Age-Related Changes in Soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR1) and Receptor 2 (sVEGFR2) in Healthy Japanese Subjects

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Abstract We measured serum soluble vascular endothelial growth factor receptor 1 (sVEGFR1) and receptor 2 (sVEGFR2) levels in healthy Japanese individuals in order to establish a reference value using a specific ELISA. Significant differences were observed in serum sVEGFR1 and sVEGFR2 levels between children and adults. To demonstrate the usefulness of the reference value for children, we measured serum sVEGFR1 and sVEGFR2 levels in children with diarrhea positive (D+) hemolytic uremic syndrome (HUS) as a preliminary study. Serum sVEGFR2 levels in children with HUS were markedly higher than those in healthy children from the onset of D + HUS. The reference value for healthy children in the present study will allow normal and pathological conditions to be discriminated from each other in future study

Keywords Vascular · Endothelial growth factor (VEGF) · Soluble VEGF receptor-1 (sVEGFR1) · Soluble VEGF receptor-2 (sVEGFR2) · Hemolytic uremic syndrome (HUS)

Introduction

Cytokines exert their biological effects through specific receptors expressed on target cells. A number of cytokine receptors not only have membrane-bound forms, but also soluble counterparts (soluble cytokine receptors) in serum, and these are formed either by the proteolytic cleavage of membrane-anchored receptors or alternative splicing of mRNA [1]. Soluble cytokine receptors retain their ligand binding capacity and act as agonists, antagonists of cytokine signaling, or carrier proteins of the ligand [2]. Thus, soluble cytokine receptors may modulate immune reactions.

Vascular endothelial growth factor (VEGF) and its receptor system are involved in the formation of blood vessels and lymph vessels in mammals from the earliest stages of embryogenesis [3]. The actions of VEGF are primarily mediated through binding to the membrane-associated tyrosine-kinase receptors, VEGFR1 (flt-1), VEGFR2 (KDR/flk-1), and VEGFR3 (flt-3) [4]. In the VEGF family, VEGF-A is mainly mediated by VEGFR1 and VEGFR2. Although the VEGF receptors are strongly related structurally, they still exhibit differences, for example, their abilities to respond to ligand binding with increased kinase activity and the spectrum of transduced biological responses [5]. Biological activities are also known to be modulated by the binding of free VEGF to circulating binding proteins, including the soluble extracellular portions of VEGF receptors (sVEGFR1 and sVEGFR2) [6]. An increase in serum sVEGFR1 and sVEGFR2 concentrations has been reported in various diseases [7, 8]. On the other hand, the pathological significance of sVEGFR1 and sVEGFR2 in pediatric patients has yet to be elucidated in detail. We speculated that there may be differences in sVEGFR1 and sVEGFR2 values

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between adults and children. We attempted to investigate the clinical significance of serum sVEGFR1 and sVEGFR2 levels in pediatric patients. The diagnostic application of measuring of serum sVEGFR1 and sVEGFR2 levels critically depends on the samples used as controls. The levels of several soluble cytokine receptors in the body fluids of healthy individuals have been shown to change with age [9]. There is currently no information on variations in sVEGFR1 and sVEGFR2 serum levels in healthy children.

To investigate the shifts that occur in serum sVEGFR1 and sVEGFR2 levels with pathological conditions in pediatric patients, we determined sVEGFR1 and sVEGFR2 serum levels in healthy Japanese subjects to obtain reference values. We also measured serum sVEGFR1 and sVEGFR2 levels in patients with diarrhea positive (D+) hemolytic uremic syndrome (HUS) [10] as a diagnostic parameter for evaluating the disease state as a preliminary application.

Materials and Methods

Healthy Japanese Subjects

Forty-one healthy Japanese children (26 boys and 15 girls, median age: 4.0 years old) and 49 healthy Japanese adults (33 men and 16 women, median age: 33.0 years old) were included in the present study. Serum samples were obtained from nonatopic healthy children undergoing a medical check for corrective surgery for a hernia or circumcision repairs, which were considered to be unrelated to diseases associated with altered immunological parameters. Some samples were collected at a group physical examination in an elementary school. Specimens from healthy Japanese adults were collected during a group physical examination.

Exclusion criteria were the use of any medication within the previous 3 months. By the time of blood sampling, none of the study subjects reported or exhibited any inflammatory or other concurrent diseases. Serum samples were frozen immediately and stored at -70°C . The purpose, procedure, and benefits of this study were explained to the participants or their parents, and their informed consent was obtained.

Patients

Eleven Japanese children with D + HUS (8 boys and 3 girls, median age; 4 years old) were included in the study. HUS was defined according to the diagnostic criteria proposed by the Japanese Society for Pediatric Nephrology [10] by the presence of hemolytic anemia (hemoglobin, $\text{Hb} < 10 \text{ g/dl}$), acute renal dysfunction (oliguria, anuria, or

an increase in the serum creatinine levels corrected for age), and thrombocytopenia (platelet count, $\text{PLT} < 10 \times 10^4/\mu\text{l}$). Serum samples, which were collected on the day of the onset of HUS, were used in the present study. The purpose, procedure, and benefits of our study were explained to the participants or their parents, and we obtained their informed consent.

Determination of Soluble VEGFR1 and VEGFR2 Levels

sVEGFR1 and sVEGFR2 levels in the serum samples were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA). This was a standard sandwich ELISA that was performed according to the manufacturer's instructions using human recombinant standards. All samples were run in duplicate with the appropriate standards on 96-well microplates. In the present study, each assay was run with same quality control samples to monitor the accuracy of the assay. The detection limit of both assays was 0.08 ng/ml.

Statistical analysis

Results were expressed as medians, and lower (Q1) and upper (Q3) quartiles. Comparisons between two groups were performed using the Mann–Whitney U rank sum test. The strength of associations between two quantitative variables was assessed by the Spearman rank correlation test. A value of $p < 0.05$ was considered to be significant. Statistical analyses were performed with the aid of the computerized statistical package, Stat View-5.0 (SAS Institute Inc., USA).

Results

Serum sVEGFR1 and sVEGFR2 Levels in a Healthy Japanese Population

The upper age limit of pediatrics ranged from 14 to 18 years old, depending on the country. The upper limit in Japan is typically 14 years old. Therefore, we divided subjects into two groups (under 14 years and over 15 years). sVEGFR1 and sVEGFR2 levels in the serum samples collect from healthy children ($n = 41$) and those from the healthy adults ($n = 49$) are summarized in Table 1. A significant difference was observed in serum sVEGFR1 or sVEGFR2 levels between the children and adults.

Scatter plots of serum sVEGFR1 and sVEGFR2 concentrations are shown in Fig. 1a, b, respectively.

Spearman's correlation rank coefficients (two-tailed) were used to evaluate the relationship between age and sVEGFR1 or sVEGFR2 levels. A correlation was observed between both receptors and age (sVEGFR1: Spearman $r = -0.291$, $p = 0.013$ and sVEGFR2: Spearman $r = -0.566$, $p < 0.001$). A positive correlation was found between serum sVEGFR1 and sVEGFR2 levels in healthy subjects (Fig. 2, Spearman $r = 0.354$, $p < 0.005$). No

significant difference was observed in either soluble cytokine receptor between the female and male groups (data not shown).

Preliminary Application to Patients with HUS

Serum levels of sVEGFR1 and sVEGFR2 and the laboratory data obtained from the 11 children with HUS and those from healthy children are summarized in Table 2.

Table 1 Summary of serum sVEGFR1 and sVEGFR2 levels in healthy subjects

	Children	Adults
Number of subjects	41	49
Male/Female	26/15	33/16
Age (years old)		
Median	4.0	33.0
Lower quartile (Q_1)	1.0	24.0
Upper quartile (Q_3)	6.0	44.0
sVEGFR1 (ng/ml)		
Median	1.7*	0.0
Lower quartile (Q_1)	0.0	0.0
Upper quartile (Q_3)	5.9	3.5
sVEGFR2 (ng/ml)		
Median	2.6**	1.7
Lower quartile (Q_1)	2.2	1.5
Upper quartile (Q_3)	3.1	2.1

Serum sVEGFR1 and sVEGFR2I levels are shown as median values, together with the lower (Q_1) and upper (Q_3) quartiles. The serum sVEGFR1 and sVEGFR2 levels in children (age: 1–14 years old) were significantly higher than those in adults (age: 22–67 years old). The Mann–Whitney U rank sum test was used to investigate significant differences. ** $p < 0.01$, * $p < 0.05$

Fig. 2 The relationship between serum sVEGFR1 and sVEGFR2 levels. The serum sVEGFR1 and sVEGFR2 levels in 41 healthy children and 49 healthy adults were plotted. Spearman's correlation test was applied to evaluate the relationship between serum sVEGFR1 and sVEGFR2 levels. A correlation was observed between sVEGFR1 and sVEGFR2 levels. (Spearman $r = 0.354$, $p < 0.005$)

Fig. 1 Age-dependent profiles of the serum levels of soluble cytokine receptors in healthy subjects. Serum sVEGFR1 (a) and sVEGFR2 (b) levels in 41 healthy children and 49 healthy adults

Table 2 Summary of soluble VEGFR1 and R2 serum levels and laboratory data in individual patients

Patient no.	Age	Sex	Day of onset						
	Years old	F/m	WBC /μl	Platelet ×10 ⁴ /μl	Hb g/dl	Na mEq/l	SCre mg/dl	SVEGFR1 ng/ml	SVEGFR2 ng/ml
HUS (n = 11)									
1	5	m	9,400	2.2	7.5	131	1.50	4.0	122.2
2	3	f	11,300	3.9	7.3	131	0.92	4.4	239.3
3	3	m	12,500	1.8	9.2	139	0.43	ND	ND
4	9	m	17,800	6.5	4.2	136	0.65	4.9	47.1
5	9	m	8,800	1.7	5.7	137	3.15	0.8	56.0
6	8	f	15,700	3.4	7.6	129	8.70	ND	67.1
7	2	m	18,800	9.4	12.5	131	2.13	9.9	402.0
8	2	f	8,300	14.9	8.5	140	0.49	2.4	ND
9	10	m	13,700	2.2	4.0	137	10.19	8.3	208.6
10	3	m	12,200	6.0	9.7	131	1.03	53.4	ND
11	6	m	11,000	0.9	7.4	137	0.78	1.0	153.6
Median	5.0		12,200	3.4	7.5	136	1.03	4.0 ^{NS}	67.1**
Lower quartile	3		10,200	2.0	6.5	131	0.72	0.9 ^{NS}	23.6**
Upper quartile (Q3)	8.5		14,700	6.3	8.9	137	2.64	6.6 ^{NS}	181.1**
Healthy children (n = 41)									
Median	4							1.7 ^{NS}	2.6**
Lower quartile (Q1)	1							0.0	2.2
Upper quartile (Q3)	6							5.9	3.1
Reference value			3,500–8,000 [#]	12–40 [#]	– [§]	135–147 [#]	0.3–1.0 [#]		

[#] Reference values of healthy children from our institution. Each reference value depended on age

[§] The reference value of hemoglobin (Hb) was 13.7–16.9 for males and 11.4–14.7 for females. We investigated significant differences between sVEGFR1 and sVEGFR2 levels in patients with HUS and healthy controls by nonparametric statistical tests (two-tailed Mann–Whitney U tests)

We considered ** $p < 0.01$ as significant

NS no significant, ND not detected, sCre serum creatinine

Hemoglobin (Hb) levels, platelet counts, and serum creatinine (sCre) levels are the markers that are used to diagnose HUS [10]. At the onset of HUS, serum sVEGFR2 levels in the patients with HUS were higher than those in the healthy group ($p < 0.01$). On the other hand, no significant difference was observed in serum sVEGFR1 levels between the patient group and the healthy group (Fig. 3).

Discussion

An appropriate evaluation of the measurement of biomarkers depends on the reference values used. Thus, we first determined the reference sVEGFRs values of healthy subjects. In the present study, we determined the serum levels of sVEGFR1 and sVEGFR2 in healthy subjects (Table 1). A significant difference was observed in the serum levels of either type of sVEGFR between the children and adults.

Previous studies, including ours, reported changes in the levels of some soluble cytokine receptors in body

fluids, such as serum and urine, with age [9]. Correlations have been observed between serum levels of either type of sVEGFR and age. Age-dependent decreases in serum levels have been found in both types of receptors. Although age-dependent changes in serum VEGF values have been reported previously [11], they have not yet been determined for soluble receptors. Thus, our results will be valuable for the evaluation of various clinical samples.

A positive correlation was observed between serum sVEGFR1 and sVEGFR2 levels in the healthy subjects (Fig. 2, Spearman $r = 0.354$, $p < 0.005$). The sVEGFR1/R2 ratio was previously reported to be reduced in some pathological cases [12]. This relationship between sVEGFR1/R2 may also be important information for diagnoses.

We determined serum sVEGFR1 and sVEGFR2 levels in patients with diarrhea positive (D+) HUS as a preliminary application of the sVEGFRs reference values of healthy children. D + HUS occurs after a prodrome of hemorrhagic colitis caused by verotoxin-producing *Escherichia coli*

Fig. 3 Soluble VEGF receptor levels in the serum of patients with D + HUS at its onset. The graphs show the serum sVEGFR1 (a) and sVEGFR2 levels (b) of 41 healthy children and 11 patients with D + HUS. The open circles indicate the healthy subjects, the closed

circles indicate individual patients, and the horizontal bars represent the median values of each group. Significant differences were determined by the Mann–Whitney U rank sum test. (** $p < 0.01$)

infection [13]. The disease causes the destruction of red blood cells, damage to blood vessel walls, and, in severe cases, kidney failure. The recovery length varies from several days to months. HUS is a rare condition that mostly affects children under the age of 10, and severe cases are life-threatening. If the child survives the initial stages of the disease, the long-term prognosis is good.

Serum VEGF levels were elevated in patients with HUS [14]. However, serum levels of sVEGFRs have not yet been clarified. We showed that the serum levels of sVEGFR2 were significantly elevated in patients with HUS, whereas, serum sVEGFR1 levels remained unchanged. Further studies are warranted to clarify the roles of sVEGFR1 and sVEGFR2 as well as their interactions with VEGF in the development of HUS

In summary, this study determined the age-related profile of serum sVEGFR1 and sVEGFR2 levels in healthy Japanese individuals. It was found that the sVEGFR1 and sVEGFR2 serum level of children (0–14 years old) was significantly higher than that of adults (over 15 years old). Thus, it is recommended that when the serum sVEGFR1 and sVEGFR2 levels of patients are evaluated, the values should be compared with those of age-matched controls. These data on healthy subjects were important for the future clinical studies on sVEGFRs.

Conflict of interest The authors declared no financial or commercial conflict of interest.

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