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Relation of Soluble Endothelial Protein C Receptor and Cytokines After Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract

Aim: The objective of this study was to elucidate the effects of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6), and interleukin 8 (IL-8) on the expression of soluble endothelial protein C receptor (sEPCR) in the pathogenesis of thrombotic complications after hematopoietic stem cell transplantation (HSCT).

Methods: The relationship between plasma concentrations of proinflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6, and IL-8) and sEPCR was evaluated in 32 consecutive allogeneic hematopoietic stem cell–transplanted patients prior to conditioning regimen and randomly once between +5 and +30 days after transplantation and compared these results with 20 healthy controls.

Results: Soluble endothelial protein C receptor levels did not indicate any significant difference between pre- and posttransplantation period, and sEPCR levels showed a significantly negative correlation between IL-6 and IL-8 (sEPCR and IL-6, $r = -.43$, $P < .01$; sEPCR and IL-8, $r = -.57$, $P < .01$). There was no correlation between sEPCR levels and TNF-α, IL-1β, or IL-2 (sEPCR and TNF-α, $r = -.13$, $P > .05$; sEPCR and IL-1β, $r = -.1$, $P \geq .05$; sEPCR and IL-2, $r = -.07$, $P > .05$).

Conclusions: Our results suggest that the production of sEPCR was not affected by allogeneic HSCT. Soluble endothelial protein C receptor did not show any positive correlation between these proinflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6, and IL-8), on the contrary a significantly negative correlation was determined between sEPCR and either IL-6 or IL-8. This negative correlation may be a protective mechanism in the pathway of protein C activation.

Keywords

cytokine, interleukin, protein C, soluble endothelial protein c receptor, thrombosis, sinusoidal obstruction syndrome

Introduction

Patients undergoing high-dose chemoradiotherapy followed by hematopoietic stem cell transplantation (HSCT) may present thrombotic complications in the early period after transplantation. Pulmonary endothelial leakage syndrome, thrombotic thrombocytopenic purpura, venous thrombosis, and hepatic sinusoidal obstruction syndrome (SOS) have been reported in this period.[1,2] Hypercoagulability after HSCT is accused of these complications. Activation of the coagulation system has been observed after HSCT in many reports. A reduction in the natural anticoagulants protein C, protein S, and antithrombin has been reported in transplanted patients.[3-7] It has been suggested that SOS results from a prothrombotic state in the posttransplant period due to a fall in natural anticoagulants, factor V Leiden, and prothrombin variant G20210A mutation.[3-9] Protein C levels appear to be significantly low in patients who developed SOS compared to non-SOS patients in posttransplant period.[3,4,10,11]

The pathway of protein C is well established as a physiologically important mechanism for inhibiting the coagulation process.[12] Not only decrease in the protein C levels but also decrease in the protein C activation rate may be responsible for thrombotic complications in the early phase posttransplant. A soluble form of endothelial protein C receptor (sEPCR) inhibits both activation of protein C and anticoagulant activity of activated protein C.[13,14] The importance of sEPCR in thrombosis is gradually increasing, and the place of sEPCR in thrombotic complications after transplantation is really unknown. Thrombin and interleukin 1β (IL-1β) can lead to the release of sEPCR, decreasing the potential of protein C activation.[15] A correlation between tumor necrosis factor α (TNF-α) and sEPCR levels among healthy controls was reported.[16] In another study, TNF-α significantly decreased the expression

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of thrombomodulin and endothelial protein C receptor.[17] The role of proinflammatory cytokines in the pathogenesis of major transplant-related complications (MTC) including SOS is previously presented. Systemic release of TNF-α, interleukin 1 (IL-1), interleukin 6 (IL-6), and interleukin 8 (IL-8) is a part of initiating these MTC.[18-22] It was hypothesized that HSCT triggers the production of sEPCR under the influence of alterations in cytokines, and this pathway is responsible for lowered activated protein C (APC) anticoagulant activity, which is important in thrombotic complications after transplantation.

We studied the relationship between plasma concentrations of proinflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6, and IL-8) and sEPCR in a group of 32 patients undergoing HSCT.

Patients and Methods

Patients and Controls

A total of 32 patients who received allo-HSCT at Ankara University Pediatric Bone Marrow Transplant (BMT) unit and 20 healthy controls were included in the study. Informed consent was obtained from all parents. The study was approved from Ethics Committee of Ankara University School of Medicine. The median age of the patients was 12 years (range 1.9-17), and 17 of them (53%) were males. The median age of the 20 controls was 10.6 years (3.1-17.7), and 11 of them (55%) were males.

Tumor necrosis factor α, IL-1β, IL-2, IL-6, IL-8, and sEPCR were evaluated in 32 consecutive allogeneic BMT patients prior to conditioning regimen and randomly once between +5 and +30 days after transplantation and these results compared with 20 healthy controls.

Patient and Transplant Characteristics

Demographic and clinical features of the patients and the characteristics of allogeneic HSCT are summarized in Table 1. All the patients underwent first HSCT, and all were related HLA identical donors. All patients received myeloablative conditioning regimen.

Table 1. Demographic and Clinical Features of the Patients and the Characteristics of Allogeneic HSCT

	N	%
Patient characteristics		
Sex	17 B/15 G	53 B/47 G
Age (years), median	12 years	
Range	1.9-17	
Diagnosis		
Hemoglobinopathy/AA	15/6	47/19
Acute leukemia	7	22
MDS/CML	2/1	6/3
FHLH	1	3
Donor		
Sibling	29	91
Parents	2	6
Cousin	1	3
Stem cell source		
BM	22	69
PB stem cell	8	25
BM and PB stem cell	1	3
BM and cord blood	1	3
Conditioning regimen		
BU + CY	19	59.2
HU + AZT + FLU + BU + CY + ATG	4	12.5
CY + ATG + FLU	3	9.4
BU + CY + VP-16	2	6.3
Melphalan + BU + CY	2	6.3
CY + ATG	2	6.3
GVHD prophylaxis		
CsA	6	19
CsA + MTX	26	81

NOTES: AA = aplastic anemia; ATG = antithymocyte globuline; AZT = azathiopurine; B = boy; BM = bone marrow; BU = busulphan; CML = chronic myeloid leukemia; CsA = cyclosprine A; CY = cyclophosphamide; G = girl; FHLH = familial hemophagocytic lymphohistiocytosis; FLU = fludarabine; GVHD = graft versus host disease; HU = hydroxyurea; MDS = myelodysplasia; MTX = methotrexate; PB = peripheral blood; VP-16 = etoposide.

Definition of SOS

Sinusoidal obstruction syndrome was diagnosed according to Seattle criteria.[23] At least 2 of the 3 following criteria were observed, within the 30 days after stem cell transplantation: (1) jaundice (bilirubin > 2 mg/dL), (2) hepatomegaly or right upper quadrant pain of liver origin, and (3) ascites and/or unexplained sudden weight gain (>2% of baseline body weight). Sinusoidal obstruction syndrome was classified as severe, moderate, and mild according to the McDonald severity criteria.[24]

Sample Collection

Blood samples were collected from patients and controls by venipuncture in tubes containing 0.11 mol/L sodium citrate. The blood samples were collected before conditioning regimen and randomly once between +5 and +30 days after the day of HSCT from patients. Platelet-poor plasma was obtained by centrifugation at 2000*g* for 20 minutes and stored at −80°C until use.

Cytokine Level Measurement

In both the groups, cytokine levels were analyzed using automated multiplex serum cytokine assay (Luminex Corporation, Austin, Texas). The sensitivity value was 3.2 pg/mL for TNF-α, IL-1β, IL-2, IL-6, and IL-8.

sEPCR Assay

The sEPCR levels were determined by enzyme immunoassay (Asserachrom sEPCR; Diagnostica Stago, Asnières, France),

Table 2. Comparison of Cytokines and sEPCR Between Patients (Prior to Conditioning Regimen) and Control Group

Parameters	Pre-HSCT (n = 32), Median (Range)	Controls (n = 20), Median (Range)	*P*
TNF-α (pg/mL)	6.8 (4.5-59.7)	9.6 (3.1-28.6)	.26
IL-6 (pg/mL)	45.6 (29.4-369.7)	19.7 (8.2-81.2)	.004[a]
IL-8 (pg/mL)	9.9 (4.3-66.5)	11.1 (6.5-15.6)	.09
IL-1β (pg/mL)	11.4 (4.6-69)	9.3 (5.5-33.7)	.004[a]
IL-2 (pg/mL)	10.2 (1.1-221.2)	20.1 (8.6-70.7)	.002[a]
sEPCR (ng/mL)	65 (13-308)	58.5 (39-456)	.30

NOTES: pre-HSCT = pretransplant; sEPCR = soluble endothelial protein C receptor; TNF-α = tumor necrosis factor α.
[a] Significant at the level .01, Mann-Whitney test.

Table 3. Comparison of Cytokines and sEPCR Results Between Pre- and Posttransplant in Patients

Parameters	Pre-HSCT (n = 32), Median (Range)	Post-HSCT (n = 32), Median (Range)	*P*
TNF-α (pg/mL)	6.8 (4.5-59.7)	5.4 (4.3-12.2)	.017[a]
IL-6 (pg/mL)	45.6 (29.4-369.7)	78.5 (32-260.4)	.001[b]
IL-8 (pg/mL)	9.9 (4.3-66.5)	18.3 (5.3-322.7)	.003[b]
IL-1β (pg/mL)	11.4 (4.6-69)	6.4 (4.9-11.2)	.017[a]
IL-2 (pg/mL)	10.2 (1.1-221.2)	9.2 (1.6-25.9)	.007[b]
sEPCR (ng/mL)	65 (13-308)	69.5 (16-208)	.106

NOTES: IL = interleukin; pre-HSCT = pretransplant; post-HSCT = posttransplant; sEPCR = soluble endothelial protein C receptor; TNF-α = tumor necrosis factor α.
[a] Significant at the level .05.
[b] Significant at the level .01, Wilcoxon signed rank test.

according to the manufacturer's instructions. Samples were assayed in duplicate. The sensitivity value was 10 ng/mL for sEPCR.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows, version 15. Statistical significance between patient and control groups was determined by Mann-Whitney test and the means of parameters for patients before and after HSCT was determined by the Wilcoxon signed rank test. Pearson correlation analysis was used to detect significant univariate associations between the parameters analyzed.

Results

There were no differences between patients and control group as regards age distribution ($P > .05$). The comparison of cytokines and sEPCR values between patients (prior to conditioning regimen) and control groups was shown in Table 2. The mean level of IL-6 at pre-HSCT was found to be higher ($P < .01$) than the levels in control group, whereas IL-1β and IL-2 levels were lower ($P < .01$ and $P < .01$). The mean values of TNF-α, IL-8, and sEPCR were not different between pre-HSCT and control groups ($P > .05$).

In patient group, the mean levels of IL-6 and IL-8 increased ($P < .01$ and $P < .01$), while the mean levels of TNF-α, IL-2, and IL-1β decreased significantly after transplantation ($P < .05$, $P < .01$, and $P < .05$, respectively). However, the mean values of sEPCR did not differ before and after HSCT ($P > .05$). Comparison of the levels of cytokines and sEPCR of pre-HSCT and post-HSCT in patients was shown in Table 3.

The difference between the levels of cytokine and sEPCR before and after transplantation in patients was analyzed using "Pearson correlation test" and shown in Table 4. As mentioned above, TNF-α, IL-1β, and IL-2 decreased in patient group after HSCT. A significantly positive correlation was determined between TNF-α, IL-1β, and IL-2 (TNF-α and IL-1β, $r = .93$, $P < .01$; TNF-α and IL-2, $r = .93$, $P < .01$; IL-1β and IL-2, $r = .99$, $P < .01$), besides the levels of IL-6 and IL-8 increased significantly after transplantation, and a significantly positive correlation was determined between IL-6 and IL-8 ($r = .58$, $P < .01$). Although there was no significant difference between the mean level of sEPCR at pre- and posttransplantation period, sEPCR showed a significantly negative correlation between IL-6 and IL-8 (sEPCR and IL-6, $r = -.43$, $P < .01$; sEPCR and IL-8, $r = -.57$, $P < .01$). However, the levels of sEPCR did not indicate any correlation between TNF-α, IL-1β, and IL-2 (sEPCR and TNF-α, $r = -.13$, $P > .05$; sEPCR and IL-1β, $r = -.1$, $P \geq .05$; sEPCR and IL-2, $r = -.07$, $P > .05$).

Clinical Evolution

Sinusoidal obstruction syndrome developed in 3 of 32 patients (9.3%) as defined by classical diagnostic criteria. Two of them with severe SOS and they died, one of them with moderate SOS and he is alive. The number of the patients with SOS was not adequate to evaluate these parameters according to SOS. When it was considered one by one, only the mean level of IL-6 showed an increase in all the patients with SOS, without a change in sEPCR. Characteristics, cytokines, and sEPCR results in patients with SOS were shown in Table 5.

Discussion

In the current study, we tried to identify the cytokines (TNF-α, IL-1β, IL-2, IL-6, and IL-8) and sEPCR interactions probably involved in the pathomechanisms of thrombotic complications after allogeneic HSCT. In our study, sEPCR mean values were not different between the pre-HSCT and control groups at baseline time, and the mean value of sEPCR did not show any significant difference before and after HSCT. Our results suggest that sEPCR production was not affected by allogeneic HSCT. Tumor necrosis factor α, IL-1β, IL-2, IL-6, and IL-8 are well-known proinflammatory cytokines, which are involved in local and systemic inflammatory reactions, have been found to be involved in many adverse conditions by HSCT.[19,21,22,25-28] A positive relationship was expected between these cytokines and sEPCR. Interestingly, sEPCR did not show any positive correlation between these proinflammatory cytokines, on the contrary sEPCR showed a significantly negative correlation

Table 4. The Correlation of the Difference Between the Levels of Cytokines and sEPCR Values Before and After Transplantation in Patients

Parameters	r/P	D TNF-α	D IL-6	D IL-8	D IL-1β	D IL-2	D sEPCR
D TNF-α	r	1	.25	.06	.93	.93	−.13
	P	-	.16	.72	.001[b]	.001[b]	.48
D IL-6	r	.25	1	.58	.20	.19	−.43
	P	.16	-	.001[b]	.26	.28	.01[a]
D IL-8	r	.06	.58	1	.02	.01	−.57
	P	.72	.001[b]	-	.91	.93	.001[b]
D IL-1β	r	.93	.20	.02	1	.99	−.10
	P	.001[b]	.26	.91	-	.001[b]	.57
D IL-2	r	.93	.19	.01	.99	1	−.07
	P	.001	.28	.93	.001[b]	-	.68
D sEPCR	r	−.13	−.43	−.57	−.10	−.07	1
	P	.48	.01[a]	.001[b]	.57	.68	-

NOTES: D = difference of pre-HSCT values and post-HSCT values; IL = interleukin; *r* = correlation coefficient; sEPCR = soluble endothelial protein C receptor; TNF-α = tumor necrosis factor α.
[a] Significant at the level .05.
[b] Significant at the level .01, Pearson correlation.

Table 5. Characteristics, Cytokines, and sEPCR Results of Patients who Developed SOS

		SOS 1	SOS 2	SOS 3
Age/sex		12.7/B	15/G	13.5/B
Diagnosis		FA	AML	TM
VOD diagnosis (day)		+11	+9	+19
Clinical outcome		Severe Died	Severe Died	Moderate Alive
TNF-α (pg/mL)	Pre	ND	5.97	4.74
	Post	ND	ND	ND
IL-6 (pg/mL)	Pre	58	ND	48.57
	Post	260.44	83.41	80.03
IL-8 (pg/mL)	Pre	6.76	ND	21.86
	Post	322.67	20	10.12
IL-2 (pg/mL)	Pre	2.56	2.56	ND
	Post	ND	ND	ND
sEPCR (ng/mL)	Pre	308	215	14.5
	Post	93	173	42

NOTES: AML = acute myeloid leukemia; FA = Fanconi anemia; IL = interleukin; ND = not detectable; sEPCR = soluble endothelial protein C receptor; SOS = sinusoidal obstruction syndrome; TM = thalassemia major; TNF-α = tumor necrosis factor α; VOD = venocclusive disease of the liver (<3.2 pg/mL; the sensitivity value).

between IL-6 and IL-8. Interleukin 6 is released in response to tissue injury and is a key cytokine regulating the acute-phase response. Wang et al[29] reported that changes in the levels of serum IL-6 were associated with changes in symptom severity during the initial weeks of allo-HSCT in their study. Interleukin 8 can be released by activated endothelial cells in response to TNF-α and is a potent activator of neutrophils, which can further contribute to tissue damage.[30] Ferra et al[27] observed a strong correlation between IL-6 and IL-8 levels after HSCT. In this study, the mean level of IL-6 of the pre-HSCT group was found to be higher, and the mean level of IL-8 was not different between pre-HSCT and control groups. However, the mean levels of IL-6 and IL-8 increased significantly after transplantation. A strong positive correlation between IL-6 and IL-8 levels was observed in this study similar to those reported by Ferra et al.[27] In other words, when IL-6 and IL-8 levels increased, sEPCR levels significantly decreased in our study. Opposite to our hypothesis, this may be a protective mechanism in the pathway of protein C activation.

Various studies have shown that increased plasma levels of TNF-α and IL-1β precede the complications of HSCT, such as graft versus host disease (GVHD) and SOS.[19,31,32] Tumor necrosis factor α infusion in humans strongly promotes procoagulant activity and suppresses anticoagulant activity of endothelial cell surface by blocking the protein C pathway through the suppression of thrombomodulin synthesis.[7] Both TNF-α and IL-1β are procoagulants.[33-35] In this study, sEPCR did not show any correlation between TNF-α, IL-1β, and IL-2. Tumor necrosis factor α values were not different between pre-HSCT and control groups at baseline time, however, TNF-α decreased significantly after allogeneic transplantation. Gugliotta et al[31] reported that both allogeneic (10 cases) and autologous (20 cases) transplantation patients showed mean TNF-α levels significantly increased with respect to normal values at baseline time. In their study, TNF-α values always remained increased compared with normal in allogeneic HSCT, whereas autologous patients showed a significant reduction.[31] All our patients were allogeneic, but interestingly TNF-α values showed significant reduction similar to autologous HSCT patients in Gugliotta's study. In our study, the mean levels of IL-1β and IL-2 of pre-HSCT group were found to be lower, and TNF-α mean values were not different between pre-HSCT and control groups. Patients' clinical condition, such as underlying disease, used drugs, and associated infections, may have a role on cytokine levels.

Tumor necrosis factor α, IL-2, and IL-1β decreased significantly after transplantation, and a significantly positive correlation was determined between TNF-α, IL-1β, and IL-2. Cytokines including TNF-α, IL-1β, and IL-2 are released from monocytes, reticuloendothelial, and endothelial cells in

response to cytotoxic drugs, infection, radiotherapy, and hypoxia.[7,36,37] In our study, blood samples were collected between +5 and +30 days after HSCT randomly. These results may be affected by hematopoietic recovery and MTC development after transplantation. Moreover, the drugs used in HSCT may have a role in cytokines and sEPCR production. However, in this study, discussing the relation between drugs and sEPCR levels or cytokines is impossible in these patients with different diagnosis.

It was aimed to evaluate the cytokines and sEPCR relations between SOS- and non-SOS-developed patients in this study. Because of limited number of the patients with SOS (n:3), we could not compare these parameters according to SOS. In this study, when it was considered one by one, only IL-6 values showed an increase in all the SOS-developed patients, without a change in sEPCR according to hypothesis we setup. The samples were taken in the 6th day after HSCT in these patients who developed SOS. There were no findings of SOS in the sampling period (SOS findings were at +9, +11, +19 days, respectively, after HSCT in these 3 patients). Cytokines in the pathogenesis of SOS is unclear. Hypercoagulable status in SOS can be induced and maintained by proinflammatory cytokines. Cytokines may induce a procoagulant environment with endothelial protein C receptor or sEPCR. It was needed for a larger series to explain these probable relationships. The difficulty with studies examining biological markers of inflammation in SOS is that it is impossible to speculate whether these changes are primary features or secondary to these disease process itself.[38] Proinflammatory cytokine release is a factor essential and common to all types of MTC, and no particular pattern was specific for SOS, endothelial leakage syndrome, idiopathic pneumonia syndrome, or acute GVHD. Major transplant-related complications can be considered the clinical correlates or end points of an exaggerated systemic inflammatory reaction with massive cytokine release.[18]

In summary, our results suggest that, sEPCR production was not affected by allogeneic HSCT. Soluble endothelial protein C receptor did not show any positive correlation between these proinflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6, and IL-8), on the contrary sEPCR showed a significantly negative correlation between IL-6 and IL-8. This negative correlation may be a protective mechanism in the pathway of protein C activation.

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Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

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