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Regular Article

Decreased thrombomodulin mRNA expression on peripheral monocytes in disseminated intravascular coagulation patients relates to poor outcomes: The *ex vivo* effects of lipopolysaccharide and thrombin on monocyte thrombomodulin and CD14 mRNA

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ABSTRACT

Background: Monocytes express substantial amounts of thrombomodulin, which is consumed throughout ongoing thrombin generation. The modulation of thrombomodulin may aggravate intravascular fibrin deposition and the clinical course of disseminated intravascular coagulation (DIC). Although thrombomodulin restoration has received considerable attention, no reports have been published on the *in vivo* expression status of thrombomodulin. CD14 expression on monocytes is important for regulation of the inflammatory response. We used an *ex vivo* stimulation study to evaluate the association of the levels of monocyte-expressed thrombomodulin and CD14 messenger RNA (mRNA) with the severity and prognosis of disseminated intravascular coagulation.

Methods: A total of 78 patients with suspected DIC were enrolled. Thrombomodulin and CD14 mRNA levels were measured in peripheral blood by real-time quantitative reverse-transcription polymerase chain reaction. Thrombomodulin and CD14 mRNA were also assessed in *ex vivo* cultures of peripheral whole blood that were stimulated by lipopolysaccharide or thrombin.

Results: The levels of monocyte-expressed thrombomodulin mRNA were significantly lower in the non-survivors than in the survivors. A low level of monocyte-expressed thrombomodulin mRNA was a significant prognostic marker, but CD14 did not possess prognostic power. Monocyte-expressed CD14 mRNA correlated significantly with the severity of DIC in survivors. In addition, stimulation of *ex vivo* cultures of whole blood demonstrated that thrombin upregulates both thrombomodulin and CD14 mRNA, and lipopolysaccharide downregulates thrombomodulin mRNA.

Conclusions: The downregulation of thrombomodulin on monocytes reflects the decompensated status of physiological defenses against hypercoagulopathy and represents the poor prognosis in DIC. The expression levels of thrombomodulin on monocytes may be a useful marker to screen for candidates eligible for recombinant thrombomodulin therapy in future.

Introduction

Thrombomodulin (TM) is a membrane glycoprotein that functions as an anticoagulant factor by activating protein C [1]. Within the vasculature, the endothelium and circulating monocytes express constant levels of TM, which maintains the normal rheological properties of blood. However, inflammatory stimuli such as endotoxin and inflammatory cytokines can suppress TM expression in the endothelium [2]. Continuous dysregulation of TM expression eventually contributes to procoagulant diathesis, which can facilitate the development of disseminated intravascular coagulation (DIC).

A monocyte is a unique blood cell that expresses TM and tissue factor [3] and thus plays an essential role in the coagulation process. For example, inflammatory cytokines upregulate the expression of monocyte tissue factor, which results in hypercoagulable diathesis [4]. With regard to monocyte surface expression of TM, we reported that TM expression on a small subset of inflammatory monocytes increases in patients with overt DIC [5]. However, until now no reports have been published on the global level of monocyte-expressed TM in patients with DIC *in vivo*.

Bacterial lipopolysaccharide (LPS) is bound to CD14 on the monocyte, and subsequently, CD14 interacts with Toll-like receptor (TLR)-4 to activate intracellular inflammatory signaling pathways [6]. The activation of these pathways triggers a cascade of various inflammatory cytokines from monocytes. Therefore, the expression level of CD14 on

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monocytes may be important to the regulation of the inflammatory response. Increased CD14 expression on monocytes has been associated with high rates of mortality in septic patients [7].

Although potential benefits of recombinant human TM for the treatment of DIC or sepsis have been suggested [8], the *in vivo* levels of TM expression in circulating monocytes has not been investigated yet in candidate patients with DIC or sepsis. Therefore, we measured messenger RNA (mRNA) expression of TM and CD14 on monocytes from suspected DIC patients to determine the association between expression of TM and CD14 and DIC severity and prognosis. Furthermore, to investigate if LPS and thrombin, which are abundant in the circulation of DIC patients, influence the mRNA expression of TM and CD14 in monocytes, we measured TM and CD14 mRNA levels in monocytes derived from peripheral whole blood that had been *ex vivo* stimulated with LPS or thrombin.

Materials and Methods

Study Population

A total of 78 patients clinically suspected of having DIC were recruited. This study was approved by the Institutional Review Board of Seoul National University Hospital and was conducted under the Declaration of Helsinki. Demographic and clinical data, including illness severity scores, were obtained from patient medical records (Table 1). Overt-DIC diagnosis was based on the International Society on Thrombosis and Haemostasis (ISTH) subcommittee scoring system [9,10]. Patients with cumulative scores less than 5 were arbitrarily defined as no overt-DIC, because they did not have overt-DIC.

Table 1
Characteristics and laboratory results of the study population.

	No overt-DIC	Overt-DIC
Number	44	34
Age, yr	57 ± 19	61 ± 17
Gender (%)		
Male	11 (25.0)	14 (41.2)
Female	33 (75.0)	20 (58.8)
Clinical diagnosis (%)		
Malignancies	19 (43.2)	14 (41.2)
Hepatic failure	10 (22.7)	7 (20.6)
Sepsis/severe infection	7 (15.9)	9 (26.5)
Others[a]	8 (18.2)	4 (11.8)
Twenty eight-day mortality (%)		
Survivors	35 (79.5)	22 (64.7)
Non-survivors	9 (20.5)	12 (35.3)
SOFA score (range)	3.0 (1.0–4.0)	4.0 (3.0–7.8) *
SAPS II (range)	28.0 (19.0–34.3)	38.0 (27.3–50.5) *
Platelets, $\times 10^9$/L (range)	143.0 (75.5–280.5)	59.0 (43.5–91.0)*
PT, sec (range)	12.0 (11.2–13.3)	19.0 (18.1–23.3)*
D-dimer, μg/mL (range)	1.0 (0.6–3.0)	5.0 (3.3–12.5)*
Fibrinogen, mg/dL (range)	335.0 (269.5–408.5)	262.0 (160.8–333.8)
Antithrombin, % (range)	79.0 (59.0–101.0)	59.0 (35.3–73.8)*
Protein C, % (range)	69.0 (49.8–101.3)	51.0 (23.3–67.0)*
IL-6, pg/mL (range)	5.5 (0.1–32.0)	24.2 (2.2–59.0)
Absolute monocyte count, $\times 10^6$/L (range)	609.1 (392.9–939.1)	347.4 (292.1–533.0)
Monocyte thrombomodulin[b] (range)	38.1 (23.9–81.0)	30.7 (8.8–55.7)
Monocyte CD14[b] (range)	16.6 (7.4–46.9)	34.7 (12.5–69.7)

Values were presented as number of patients (percentage) or mean ± standard deviation or median (interquartile range).
DIC, disseminated intravascular coagulation; SOFA, sequential organ failure assessment; SAPS II, simplified acute physiology score II; PT, prothrombin time.
[a] Others refers to post-surgery (n = 6), renal failure (n = 2), fulminant autoimmune disease (n = 1), mitral regurgitation (n = 1), multiple sclerosis (n = 1), and severe pre-eclampsia (n = 1).
[b] Monocyte thrombomodulin and CD14 mRNA levels were scaled in a relative ratio by comparison with mRNA GAPDH.
* $p < 0.05$ versus no overt-DIC.

Blood Sampling

Peripheral blood was collected in sodium citrate tubes (Becton Dickinson, San Jose, CA, USA). The samples of whole blood were centrifuged for 15 min at 1550 *g* within 2 h of sample collection. The buffy coat layer, which contains monocytes, was aliquoted and stored at –70 °C.

Coagulation and Cytokine Assays

A standard clotting assay was used to evaluate prothrombin time (PT) and fibrinogen on an ACL 3000 (Beckman Coulter, Fullerton, CA, USA)). The ACL 3000 also was used to measure D-dimer by an immunoturbidimetric assay and protein C and antithrombin by a chromogenic assay. IL-6 was quantified using an IL-6 ELISA kit (Pierce Biotechnology, Rockford, IL, USA).

Quantitative RT-PCR Analysis of TM and CD 14 Expression on Monocytes

Total RNA was extracted from the buffy coat layer of peripheral blood by using TRIzol (Life Technologies, Gaithersberg, MD, USA) reagent according to the manufacturer's instructions. Reverse transcription was performed with a reverse transcription kit (Invitrogen, Grand Island, NY, USA)) using a total of 1 μg of RNA in a final volume of 20 μl. Real-time polymerase chain reaction (PCR) amplification was conducted with the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Lincoln, CA, USA). Gene expression was quantified by using the TaqMan Universal PCR Master Mix, TM-specific primer, CD14-specific primer, and FAM-labeled probe sets (Applied Biosystems). The expression levels of the TM and CD14 genes were normalized by comparison against internal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer/probe pair levels (VIC MGB probe, primer limited). Expression levels were presented as relative values compared to the level of GAPDH mRNA. Reference values of monocyte TM and CD14 mRNA levels obtained from 16 healthy individuals were 314.0 ± 172.5 (mean ± standard deviation) and 94.7 ± 53.5, respectively.

Ex Vivo Experiments on Monocyte-Expressed TM and CD 14

Peripheral whole blood was collected in sodium citrate tubes from 3 healthy volunteers (1 man and 2 women; mean age, 35 y) who provided informed consent. The samples of whole blood were incubated with either vehicle (phosphate buffered saline), 100 ng/ml LPS (Sigma Aldrich), or 0.3 U/ml thrombin (Sigma Aldrich). After 2 h and 4 h of incubation, TM and CD14 mRNA were measured by quantitative RT-PCR analysis (see above).

Statistical Analysis

All statistical analyses were performed using SPSS 12.0 K for Windows (SPSS; Chicago, IL, USA). Continuous data comparisons were performed using the Mann-Whitney U and Rank-Sum tests, and the correlations were analyzed using the Spearman's correlation coefficient. The chi-square test was used to compare categorical variables. Kaplan-Meier analysis by the log-rank method was used to analyze survival. Parameters were identified by multivariate Cox regression analyses adjusted for age and gender. The optimal cutoff and diagnostic values for each parameter were set based on receiver operating characteristic (ROC) curve analysis using MedCalc (MedCalc Software; Mariakerke, Belgium). A probability level of less than 0.05 ($p < 0.05$) was considered statistically significant.