Molecular Evaluation of Circulating Endothelial Progenitor Cells in Children Undergoing Hemodialysis and After Kidney Transplantation

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ABSTRACT: Increased risk of cardiovascular disease in end-stage renal disease (ESRD) has been explained by accelerated atherosclerosis and impaired angiogenesis, in which endothelial progenitor cells (EPC) may play key roles. Circulating cells with endothelial progenitor phenotype have not been evaluated in children with ESRD. Using a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) approach, we measured endothelial-specific and progenitor-associated genes VE-cadherin (VE-C), CD146, CD31, tyrosine-protein kinase receptor (Tie-2), Flk1, CD133, and growth factors promoting EPC function, vascular endothelial growth factor (VEGF), erythropoietin (EPO), and stromal cell-derived factor-1 (SDF-1) in the blood of pediatric patients undergoing hemodialysis and after transplantation. Patients' metabolic parameters were correlated with EPC marker gene levels. Compared with controls, circulating VE-cadherin, CD146, Flk1, VEGF, and EPO RNA levels were decreased in ESRD and normalized in transplanted patients. Levels of VE-cadherin, which were the most significantly reduced in ESRD ($p = 0.001$) inversely correlated in all of the patient population with serum urea and creatinine concentration, whereas among the ESRD group showed an inverse correlation with diastolic blood pressure (BP), interventricular septum thickness (IVST), and left ventricular mass index. Pediatric ESRD patients may have lower angiogenic potential and increased cardiovascular morbidity, because of decreased expression of circulating endothelial cell specific transcripts. Prospective studies are required to link this expression pattern and its restoration in transplanted patients to cardiovascular outcome. (*Pediatr Res* 65: 221–225, 2009)

Chronic kidney disease/uremia is a state of endothelial dysfunction, accelerated progression of atherosclerosis and high cardiovascular risk, as well as a state of reduced hematopoiesis (1–3). The maintenance of endothelial integrity plays a critical role in preventing atherosclerotic disease progression. Accumulating evidence suggests that circulating peripheral blood endothelial cells (CECs) and/or their putative progenitor subset endothelial progenitor cells (EPCs) may contribute to endothelial replacement in areas of postnatal

neovascularization, including the kidney, by incorporation into newly formed capillaries or by releasing factors, which act in a paracrine manner to support local angiogenesis (4–8). EPCs exhibit a high-expression of pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF), which enhances function and migration of mature endothelial cells and tissue residing progenitor cells (6). Circulating endothelial cells and EPCs have been traditionally evaluated by flow cytometry, which requires a complex four-color approach. RT-PCR is widely used for detection of circulating tumor cells in the peripheral blood (9,10). To date, however, RT-PCR is used infrequently to study endothelial cells in peripheral blood, which is likely due to the lack of suitable and tested markers (11,12). Recently, it has been demonstrated that quantification of endothelial progenitor cell numbers by flow cytometric analysis is highly consistent with the analysis of endothelial molecular markers by quantitative RT-PCR (13–15).

Circulating EPCs have yet been evaluated in the end-stage kidney pediatric population. We therefore conducted an observational study to test the following hypotheses: 1) levels of EPC marker molecules [VEGF receptor 2 (*e.g.* Flk-1), tyrosine-protein kinase receptor (Tie-2), CD133 (prominin-1), CD146, platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31), and vascular endothelial cadherin (VE-C)] are altered in children undergoing hemodialysis and restored after renal transplantation; and 2) Levels of EPC markers are directly related to cardiovascular, metabolic, and biochemical parameters in our patient population.

MATERIALS AND METHODS

Patients. This study was approved by local Helsinki committee of the Schneider Medical Center. Informed consent has been obtained from patients. At the time of the study, we analyzed 13 patients undergoing hemodialysis at

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Abbreviations: BP, blood pressure; CT, cycle threshold; EPC, endothelial progenitor cells; EPO, erythropoietin; ESRD, end stage renal disease; IVST, inter-ventricular septum thickness; LVMI, left ventricle mass index; PECAM-1 (CD31), platelet/endothelial cell adhesion molecule-1; SDF-1, stromal cell-derived factor-1; Tie-2, tyrosine-protein kinase receptor; VE-C, vascular endothelial cadherin; VEGFR-2/Flk1, vascular endothelial growth factor receptor 2

Table 1. Patients' characteristics

	Hemodialysis (n = 13)	Transplant (n = 8)
Age, yr	15.9 ± 3.5	16.8 ± 1.4
Gender (M:F)	7:6	4:4
Etiology of ESRD	Unknown 1 Neurogenic bladder 2 Dysplastic kidney 5 Congenital nephritic syndrome 2 Focal segmental glomerulosclerosis 1 Mesangioproliferative glomerulonephritis 1 HUS 1	Unknown 2 Neurogenic bladder 1 Dysplastic kidney 3 Congenital nephritic syndrome 1 Focal segmental glomerulosclerosis 1
Anti-HTN treatment	10	5

Values are means ± SD.

M, male; F, female; ESRD, end-stage renal disease; HTN, hypertension; HUS, hemolytic uremic syndrome.

Table 2. Clinical characteristics

	Hemodialysis (n = 13)	Transplant (n = 8)
Height (cm)	131.7 ± 16	140.9 ± 25.9
Weight (kg)	31.5 ± 12	47.5 ± 22.7
BMI (kg/m ²)	16.4 ± 1.9	22.4 ± 5.5
Systolic BP (mm Hg)	122.8 ± 15.6	116.1 ± 12.4
Diastolic BP (mm Hg)	68.8 ± 14.2	71.9 ± 14.4
IVST (cm)	0.82 ± 0.15	0.75 ± 0.16
LVMi (g/m ²)	104.2 ± 49	71.2 ± 27
FK level (mg/L)	—	7.4 ± 2
Hemoglobin (g/dL)	10.9 ± 1.4	12 ± 1.6
Iron (mg/dL)	65.3 ± 35	64 ± 26
Calcium (mg/dL)	9.26 ± 0.99	9.48 ± 0.43
Phosphorus (mg/dL)	6.05 ± 1.63	4.73 ± 0.69
PTH (pg/mL)	424 ± 324	—
Cr (mg/dL)	7 ± 1.4	0.97 ± 0.42
Urea (mg/dL)	142 ± 44	41 ± 19
Albumin (g/dL)	4.13 ± 0.5	4.25 ± 0.2
Cholesterol (mg/dL)	164 ± 42	150 ± 32
HDL (mg/dL)	42 ± 11	44 ± 5.5
Triglycerides (mg/dL)	194 ± 74	133 ± 54
PH blood gases	7.34 ± 0.03	7.315 ± 0.05
HCO ₃ ⁻ (mmol/L)	21.1 ± 2.9	22.1 ± 2.4

Values are means ± SD.

Cr, creatinine; BMI, basal metabolic index; BP, blood pressure; LVMi, left ventricle mass index; PTH, parathyroid hormone; IVST, interventricular septum thickness.

the Schneider Children's Medical Center, Petach-Tikva, Israel. Eight of them received a kidney graft and were further analyzed in the 1st y after transplantation. The transplanted children were on standard immunosuppressive regimen, including steroids, tacrolimus, and mycophenolate mofetil. The demographic characteristics and clinical data for all end-stage renal disease (ESRD) patients undergoing hemodialysis and after transplantation are shown in Tables 1 and 2. Ten age-matched children followed in nephrology clinic for mild hydronephrosis served as the control group. In the patients with dialysis, blood samples of pediatric CRF patients were drawn just before the beginning of hemodialysis and at the end of the dialysis session. In transplanted patients and control group, blood was obtained as a part of routine sampling.

RNA isolation and cDNA preparation. Blood samples were lysed by Puregene RBC Lysis Solution (Gentra systems) to remove red cells and total RNA was isolated using Trizol reagent (Invitrogen). cDNA was synthesized from total RNA using the Super Script First-Strand Synthesis System for PCR-RT kit (Invitrogen).

Quantitative real-time reverse transcription-PCR. Real-time PCR was performed as previously described (16) using a SYBR Green PCR Master Mix (Applied Biosystems). Table 3 summarizes the sequences of the primers used in the present study. Each PCR reaction contained 100 ng cDNA; Primer's final concentration was 500 nM each (forward and reverse). Amplification and detection were performed with the ABI7900HT sequence detection system (Applied Biosystem) and analyzed by the SDS 2.1 program. The thermal cycle used was 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s denaturation at 95°C with 1 min annealing at 60°C.

RNA quantification. To evaluate target genes expression, respiratory quotient (RQ) values were calculated for each sample. The RQ value was the Δ CT value of the target gene (as measured by the ABI7900HT) normalized against the Δ CT of the reference gene (β -actin) and compared with reference samples (control samples). Figure 1 is a representative output of real-time qRT-PCR results in ABI7900HT as represented by the SDS 2.1 program. The figure demonstrates how differences in gene expression are represented graphically by the ABI7900HT program. When gene expression in samples is similar, graphs tend to overlay; hence in the case of β -actin (A and B) and PECAM/CD31 (A), graphs are inseparable. In contrast, in the case of CD-146 (B) gene expression differs, and overlay of graphs is not observed.

Statistical analysis. Values are given as Mean ± Standard Deviation. All statistical analyses were performed using Statistical Package for the Social Sciences for Windows (SPSS, version 10.0, Chicago, IL, USA). Differences in gene expression between tested groups were analyzed using One-Way-ANOVA. Data were analyzed by nonparametric tests, followed by the One-Sample Kolmogorov-Smirnov Test procedure to compare the observed cumulative distribution function for a variable with the Normal distribution. The bivariate Spearman correlation coefficient was calculated to demonstrate the linear relationship between EPC markers and metabolic parameters. All *p* values <0.05 were considered statistically significant.

RESULTS

RNA levels of EPC marker molecules were markedly reduced in ESRD patients undergoing hemodialysis compared with control patients [(VE-C: -3.167 ± 0.724 versus -2.077 ± 0.385 , $p < 0.001$; Flk1: -4.182 ± 1.031 versus -2.916 ± 0.564 , $p < 0.004$; CD146: -3.477 ± 0.852 versus -2.906 ± 0.550 , $p < 0.05$ (Fig. 1). Interestingly, these alterations in EPC marker

Table 3. Primer sequences for real-time PCR

Gene name	Forward	Reverse
VE-Cadherin	5'-GGCATCTTCGGGTGATCCT-3'	5'-CCGACAGTTGTAGGCCCTGTT-3'
FLK1	5'-CACCACCTCAAACGCTGACATGTA-3'	5'-GCTCGTTGGCGCACTCTT-3'
CD31	5'-CCCAGTTTGAGGTCAATAAAGGA-3'	5'-AGGCAAAGTTCCTGATCGA-3'
CD146	5'-TGGTTTGACACCTTGACAGTATTC-3'	5'-TGGGACGCCGGTAGTTGA-3'
TIE2	5'-GCTTGCTCCTTCTTGGAAGTGT-3'	5'-CGCCACCCAGAGGCAAT-3'
AC133	5'-TGGATGCAGAACTTGACAACGT-3'	5'-ATACCTGCTACGACAGTCGTGGT-3'
VEGF	5'-AGCCTTGCTTGTGCTGCTCTA-3'	5'-GTGCTGGCCTTGGTGAGG-3'
SDF1	5'-GACCCGCGCTCGTCCGCC-3'	5'-GCTGGACTCCTACTGTAAGGG-3'
EPO	5'-ACCAACATTGCTTGTCAC-3'	5'-TCTGAATGCTTCCTGCTCTGG-3'
B-ACTIN	5'-CCTGGCACCCAGCACAAT-3'	5'-GCCGATCCACACGGAGTACT-3'

Figure 1. Representative real-time quantitative reverse transcription-PCR evaluation of β -actin (A and B), PECAM/CD31 (A) and CD-146 (B) in peripheral blood samples of children with ESRD undergoing hemodialysis, post kidney transplantation (Trans), and control. x-axis is the number of PCR cycles and y-axis (ΔRn) is the florescence read as measured by the ABI7900HT. Real time PCR reaction was performed using SYBER green reaction mix.

molecules were observed along with reduction in circulating angiogenic growth factor levels (VEGF: -5.232 ± 1.022 versus -3.884 ± 0.126 , $p < 0.002$ and erythropoietin (EPO): -4.080 ± 0.564 versus 2.854 ± 0.751 , $p < 0.008$), which are known to favorably modulate EPC survival and function (Figs. 2 and 3). In contrast, transplanted patients exhibited normal gene levels of EPC marker molecules and angiogenic growth factors compared with healthy controls whereas VE-C and stromal cell-derived factor-1 (SDF-1) were significantly elevated (VE-C:

7.9897 ± 4.021 versus -2.077 ± 0.385 , $p > 0.0001$; SDF-1: -5.496 ± 1.007 versus -6.347 ± 0.346 , $p > 0.05$) (Fig. 1), indicating that within 12 mo after kidney transplantation, alteration in EPC marker levels was mostly restored in this small sample population.

After the observation that EPC marker molecules are decreased among the pediatric ESRD patients, we determined whether they correlate with metabolic parameters. In all of the patient population, VE-C RNA levels inversely correlated with urea and creatinine concentration ($r = -0.673$, $p < 0.001$; $r = -0.712$, $p < 0.002$, respectively). In addition, in ESRD patients, VE-C RNA levels inversely correlated with left ventricle mass index (LVMI) ($r = -0.667$, $p < 0.05$), interventricular septum thickness (IVST) ($r = -0.729$, $p < 0.02$), and diastolic blood pressure (BP) ($r = -0.731$, $p < 0.04$) and positively correlated with children age ($r = 0.483$, $p < 0.05$), height ($r = 0.616$, $p < 0.05$), and iron levels ($r = -0.635$, $p < 0.04$). Interestingly, levels of VE-C highly correlated with those of the EPC marker Flk1 ($r = 0.891$, $p < 0.0001$) and to a lesser degree with CD146 ($r = 0.532$, $p < 0.04$). Similar to VE-C, among the pediatric ESRD patients, Flk1 RNA levels inversely correlated with IVST ($r = -0.667$, $p < 0.05$) and positively correlated with children age ($r = 0.553$, $p < 0.03$).

DISCUSSION

In the present study, we evaluated for the first time the angiogenic potential in pediatric patients undergoing hemodialysis and after renal transplantation by quantitating the mRNA expression of EPC-specific molecules, including Flk-1, Tie-2, CD133, VE-C, CD146, and CD31/PECAM-1 and of growth factors promoting EPC function, VEGF, EPO, and SDF-1 in their peripheral blood as compared with healthy controls. The analysis of the molecular markers has been shown to be highly consistent with quantification of progenitor cell/EPC numbers by flow cytometric analysis (13–15). Quantitative molecular evaluation offers some distinct advan-

Figure 2. Evaluation of circulating VE-C (A), CD146 (B), Flk1 (C), PECAM/CD31 (D), Tie-2 (E), and AC133 (F) mRNA levels in healthy controls (□), ESRD patients undergoing hemodialysis (■), and after renal transplantation (▨). Results are expressed as means $\pm$ SD; VE-C, CD146, and Flk1 are significantly reduced in ESRD, compared with controls; VE-C, $p < 0.001$, CD146, $p < 0.05$, and Flk1 $p < 0.01$. After transplantation, CD146 and Flk1 are normalized whereas VE-C is elevated significantly, $p < 0.0001$ compared with other patient groups. ‡ $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Figure 3. Evaluation of circulating VEGF (A), EPO (B), and SDF-1 (C) mRNA levels in healthy controls (□), ESRD patients undergoing hemodialysis (■) and after renal transplantation (▨). Results are expressed as means $\pm$ SD; VEGF and EPO are significantly reduced in ESRD patients, compared with controls; VEGF and EPO, $p < 0.01$. After transplantation, VEGF and EPO are normalized whereas SDF-1 is elevated significantly, $p < 0.05$ compared with other patient groups. ** $p < 0.01$, * $p < 0.05$.

tages, namely, RNA enumeration can be easily performed in a large series of frozen samples. In addition, inter-laboratory standardization can be more easily achieved (13).

A limitation of our analysis is the small sample sizes (13 hemodialyzed pediatric patients of which eight were transplanted), having a direct influence on the statistical power of the study. Nevertheless, although preliminary, there are several novel findings in our study. First, compared with control patients, pediatric ESRD patients on hemodialysis already demonstrate a significant reduction in EPC marker molecules (VE-C, Flk1, and CD146) as well of EPC-promoting and angiogenic growth factors (VEGF, EPO). These results are in accordance with those obtained in adult patients with advanced renal failure, and dialysis demonstrating overall impaired EPC capacity and fewer numbers of circulating EPCs compared with a control group (17–19). Because both VEGF and EPO (in spite of application of exogenous EPO) are known to augment EPC number and function (6,20), their deficiency may, in turn, affect EPCs as evident by reduced marker levels.

Second, pediatric renal transplantation and the disappearance of the uremic state resulted in the restoration of these marker molecules to levels observed in the normal control population and even to an exaggerated response of several molecules (VE-C, SDF-1). Similarly, de Groot *et al.* (21) have shown that EPC numbers in stable adult renal transplant recipients are comparable to those found in healthy subjects. Although, these and our results do not rule out impairment of EPC capacity evident by diminished function but not levels, Herbrigg *et al.* (22) have recently demonstrated improved EPC function after transplantation.

Third, the EPC marker found to be most significantly reduced in pediatric ESRD, VE-C, related to urea and creatinine concentration in the study population with an inverse correlation to cardiovascular parameters such as diastolic BP, LVMI, and IVST among ESRD patients. Flk1, whose levels closely correlated to those of VE-C, demonstrated similar inverse correlation to IVST. Interestingly, in children undergoing hemodialysis, VE-C positively correlated with height, possibly indicating that improved endothelial/vascular function is associated with increased tissue growth. Clearly, these highly significant correlations observed with Spearman analysis and univariate relationship should be confirmed in a larger cohort of patients using multivariate analysis.

There is growing evidence showing over the past several years that children and young adults with ESRD also experi-

ence significant cardiovascular complications. Moreover, studies in the United States and Europe have demonstrated that cardiovascular disease is a leading cause of death in young adults diagnosed with ESRD in childhood (23,24). Our observations are the first to link EPC marker levels and adverse cardiovascular parameters in pediatric ESRD and suggest that reduced EPC number in childhood might be related to the high cardiovascular risk in this patient population. Taking this into account, the recent study in adult patients with ESRD showing beneficial effects of nocturnal hemodialysis on EPC levels and function compared with conventional hemodialysis (25) might also be of importance to the pediatric population.

Cardiovascular events are the leading cause of morbidity and mortality in ESRD patients. The mechanism of cardiovascular pathology in ESRD is complex, involving ongoing myocardial injury compounded by volume and pressure overload, endothelial dysfunction, and progressive uremic arteriopathy (25). Endothelial dysfunction and vascular damage begin early in the course of chronic kidney disease. They are more severe in children on dialysis (26–29). In recent years, attention has focused on the mechanisms of the development and progression of uremic vascular disease, especially on its correlation to disturbed Ca-P metabolism. There are few studies that deal with other determinants of cardiovascular complications of chronic kidney disease. Because EPCs maintain endothelial function in mature blood vessels, reduced EPC levels (indicated by reduced marker molecules) are likely to be related to failure of this function leading to endothelial dysfunction and abnormal vasoreactivity (30). This, in turn, may manifest in abnormal clinical variables that were found in our study to be related, elevated BP and LVMI. Recently, the measurement of carotid artery intima-media thickness using high-resolution ultrasonography has been suggested as an excellent marker of uremic vasculopathy in pediatric patients (29,31). In addition, Doppler assessment of brachial artery flow and flow-mediated dilatation (FMD) has been demonstrated as a measure of endothelial dysfunction in pediatric chronic renal failure (32). Future studies aimed at finding an association between EPC marker gene levels and other parameters of endothelial dysfunction assessed by such methodologies are therefore warranted.

In summary, we revealed that angiogenic potential as evaluated by the expression of EPC specific-molecules was relatively low in children with ESRD undergoing hemodialysis and mostly restored after renal transplantation. Measurements

of VE-C and Flk1 RNA levels may be helpful in monitoring endothelial dysfunction and identifying children prone to cardio-vascular morbidities in this clinical setting. Prospective studies should determine whether the increase in EPC marker molecules affects cardiovascular outcome in renal transplant recipients.

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