

# Nephrotic syndrome unfavorable course correlates with downregulation of podocyte vascular endothelial growth factor receptor (VEGFR)-2

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**Abstract:** Idiopathic nephrotic syndrome (INS) in children is most commonly caused by primary glomerulopathies. Morphological lesions observed in INS might be secondary to inflammatory factors of mainly extra-renal origin. The vascular endothelial growth factor (VEGF) family is regarded as playing a crucial role in this pathomechanism. The aim of the present work was to analyze the possible relation between VEGF-C and VEGF receptor (VEGFR)-2 expressions at electron microscopy level in different INS cases. The study group comprised 18 children with minimal change disease (MCD), 30 patients diagnosed with diffuse mesangial proliferation (DMP) and 11 subjects with focal segmental glomerulosclerosis (FSGS). An indirect immunohistochemical assay employing monoclonal anti-VEGF-C and anti-VEGFR-2 antibodies was applied in the study. The immunohistochemical expression of VEGF-C within podocyte cytoplasm was significantly increased in DMP subjects who were resistant to steroids and in all FSGS patients compared to MCD children and controls ( $p < 0.05$ ). VEGF-C over-expression in these cases was followed by downregulation of VEGFR-2. Nephrotic syndrome progression correlates with the downregulation of podocyte VEGFR-2. For this reason, decreased VEGFR-2 expression in the podocyte processes of children with idiopathic nephrotic syndrome might be regarded as a potent factor of unfavorable prognosis. (*Folia Histochemica et Cytophiologica* 2011; Vol. 49, No. 3, pp. 472–478)

**Key words:** VEGFR-2, nephrotic syndrome, electron microscopy, children

## Introduction

The vascular endothelial growth factor (VEGF) family is composed of five members: VEGF (sometimes called VEGF-A), placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D [1]. VEGFs belong to the platelet-derived growth factor (PDGF)/VEGF

superfamily of secreted dimeric glycoprotein growth factors that contain a cysteine knot motif [1]. The VEGF ligands bind with differing specificities to three mostly endothelial transmembrane tyrosine kinase receptors including: VEGFR-1/fms-like tyrosine kinase 1 (Flt1), VEGFR-2/human kinase insert domain receptor (KDR)/mouse fetal liver kinase 1 (Flk1) and VEGFR-3/fms-like tyrosine kinase 4 (Flt4) [1].

VEGFR-2 manifests the widest spectrum of binding ligands. It might process with all the VEGF family members except PlGF and VEGF-B [2]. Interestingly, VEGF signaling through VEGFR-2 is considered as a major angiogenic pathway [1]. VEGFR-1

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seems to act as a negative regulator of VEGF-mediated angiogenesis during development, and as a stimulator of pathological angiogenesis when activated by its specific ligands: PlGF and VEGF-B [1, 2]. For VEGF-B, with very limited angiogenic potential, a new role has been identified in the regulation of lipid metabolism in the cardiac muscle [2]. VEGF-C and VEGF-D are believed to induce lymphangiogenesis via VEGFR-3. They have been proven to activate the development of lymphatic vessels in different tumors and, consequently, stimulate metastases [3]. VEGF-C seems to be a much more pleiotropic molecule than VEGF-D. It is expressed in bone marrow hematopoietic cells [4, 5], developing nerve fibers [6] and the kidneys [7].

The most frequent reason for primary glomerulopathies in children is idiopathic nephrotic syndrome (INS), reflecting impaired glomerular permeability [8]. Morphological lesions observed in INS might be secondary to inflammatory factors of mainly extra-renal origin.

Some (e.g. interleukins and VEGF-C) are regarded as playing a fundamental role in this pathomechanism [7, 9, 10]. Interestingly, VEGF-C could be expressed both in cultured and tissue podocytes. In cultured podocytes, VEGF-C acts in an autocrine manner to promote their survival [10]. In other studies, VEGF-C has been found to increase filtration barrier permeability and has been regarded as a factor of unfavorable prognosis in nephrotic syndrome children [7]. This apparent discrepancy may be explained by detailed, molecular studies of VEGF-C receptors expressions in filtration barrier elements (podocytes, basement membrane and endothelial cells). Studies performed by Foster et al. revealed VEGF-C does not act in cultured podocytes via VEGFR-3 [10]. They concluded that the receptor or receptor complex activated has yet to be elucidated. Because VEGF-C is able to induce its biological effects via VEGFR-2 or VEGFR-3, the aim of the present study was to evaluate both VEGFR-2 and VEGFR-3 expressions in filtration barrier elements at electron microscopy (EM) level using the immuno-gold technique for the detection of antigen-antibody complexes in biopsy material.

## Material and methods

**Patients.** The study group was composed of children diagnosed with INS, hospitalized between 2003 and 2006 in the Department of Pediatric Cardiology and Nephrology, Poznan University of Medical Sciences. Two hundred and eighty-six children were referred to our clinic, and evidence of proteinuria was found in 146 of them. Eighty-four patients (44 boys and 40 girls), following International Study

for Kidney Diseases in Children recommendations, underwent a biopsy [11]. The indications included primary idiopathic glomerulonephritis (n = 36), steroid-resistant nephrotic syndrome (SRNS) (n = 23) and secondary glomerulonephritis (n = 25, who were excluded from further analysis). Kidney biopsy was performed after eight weeks of high dose steroid treatment in SRNS children and in all the children recognized with primary idiopathic glomerulonephritis before commencement of the treatment. No study participants were administered at the time of kidney biopsy any anti-hypertensive drugs or immunosuppressants, apart from steroids.

Diagnoses of minimal change disease (MCD), diffuse mesangial proliferation (DMP) and focal segmental glomerulosclerosis (FSGS) were established according to WHO criteria and were based on the number of cells per mesangial area (four or more cells for DMP, n = 30 patients) or the presence of at least one sclerotic focus in kidney biopsy material for FSGS (n = 11 subjects) [12]. Eighteen children were diagnosed with MCD. In all, the study group was composed of 59 participants.

INS has been classically characterized by the absence of significant deposits in immunofluorescence microscopy (IF), except for FSGS and/or hyalinosis in FSGS, which bind IgM and C3 antiserum [13]. There were no significant differences between the various categories defined by IF [14]. In 25 patients with DMP, the presence of IgM and/or IgG within the mesangial area was observed without electron-dense deposits. No IgA nephropathy was diagnosed in the subjects. The relevant data is presented in Table 1.

The Ethics Committee of Poznan University of Medical Sciences approved the research protocol and the parents of all study participants gave informed consent for the investigation.

**Controls.** Ten age- and sex-matched children in whom total nephrectomy due to the presence of Wilms tumor had been performed served as a control group. All of these subjects had a normal renal function. Macroscopically normal-appearing kidney tissue was rinsed with PBS, fixed in PBS-buffered 4% paraformaldehyde with 0.05% glutaraldehyde for 1 h on ice, and subsequently fixed in 1% glutaraldehyde for 0.5 h at room temperature. The material was then saved for the subsequent procedure.

**Immunoelectron microscopy (post-embedding technique).** Similarly to control tissue, kidney biopsy specimens were fixed in PBS-buffered 4% paraformaldehyde with 0.05% glutaraldehyde and subsequently in 1% glutaraldehyde. The material was then incubated in the same buffer containing 2.3 M sucrose overnight, and subsequently embedded in Lowicryl HM 20 (Polysciences Europe, Eppenheim, Germany).

For immuno-gold labeling of VEGF-C, VEGFR-2 and VEGFR-3, ultrathin sections were transferred to 200-mesh nickel grids coated with 3% collodion. After blocking of nonspecific labeling with 50 mM glycine and 2% gelatin,

0.5% acetylated BSA ultrathin sections were incubated overnight at 8°C with anti-VEGF-C antibody (NB110-61022, Novus Biological, Cambridge, UK; diluted 1:20), with anti-VEGFR-2 antibody (NB100-686, Novus Biological; diluted 1:50) or with anti-VEGFR-3 antibody (NBP1-03254, Novus Biological; diluted 1:20). All of them were subsequently detected with goat anti-rabbit IgG (NB100-2332, Novus Biological; diluted 1:50) and coupled to 20-nm gold particles (Dianova, Hamburg, Germany). Sections were counterstained with 2% uranyl acetate and Reynold's lead citrate, and examined with a Zeiss EM 900.

Specificity controls were performed by omission of the primary antibody using in place of normal goat IgG serum (sc-3887, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:50) or PBS. Positive immuno-labeling was performed using VEGF-C, VEGFR-2 and VEGFR-3 antibodies incubated in appropriate positive tissues (neuroblastoma cancer cells, blood vessel endothelial cells and lymphatic endothelial cells respectively) as well as with anti-podocalyxin primary antibodies (sc-33138, Santa Cruz Biotechnology; diluted 1:50) [15].

Finally, 5–6 EM images demonstrating individual immuno-gold incubations were analyzed for each study participant (patient or control).

**Quantitative and statistical analysis.** The analysis was performed blind on coded samples. Continuous or interval-related variables were expressed as mean  $\pm$  SD. Data was compared using Student's *t*-test, or one-way analysis of variance test (ANOVA) where appropriate. Variations in antigen distribution within renal glomeruli were evaluated using MicroImage v. 4.0 morphometric software (Olympus, MS Windows XP). An average density of marker expression on selected area (number of gold particles per  $1 \mu\text{m}^2$  using MicroImage v. 4.0) was measured and compared to results obtained in the control group as well as among MCD, DMP and FSGS patients. Immuno-gold stained slides were examined field per field by two experi-

enced investigators to identify the area showing the most intense concentration of gold particles ('hot spot'). The selection of hot spots was based on observing restricted areas with an impression of a higher density of gold particles. Three hot spots from each image were then photographed, and an average of gold particles per  $1 \mu\text{m}^2$  was counted for consequent statistical analysis. Any black dot that was clearly separated from adjacent tissues was considered as a single, countable gold particle. Differences between groups were then evaluated by ANOVA test. To determine the significance between group means in the analysis of variance, the nearest significant difference test (Tukey test) was used as the multiple comparison analysis. Significance was set at  $p < 0.05$ .

## Results

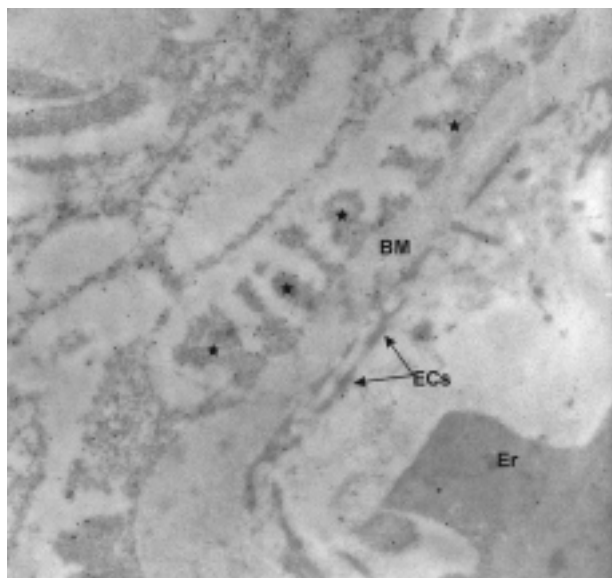
### Clinical course

According to the histological evaluation of the kidney biopsy, the patients comprising the study group were divided into MCD, DMP and FSGS subjects. All MCD children manifested steroid sensitive course of nephrotic syndrome (SSNS), while all FSGS subjects revealed steroid resistant nephrotic syndrome (SRNS). Eighteen children diagnosed with DMP developed SSNS (DMP-s subgroup) and 12 patients demonstrated SRNS course (DMP-r subgroup). All the study participants observed with SRNS had a significantly higher proteinuria and significantly lower concentration of serum albumin. Moreover, there was a significantly higher percentage of patients with evaluated erythrocyturia in DMP-s and FSGS patients compared to MCD and DMP-r children. Finally, FSGS children had a significantly lower glomerular filtration rate (GFR). Detailed information is presented in Table 1.

**Table 1.** Clinical and biochemical characteristics of the study population at the time of kidney biopsy (after eight-week steroid treatment)

Characteristics	Study group			
	MCD	DMP-s	DMP-r	FSGS
Number of patients	18	18	12	11
Age (years)	10.7 (6–18)	10 (5–16)	10 (5–14)	11.5 (6–16)
Sex (M/F)	10/8	8/10	8/4	6/5
Proteinuria [mg/kg/24 h]	41 $\pm$ 8	30 $\pm$ 10	90 $\pm$ 23**	56 $\pm$ 11*
Erythrocyturia (% of patients)	22.2	50.0*	33.4	72.7**
GFR [ml/min/1.73 m <sup>2</sup> ]	98 $\pm$ 8	105 $\pm$ 11	108 $\pm$ 15	85 $\pm$ 12*
Serum albumin [g/dl]	2.9 $\pm$ 0.8	3.1 $\pm$ 0.8	2.5 $\pm$ 0.3	2.2 $\pm$ 0.5

MCD — minimal change disease; DMP-s — diffuse mesangial proliferation (sensitive to steroids); DMP-r — diffuse mesangial proliferation (resistant to steroids); FSGS — focal segmental glomerulosclerosis; erythrocyturia — number of red blood cells in urine  $> 2$  cell per high power field; GFR — glomerular filtration rate; \* $p < 0.05$ ; \*\* $p < 0.0001$



**Figure 1.** Podocalyxin immuno-gold labeling in the control kidney specimen. The presence of gold particles is visible within glomerular endothelial cells (ECs) and podocytes (asterisks). Basement membrane (BM) is immuno-negative; Er — erythrocyte; original magnification  $\times 21,560$

**Immunoelectron microscopy**

Expressions of both VEGFR-2 and podocalyxin were demonstrated in all the children comprising the control group. VEGFR-2 was present within endothelial cells (ECs) of filtration barrier while podocalyxin was expressed both in ECs and podocyte processes (Figure 1). These subjects presented also single gold particles demonstrating VEGF-C and VEGFR-3 expressions within glomerular ECs (data not shown). A similar expression of VEGFR-2 within ECs, as well as podocalyxin in ECs and podocytes were revealed in all the patients comprising the study group. None of the studied children, however, demonstrated the im-

munohistochemical expression of VEGFR-3 within filtration barrier elements (Table 2).

Interestingly, all the patients comprising the study group expressed VEGF-C in podocyte processes while VEGFR-2, except for ECs, was also present within visceral cells of Bowman capsule. The density of gold particles differed, however, between subsequent sub-groups.

SSNS children (MCD + DMP-s) were evaluated with a relatively high expression of VEGFR-2 in podocytes (Figure 2) followed by a significantly lower expression of VEGF-C (Figure 3).

In SRNS patients (DMP-r + FSGS), the situation was exactly the opposite: a relatively low expression of VEGFR-2 in visceral cells of Bowman capsule (Figure 4) was followed by a definitely higher expression of VEGF-C within podocytes (Figure 5). VEGF-C, as presented in Figure 4, was also expressed within glomerular ECs. Relevant data and statistical analysis are summarized in Table 2.

In addition, Figure 2 demonstrates the presence of VEGFR-2 related gold particles not only within podocyte foot processes and glomerular ECs, but also in glomerular basement membrane (GBM). Moreover, the concentration of gold particles within GBM was equal to the area concentration of VEGFR-2 related gold particles in glomerular ECs.

Taken together, an unfavorable course of INS (defined by resistance to steroids) correlated to an increased expression of VEGF-C within podocytes and decreased (as compared to SSNS patients) expression of VEGFR-2 within visceral cells of Bowman capsule.

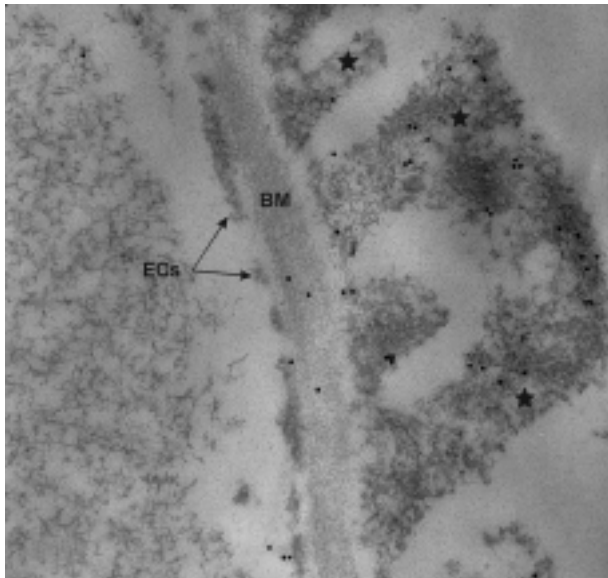
**Discussion**

Our study has demonstrated — for the first time — expression of VEGF-C at electron microscopy level within tissue podocytes of children diagnosed with INS. Although VEGF-C presence was also detect-

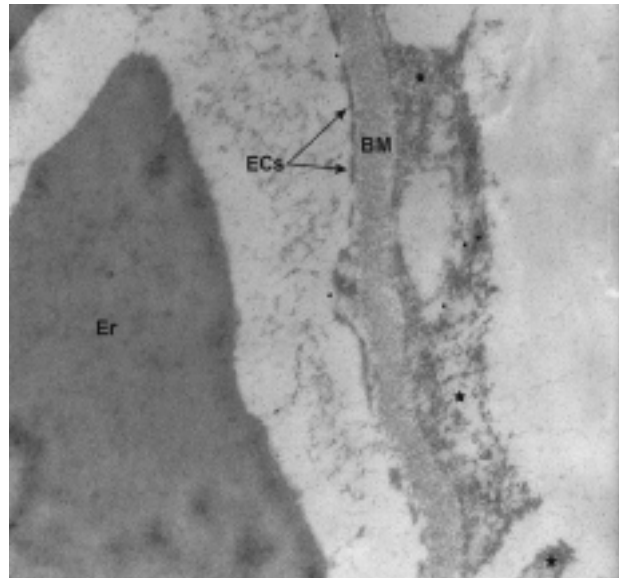
**Table 2.** Immunohistochemical expression of studied antigens within filtration barrier elements at the electron microscopy level

Antigen	Localization	Density of marker expression				Controls
		MCD	DMP-s	DMP-r	FSGS	
VEGF-C	Podocyte	0.7 ± 0.2	0.9 ± 0.2	1.8 ± 0.6*	1.9 ± 0.7*	0,0
	Endothelial cells	0.0	0.0	0.9 ± 0.4**	0.8 ± 0.3**	0.3 ± 0.1
VEGFR-2	Podocyte	1.1 ± 0.4	1.3 ± 0.4	0.5 ± 0.1*	0.4 ± 0.1*	0,0
	Endothelial cells	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.5 ± 0.1	0.5 ± 0.1
Podocalyxin	Podocyte	3.1 ± 0.9	3.4 ± 0.8	2.9 ± 1.0	3.2 ± 0.9	2,8 ± 0,7
	Endothelial cells	2.8 ± 0.6	2.9 ± 0.4	2.7 ± 0,5	2,8 ± 0,5	2,6 ± 0,7
VEGFR-3	Endothelial cells	No expression				0.4 ± 0.1

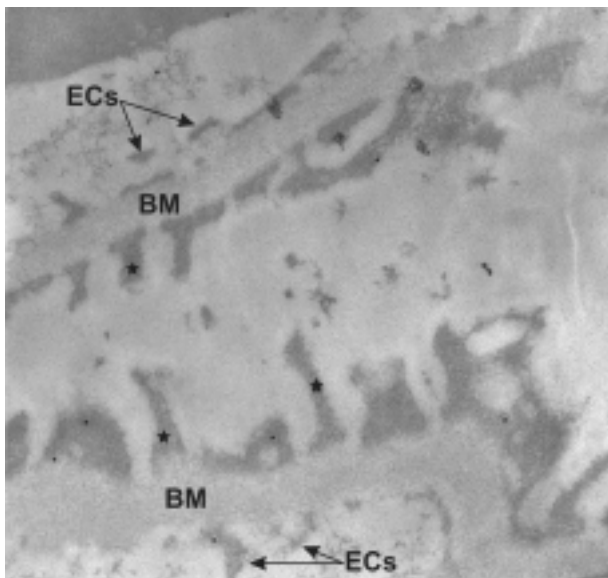
Density of marker expression — number of gold particles per  $1 \mu\text{m}^2$ : MCD — minimal change disease; DMP-s — diffuse mesangial proliferation (sensitive to steroids); DMP-r — diffuse mesangial proliferation (resistant to steroids); FSGS — focal segmental glomerulosclerosis; VEGF — vascular endothelial growth factor; VEGFR — vascular endothelial growth factor receptor; \*p < 0.05; \*\*p < 0.0001



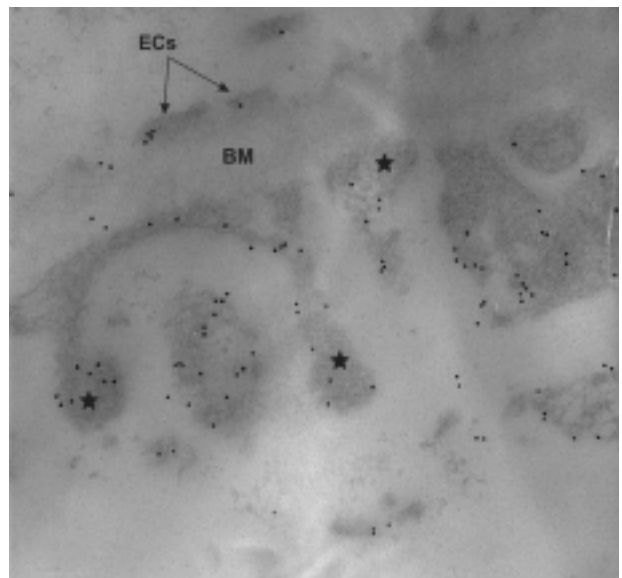
**Figure 2.** VEGFR-2 expression within filtration barrier elements in a six year-old child diagnosed with minimal change disease. The presence of gold particles is clearly visible within glomerular endothelial cells (ECs) as well as within podocytes (asterisks). Few dots visible within basement membrane might refer to unspecific staining; original magnification  $\times 35,970$



**Figure 3.** Electron microscope immuno-gold labeling of VEGF-C in a six year-old child diagnosed with minimal change disease. The marker is exclusively present within podocytes (asterisks). Basement membrane (BM) as well as glomerular endothelial cells (ECs) are immuno-negative. Er — erythrocyte; original magnification  $\times 27,800$



**Figure 4.** A nine year-old child with a diagnosis of resistant to steroids diffuse mesangial proliferation. Similarly to Figure 2, VEGFR-2 expression is present within glomerular endothelial cells (ECs). Immunoelectron microscopy (post-embedding technique); original magnification  $\times 27,800$



**Figure 5.** Immunocytochemical expression of VEGF-C at electron microscopy level in kidney biopsy of a nine year-old child recognized with resistant to steroids diffuse mesangial proliferation. Numerous VEGF-C related gold particles visible within podocytes (asterisks) and glomerular endothelial cells (ECs). Basement membrane (BM) is free of VEGF-C expression; original magnification  $\times 46,460$

ed in control tissue, its expression was significantly higher in all the patients who developed resistance to steroids. This observation corresponds with previous reports by Foster et al. who proved expression of VEGF-C and VEGFR-3 under normal conditions

in the human kidney by immunohistochemistry and the immuno-gold technique [9, 10]. Our previous studies, based on classical bright field microscopy,

have revealed VEGF-C might be regarded as a factor of unfavorable prognosis in patients diagnosed with INS [7].

On the other hand, increased VEGF-C expression in SRNS children has, most probably, no nephro-protective effect that could be expected analyzing its biological meaning in cultured podocytes [10]. The studies performed by Foster et al. reported that VEGF-C is a factor promoting cultured podocyte survival but not acting via VEGFR-3 [10]. The authors suggested there was another receptor binding VEGF-C in podocytes. The same scientific team, but in a different publication, proved VEGF-C to increase glomerular cell monolayer integrity via VEGFR-2, and observed glomerular ECs to express VEGFR-3 in tissue sections and culture [16].

These results, in part, supplement our observations. The limit of all immunocytochemical techniques, including the immuno-gold procedure, is the impossibility of differentiating whether VEGF-C is expressed in tissue *per se* or if it is bound to one of its receptors. For this reason, a much more credible factor demonstrating a tissue status of VEGF-C/VEGFR-2 interaction is VEGFR-2. We suggest that VEGFR-2 is an essential receptor for VEGF-C, acting both in podocytes as well as in glomerular ECs. That hypothesis is most of all based on our remark indicating podocyte VEGFR-2 expression is downregulated when VEGF-C becomes more strongly expressed. Interestingly, VEGFR-2 expression in glomerular ECs is constant (no significant differences between the control and the study group, as well as among subsequent studied sub-groups). Only podocyte VEGFR-2 expression varied between SSNS and SRNS patients. The exact mechanism of this process is yet to be investigated.

It must be emphasized that VEGFR-2 might be downregulated by immunosuppressive drugs, including steroids [17]. Several reports have described the VEGFR-2 downregulation process during neuroblastoma progression [18], in pancreatic-islet tumor [19] as well as in different non-cancerous models of angiogenesis [20]. Interestingly, SRNS patients expressed VEGF-C not only within podocytes but also in glomerular ECs (VEGF-C was not expressed in glomerular ECs in SSNS subjects nor in controls). This might suggest an autocrine VEGF-C alternating activity of glomerular ECs.

The question, however, as to whether VEGF-C improves ECs monolayer or increases vascular permeability, remains open. Similarly, a potent VEGF-C/VEGFR-2 interaction in regulating podocyte function and glomerular filtration barrier integrity in SSNS and SRNS is not yet clear and cannot be explained by the present report.

VEGF-C expression during embryonic development is observed both in places where the first lymph sacs develop and in regions of lymph vessel sprouting [21, 22]. The binding affinity of VEGF-C for its receptors is regulated by proteolytic processing of the pro-peptides. Its affinity for VEGFR-3 increases with processing, while only the mature forms of VEGF-C bind VEGFR-2 [23, 24]. It seems to be also possible that the mature form of VEGF-C has a much more expanded tissue activity, not only restricted to developing lymph vessels. This significant pleiotropy is, however, dependent on the type of receptor present in a tissue [2]. For this reason, VEGF-C/VEGFR-2 [25, 26] and VEGF-C/VEGFR-3 signaling pathway dualism [26, 27] might explain a binary identity of VEGF-C manifestation.

Our observation might also be interesting from another point of view. Some of the anti-angiogenic drugs are directed on blocking their specific receptors [28, 29]. If our elaboration could be expanded in the future to VEGF-C/VEGFR-2 activity in neoplasms, it might significantly influence the future anti-angiogenic therapy and explain some of the observed medication discrepancy in these patients.

In conclusion, nephrotic syndrome progression correlates with the downregulation of podocyte VEGFR-2 expression. For this reason, VEGFR-2 decreased expression in the podocyte processes of children with idiopathic nephrotic syndrome might be regarded as a potent factor of unfavorable prognosis.

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