cells in FSGS are not podocyte cells but parietal epithelial cells.

The podocyte is a unique glomerular cell because its growth response to injury differs from the mesangial or endothelial cell by virtue of its apparent inability to proliferate [13]. The podocyte cell plays an important role in progressive glomerulosclerosis and subsequent chronic renal failure [20–23]. In childhood INS, the injury to the podocyte cell is an acquired immunologic injury, whereas in Alport syndrome (AS) the podocyte injury is subsequent to a genetic mutation in  $\alpha$ -5 of collagen type IV of the glomerular basement membrane [24]. We studied the cell cycle regulatory protein such as p27, p57, p21, cyclin D, and cyclin A expression in podocytes in both INS and AS to answer the question as to whether the alterations in protein expression is a generalized response to podocyte injury or whether it is exclusive to INS.

## METHODS

Kidney biopsies from children with INS were included in the study only if the histology was consistent with MCD, DMH, or FSGS as described by the International Study of Kidney Disease in Children (ISKDC) [25] and the tissue in the archived paraffin block had at least five non-sclerosed glomeruli. Control tissue (normal) was obtained

from children who had normal renal biopsy following evaluation of loin pain-hematuria syndrome. A total of 42 kidney biopsies were investigated: 14 with MCD, seven with DMH, 12 with FSGS, four with AS, and five whose biopsies were normal. The age at the time of biopsy was noted. Serial 3  $\mu$ m sections were obtained from the cases listed above, air-dried, and heat fixed on slides. The sections were deparaffinized with xylene and iodine and rehydrated in graded series of alcohol. The sections were treated with Target Retrieval Solution (Dako #S1700, Dako Corp., Carpinteria, CA, USA) in a steamer at 90°C to 95°C for 20 minutes and then cooled for 15 minutes. The endogenous avidin and biotin activity was blocked by egg white and skimmed milk as described by Miller et al [26]. The sections were examined by immunohistochemistry using dual staining method. Podocyte cells were identified in the biopsy by Wilm’s tumor-1 (WT-1) staining as it is exclusively expressed in the podocyte cells in the glomerulus [27]. Antihuman WT-1, a mouse monoclonal antibody (Dako Corp.) was used in 1:100 dilution for WT-1 staining. The Universal Dako’s LSAB + immunostaining system with streptavidin-biotin conjugated to alkaline phosphatase was used for the immunohistochemical reaction. Vector red (Vector Laboratories, Inc., Burlingame, CA, USA) was used as the chromogen, which stained WT-1 antigen red. The cell-cycle regulatory proteins used in the study were as follows: (1) p27 staining was performed with a mouse monoclonal antibody (sc-1641; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:50 dilution, (2) p21 staining with a mouse monoclonal antibody (sc-817; Santa Cruz Biotechnology Inc.) at 1:100 dilution, (3) p57 staining with a goat polyclonal antibody (sc-1040G; Santa Cruz Biotechnology Inc.) at 1:50 dilution, (4) cyclin D1 staining with a mouse monoclonal antibody (sc-8396; Santa Cruz Biotechnology Inc.) at 1:50 dilution, and (5) cyclin A staining with a rabbit polyclonal antibody (sc-751; Santa Cruz Biotechnology Inc.) at 1:100 dilution. The Universal Dako’s LSAB + immunostaining system with streptavidin-biotin conjugated to horseradish peroxidase was used in the immunohistochemical reaction for cell-cycle regulatory proteins. Diaminobenzidine with 3% cobalt was used as the chromogen, which gave a brown-black color to cell-cycle antigen. In addition, a negative control using an irrelevant antibody, a positive control for single WT-1 immunohistochemical reaction, a positive control for single cell-cycle regulatory protein immunohistochemical reaction and a positive control for dual WT-1 and cell-cycle regulatory protein immunohistochemical reaction were also performed. For control immunohistochemical reactions, a control kidney tissue was used for p27, p57, and cyclin D, and a lymphoma tissue was used for p21 and cyclin A. In addition to the above controls for the experiment, positive expression of cell-cycle regulatory

**Table 1.** Expression of p27, p21, p57, and cyclin D in podocyte cells of children with idiopathic nephrotic syndrome (INS). The results are expressed as mean  $\pm$  SD

	Percent-positive glomeruli				Percent-positive podocyte			
	Normal (N = 5)	MCD (N = 14)	DMH (N = 7)	FSGS (N = 12)	Normal (N = 5)	MCD (N = 14)	DMH (N = 7)	FSGS (N = 12)
p27	100.0 $\pm$ 0.0	66.9 $\pm$ 39.5	45.6 $\pm$ 37.4	39.7 $\pm$ 35.3	100.0 $\pm$ 0.0	45.9 $\pm$ 31.5	22.4 $\pm$ 28.0	16.7 $\pm$ 18.9
Normal vs. (P value)	—	0.291	0.058	0.015	—	0.001	<0.001	<0.001
MCD vs. (P value)	—	—	0.565	0.224	—	—	0.203	0.028
p21	100.0 $\pm$ 0.0	9.2 $\pm$ 15.2	0.0 $\pm$ 0.0	2.8 $\pm$ 6.7	66.6 $\pm$ 12.5	2.3 $\pm$ 4.3	0.0 $\pm$ 0.0	0.7 $\pm$ 1.7
Normal vs. (P value)	—	<0.001	<0.001	<0.001	—	<0.001	<0.001	<0.001
MCD vs. (P value)	—	—	0.222	0.396	—	—	0.557	0.686
p57	100.0 $\pm$ 0.0	99.1 $\pm$ 2.0	86.3 $\pm$ 24.9	96.4 $\pm$ 7.1	60.6 $\pm$ 7.0	44.7 $\pm$ 18.7	37.0 $\pm$ 21.5	45.0 $\pm$ 18.2
Normal vs. (P value)	—	0.999	0.243	0.945	—	0.351	0.138	0.385
MCD vs. (P value)	—	—	0.095	0.931	—	—	0.795	0.999
Cyclin D	23.4 $\pm$ 11.1	8.4 $\pm$ 14.9	8.2 $\pm$ 11.7	0.0 $\pm$ 0.0	3.1 $\pm$ 2.8	1.6 $\pm$ 3.7	0.7 $\pm$ 1.0	0.0 $\pm$ 0.0
Normal vs. (P value)	—	0.097	0.147	0.005	—	0.752	0.452	0.171
MCD vs. (P value)	—	—	0.999	0.243	—	—	0.852	0.359

Abbreviations are: MCD, minimal change disease; DMH, diffuse mesangial hypercellularity; and FSGS, focal segmental glomerulosclerosis.

protein in tubular cells acted as controls within each section.

Kidney biopsies stained for WT-1 and one of the cell-cycle regulatory proteins were first examined by light microscopy. On light microscopy, evaluation of each glomerulus was done for cells expressing WT-1 and cell-cycle regulatory protein (i.e., podocyte cell expressing the protein under study), WT-1 protein only (i.e., podocyte cell not expressing the protein under study), and only cell-cycle regulatory protein but not WT-1 (non-podocyte glomerular cell expressing the protein under study). Following an initial qualitative assessment, a quantitative analysis for cell-cycle regulatory protein expression was performed. First, the percent of glomeruli that stained positive for the cell-cycle regulatory protein in the biopsy section was obtained. Next, in glomeruli that stained positive for cell-cycle regulatory protein, the percent of podocyte cells that express the cell cycle regulatory protein was obtained. The product of the above two values gave the proportion of total podocyte cells that expressed the cell-cycle regulatory protein. To compare cell-cycle regulatory protein expression and age among the four groups of normal, MCD, DMH, and FSGS children, univariate analysis of variance (ANOVA) followed by Tukey HSD test was used. Student *t* test was used to compare normal with AS.

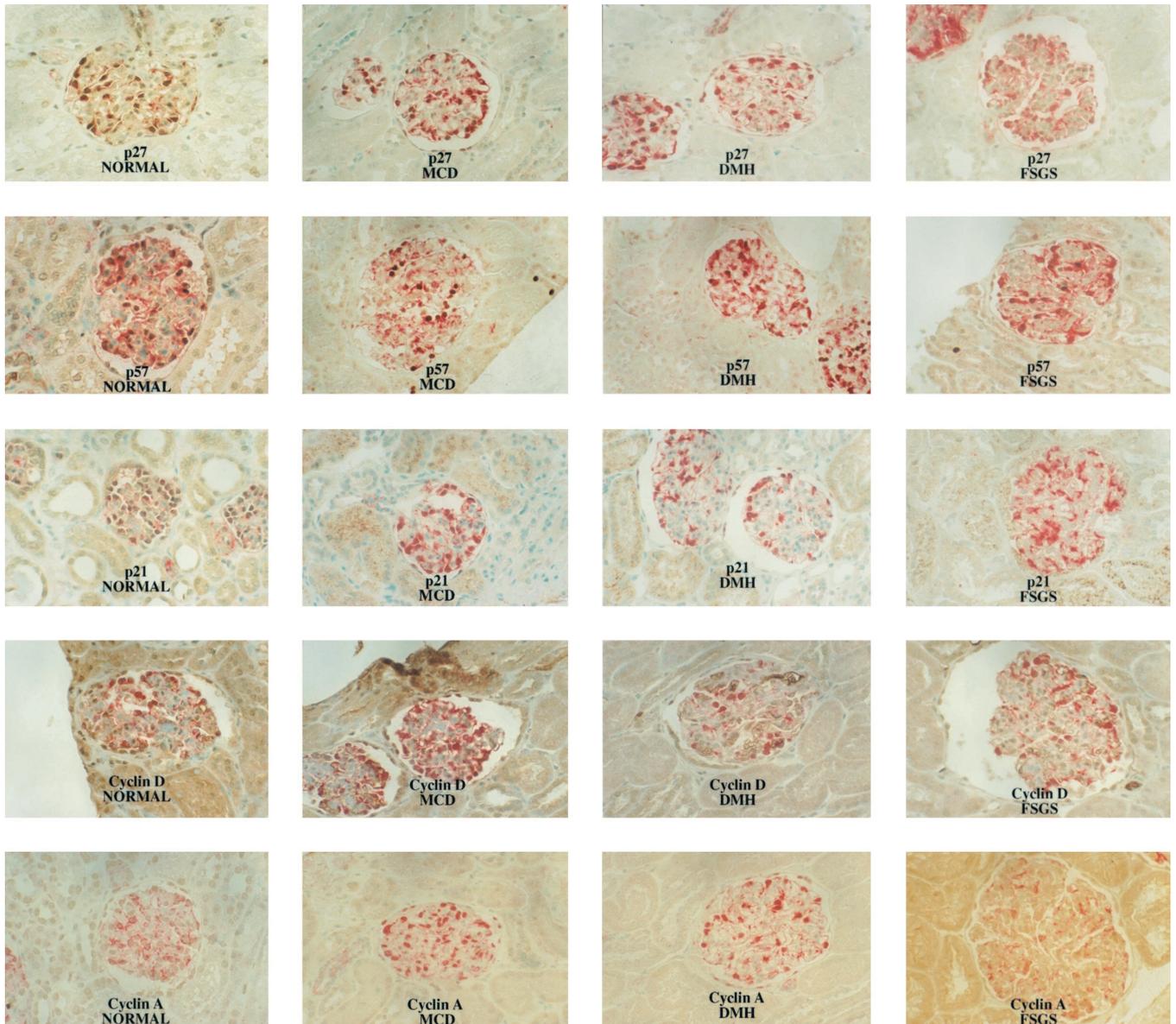
## RESULTS

The mean  $\pm$  SD for age in years for normal (9.7  $\pm$  4.0) was not different from MCD (5.4  $\pm$  3.2, *P* = 0.13), DMH (5.3  $\pm$  3.6, *P* = 0.18), FSGS (9.1  $\pm$  4.0, *P* = 0.98), or AS (10.4  $\pm$  2.2, *P* = 0.78). The age difference among children with INS (MCD, DMH, and FSGS) was not statistically different either. The biopsies were performed for associated hematuria in seven (21.2%), for steroid sensitive frequently relapsing/steroid dependent

nephrotic syndrome in 14 (42.4%) and for steroid-resistant nephrotic syndrome in 12 (36.4%). The subjects included 19 boys and 14 girls. There were 27 Caucasian, four African-American, and two Asian children in the study. Sixteen children went into remission with steroid therapy with/without an 8-week course of cyclophosphamide, while the other 17 children were either steroid-resistant or steroid-dependent needing additional immunosuppressive therapy. The total podocyte cell count per glomerulus for normal children (24.0  $\pm$  2.5) was not different from MCD (19.6  $\pm$  3.2, *P* = 0.47), DMH (23.2  $\pm$  10.1, *P* = 0.99), FSGS (17.3  $\pm$  3.6, *P* = 0.13), or AS (18.1  $\pm$  4.9, *P* = 0.42).

## Normal and INS

On light microscopy, all podocyte cells in all glomeruli expressed p27 protein in normal children. The percent of positive glomeruli and podocyte cells expressing p27 decreased in children with INS; the expression decreasing in order from normal to MCD to DMH to FSGS (Table 1 and Fig. 1). The decrease in p27 expression in podocyte cells reached statistical difference between normal and MCD, DMH and FSGS (Table 1). The decrease was significantly more marked in FSGS compared to MCD (Table 1). In normal children, podocyte cells expressing p21 was observed in all glomeruli. In a given glomerulus not all podocyte cells expressed p21 (Table 1 and Fig. 1). The percent of positive glomeruli and positive podocyte cells expressing p21 were markedly reduced in children with INS (Table 1 and Fig. 1). The decrease in percent positive glomeruli and podocyte cells for p21 expression reached statistical difference between normal and MCD, DMH, and FSGS (Table 1). There was no difference within the INS group of MCD, DMH, and FSGS (Table 1). In normal children, all glomeruli had podocyte cells that expressed p57 protein but like p21 not all podocyte cells in the glomeruli expressed p57



**Fig. 1.** Photomicrograph shows the expression of p27, p21, p57, cyclin D, and cyclin A in podocyte cells of normal and children with minimal change disease (MCD), diffuse mesangial hypercellularity (DMH), and focal segmental glomerulosclerosis (FSGS) in kidney tissue. The podocyte cell is identified by Wilm’s tumor (WT-1) expression (stained red with Vector red) and the cell-cycle regulatory protein is stained brown-black with diaminobenzidine with 3% cobalt. Only podocyte cells that showed homogenous and intense expression of cell cycle regulatory protein above the background were accepted as positive expression. The expression for p27 decreased in order from normal to MCD to DMH to FSGS. p21 expression was markedly decreased in children with MCD, DMH, or FSGS compared to normal. The expression for p57 remained unchanged between normal, MCD, DMH, and FSGS. Cyclin D expression was similar between normal, MCD, DMH, and FSGS. Cyclin A expression was not observed in either normal, MCD, DMH, or FSGS.

(Table 1 and Fig. 1). There was no difference in percent of positive glomeruli or percent positive podocyte cells expressing p57 in normal and children with INS and within the INS group of MCD, DMH, and FSGS (Table 1). On light microscopy, cyclin D was predominantly observed in endothelial and mesangial cells in the glomeruli although a small percentage of glomeruli and podocyte cells did express cyclin D in normal children (Table 1 and Fig. 1). There was no difference in cyclin

D expression in normal and children with INS and within the INS group of MCD, DMH, and FSGS (Table 1). On light microscopy, cyclin A was not expressed in the podocyte cells of either normal children or children with INS (Fig. 1).

**Normal and AS**

The podocyte expression of p27 and p21 was significantly decreased in children with AS (p27,  $63.5 \pm 17.0$ ;

p21,  $18.9 \pm 13.5$ ) when compared with normal children (p27,  $100 \pm 0.0$ ,  $P = 0.04$ ; p21,  $66.6 \pm 12.5$ ,  $P = 0.01$ ). There was no difference in podocyte expression of p57 and cyclin D between normal children (p57,  $60.6 \pm 7.0$ ; cyclin D,  $3.1 \pm 2.8$ ) and AS (p57,  $58.4 \pm 10.4$ ,  $P = 0.68$ ; cyclin D,  $6.3 \pm 1.5$ ,  $P = 0.14$ ). Cyclin A was not expressed in the podocyte cells of either normal or children with AS. The pattern of changes observed in AS biopsies was thus similar to those with INS.

## DISCUSSION

In recent times, cell-cycle regulatory proteins have become an area of intense research in order to understand the changes that occur in various renal diseases. The roles of these proteins in renal diseases have been addressed in several reviews [11–15]. Our earlier work on podocyte cells in childhood INS indicated that podocyte dysfunction/injury is clinically manifested as proteinuria, while at the molecular level, podocyte proteins such as synaptopodin, GLEPP-1, and nephrin become disorganized [9, 10]. Thus, we believe that understanding the podocyte's response following injury is critical to our understanding of the pathophysiology of INS. In adult subjects with cellular and collapsing variants of FSGS and HIVAN, the podocyte cells were observed to have decreased p27, p57, and cyclin D expression and positive staining for p21, cyclin A, and Ki-67 suggestive of a dysregulated podocyte phenotype [17, 18]. In contrast, Nagata et al [19] has contested that the proliferating epithelial cells in FSGS are not podocyte cells but parietal epithelial cells. These observations led us to investigate for alterations in expression of cell-cycle regulatory proteins in podocyte cells in children with INS. To overcome the issue of podocyte cell versus parietal epithelial cell, we identified the podocyte cell by WT-1 expression, based on Mundlos et al [27] observation that WT-1 protein is exclusively expressed in podocyte cells. In our earlier study using the same biopsy material, we had shown normal WT-1 expression in podocyte cells of children with INS [9]. This technique allows study of the cell-cycle regulatory proteins expressed solely in podocytes excluding other resident cells of the glomerulus.

p27 is believed to play a role in differentiation of cells, maintain podocyte cells in quiescence by cell cycle arrest in G<sub>1</sub> phase, and modulate apoptosis and cell-cycle exit response to antimitogenic cues [16, 28]. In p27 knockout mice, the kidneys are 25% larger due to a proportionate increase in all resident cell types, no renal histologic changes are observed by light or electron microscopy, and renal function is normal [29–32]. In animal models of glomerulonephritis, the changes in p27 are variable, it is decreased in Thy-1 model, and increased in passive Heymann nephritis [33, 34]. Although the complete absence of p27 does not have an impact on renal develop-

ment or function, it does get up- or down-regulated in response to an exogenous insult. In adults, p27 is reported to be decreased in cellular and collapsing variant of FSGS and HIVAN, but unchanged in MCD [17]. In our study we observed a significant decrease in p27 expression in podocyte cells in children with INS, decreasing in order from normal to MCD to DMH to FSGS. The decrease in p27 expression observed in our study is proportional to the severity of the injury. Unlike Shankland et al [17], we found a difference between MCD and normal, which could be attributed to a quantitative assessment performed by us rather than a qualitative assessment for p27 expression.

Could the observed difference in p27 expression between INS and normal children be due to loss of podocyte cells from the glomerulus or be age-related? Loss of podocytes from the glomerulus cannot explain the decrease in p27 expression, as unlike children with inflammatory glomerulonephritis, loss of podocytes in the urine is not observed in children with MCD [35]. The total podocyte count per glomerulus was not different among the four groups. WT-1 and p57 are expressed only in the podocyte cells of the glomerulus [27, 36]. The normal expression of WT-1 as observed in our earlier study and p57 in this study, in both normal and children with INS supports the fact that the observations are not related to loss of podocyte cells [9]. There was no significant difference between the ages of children with MCD, DMH, FSGS, and normal children. Thus, the changes observed in p27 expression could not be attributed to the age of the patient.

The p21 protein is thought to play an important role at the G<sub>1</sub> checkpoint [37]. In our study we observed p21 expression in normal kidney unlike Shankland et al [17] who had not detected p21 in normal tissue, and a significant decrease in p21 expression in podocyte cells in children with INS. The p21 CKI is rapidly induced in response to physiologic and chemical inducers of differentiation and p21 gene is a candidate gene linking differentiation signals to G<sub>1</sub> arrest in multiple cell lines [38]. The p21 expression is believed to be regulated by both a p53 dependent and a p53 independent pathway [38]. Englert et al [39] have shown that WT-1 gene induces an increase expression of p21 mRNA independent of p53, and in the kidney p21 is expressed in the differentiating glomerular podocytes along with WT-1, which, in turn, may contribute to WT-1-dependent differentiation pathways in the kidney. Englert et al [39] study suggests a close functional relationship between WT-1 and p21. WT-1 and p21 play an important role for cell differentiation and G<sub>1</sub> cell arrest. The podocyte cell is characterized by arrest in G<sub>1</sub> phase and abundant expression of WT-1. Thus, our finding of p21 in podocytes in normal kidney unlike Shankland et al [17] was not an unexpected observation based on earlier studies showing close functional relationship

between WT-1 and p21 in cell differentiation and G<sub>1</sub> cell arrest. We could speculate that difference in p21 expression between Shankland study [17] and ours could be related to the epitope recognition site of the antibody used in the two studies. The antibody used by us was clone 187, produced by immunization with full-length p21 of human origin. The antibody used by Shankland et al [17] was from clone SX 118, which is raised against a purified recombinant human p21-glutathione-S-transferase (GST) fusion protein and only recognizes the proliferating cell nuclear antigen (PCNA)-binding domain of p21. It is possible that our antibody recognizes an epitope of p21 different from the PCNA binding site as we found consistent results with normal kidney tissue from children with Wilm's tumor (data not shown) and abnormal tissue from children with congenital nephrotic syndrome of Finnish type (data not shown).

p57 is believed to play a role in the terminal differentiation of podocyte cell and overexpression of p57 arrests the cell in G<sub>1</sub> phase [40, 41]. The p57 knockout mice develop noncystic medullary dysplasia in the kidneys and have many features that resemble Beckwith-Weidermann syndrome [36]. Although p57 was reported decreased in cellular and collapsing variant of FSGS, we did not observe any difference in p57 expression in podocyte cells in normal and children with INS [17, 18]. The cellular and collapsing variant of FSGS is a small subset of FSGS in adults [42]. In our own series of 34 children with FSGS from 1984 to 1995 seen at our institution, we did not find a single child with cellular or collapsing variant of FSGS, which is a rare entity in children [1]. In the present study, children had classic FSGS. Currently, there are no studies available either in adults or children with podocyte expression of p57 in subjects with classic FSGS to make a suitable comparison. The dysregulated podocyte phenotype as described in adults for the subset of cellular and collapsing FSGS was not observed in children with classic FSGS. Yang, Gubler, and Beauflis [43] using other proliferation and podocyte markers found an abnormal distribution of WT-1 and PAX-2, and extensive loss of podocyte markers in idiopathic collapsing glomerulopathy and HIVAN; this dysregulation was associated with podocyte proliferation without detectable apoptosis, while in FSGS, proliferation was not observed, which indirectly supports our finding of absence of a proliferative podocyte phenotype in childhood INS. In addition, Nagata et al [19] have suggested that the hyperplastic cell in FSGS is the parietal epithelial cell and not podocyte cell. Thus, the above studies, combined with our own study would suggest that the subset of collapsing and cellular FSGS and HIVAN may have a different pathogenic pathway from INS, where proliferation is absent. The absence of proliferative dysregulated podocyte phenotype in childhood INS is further supported by our data on cyclin D and cyclin A (*vide infra*).

Cyclin D is required for cell proliferation and G<sub>1</sub>/S transition and works through inactivation the retinoblastoma protein [13]. In Thy-1 and passive Heymann nephritis models of glomerulonephritis, cyclin D is unchanged [33, 34]. In our study, we did not observe any difference in cyclin D expression in podocyte cells in normal and children with INS, which is consistent with animal data, but stands in contrast to Barisoni et al [18] who found cyclin D to be decreased in collapsing FSGS. We found cyclin D to be predominantly expressed in the endothelial and mesangial cells of the glomerulus. Cyclin A increase in late G<sub>1</sub>, and peaks in S and G<sub>2</sub> phase, and is required for onset of DNA synthesis and to overcome G<sub>1</sub>/S block [13]. In our study, we did not observe any expression in cyclin A in podocyte cells in normal and children with INS. The difference in our observation from Barisoni et al [18] is discussed above.

Children with AS had similar changes in cell-cycle regulatory proteins as those with INS, with decrease in p27 and p21 expression, unchanged expression of p57 and cyclin D, and absent cyclin A. Thus, it appears that the changes observed following an acquired immunologic injury to podocyte cell in children with INS are similar to the changes observed in AS, which results from a genetic mutation in  $\alpha 5$  of collagen type IV of the glomerular basement membrane. In our earlier studies we had found the expression of synaptopodin, GLEPP-1, and nephrin to be decreased in both children with INS (acquired immunologic injury to podocyte cell) and children with Galloway-Mowat syndrome and congenital nephrotic syndrome of the Finnish type (podocyte dysfunction following genetic mutations) [9, 10]. Thus, the changes in the cell-cycle regulatory proteins observed by us might indicate a final common pathway to different injury processes to the podocyte cell.

There was no statistical difference between expression of podocyte cell-cycle regulatory proteins and clinical response to steroids, nor was there statistical difference in expression of cell-cycle regulatory proteins between Caucasian and African American children (data not shown). Only four children in our series had either elevated serum creatinine or had progressed to end stage renal failure, hence we did not correlate cell-cycle regulatory protein expression and renal outcome as the sample size was too small. The study was limited, as children with mild MCD are not routinely biopsied; thus, our MCD group was skewed toward moderate to severe MCD. We can cautiously speculate that inclusion of mild MCD cases would not have altered our results much, as the difference between normal and MCD showed a dichotomous pattern in which either marked difference exists, as in the case of p21 and p27, or no difference is seen as in the case of p57, cyclin D, and cyclin A.

## CONCLUSION

In our study we observed CKIs p27, p57, and p21 to be present in normal kidneys, which keep the podocyte cell differentiated and in G<sub>1</sub> arrest. We found that in children with INS, the podocyte cell down-regulates expression of CKIs such as p21 and p27 but not p57, and fails to up-regulate cyclin D and cyclin A (marker of “S” and “G<sub>2</sub>” phase), which are needed to overcome the G<sub>1</sub>/S transition and move the cell forward in the cell-cycle process. This results in the podocyte cell remaining trapped in the G<sub>1</sub> arrest phase. The observed changes in cell-cycle regulatory proteins were observed in both children with INS and AS. In adults with cellular and collapsing variant of FSGS and HIVAN, alterations have been observed in p27, p57, p21, cyclin D, and cyclin A expression in the podocyte cell, which suggests a dysregulated phenotype that is more dedifferentiated and immature and permissive for podocyte proliferation [17, 18]. We did not observe the proliferative dysregulated podocyte phenotype in children with INS as described in the above adult studies for cellular and collapsing variant of FSGS and HIVAN. Yang, Gubler, and Beauvils [43] and our study would suggest that the subset of collapsing and cellular FSGS may have a different pathogenic pathway with proliferation as an important feature in the podocyte cell unlike childhood INS or AS where proliferation is absent in the podocyte. An injured cell as a response to injury can either undergo proliferation and/or hypertrophy. Our data suggest that the podocyte cell in INS does not respond with proliferation as suggested by earlier studies. The current study does not allow us to infer for cellular hypertrophy. The most important aspect of this study is that it provides us with an insight into the changes that are occurring in the cellular machinery of podocyte cell in INS. Our data are the first report on the changes that occur in the cell-cycle regulatory proteins in podocyte cells of children with INS. It, thus, seems that more studies are needed with special attention to cell hypertrophy markers to appreciate the role of cell-cycle regulatory proteins in the podocyte in both adult and childhood nephrotic syndrome.

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