Thrombosis Research 134 (2014) 1130–1134

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

# Differential expression of leukocyte receptors in disseminated intravascular coagulation: Prognostic value of low protein C receptor expression

Ji Yeon Sung [a,1], Ji-Eun Kim [a,b,1], Ki Seok Kim [a], Kyou-Sup Han [a], Hyun Kyung Kim [a,b,*]

[a] Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul, Korea
[b] Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea

ARTICLE INFO

*Article history:*
Received 30 May 2014
Received in revised form 9 August 2014
Accepted 26 August 2014
Available online 6 September 2014

*Keywords:*
Protein C receptor
Toll-like receptor-4
L-selectin
Disseminated intravascular coagulation

ABSTRACT

*Background:* The protein C receptor (PROCR), toll-like receptor (TLR) and L-selectin leukocyte receptor play roles in systemic inflammatory response including disseminated intravascular coagulation (DIC). Expression of these receptors, which mediate systemic immune or coagulation responses, is tightly regulated by physiologic or pathologic signals. We investigated whether the expressions of 3 leukocyte receptors (PROCR, TLR4, L-selectin) was related to clinical outcomes in patients suggestive of having DIC.
*Methods:* RNA was extracted from the peripheral blood buffy coats of patients suggestive of having DIC. After reverse transcription, mRNA expression levels of PROCR, TLR4, and L-selectin were measured using Taqman Gene Expression Assays. The 28-day hospital mortality rate was used as a clinical outcome.
*Results:* The expression level of PROCR mRNA in leukocytes was lower in those with overt-DIC as compared to those without overt-DIC, however, this difference was not statistically significant. As for TLR4 and L-selectin mRNA expression, there were no significant differences observed between those with and without overt-DIC. A Kaplan-Meier survival analysis revealed that patients with low PROCR mRNA expression levels showed significantly lower survival rates than those with high expression levels. On multivariate cox regression analysis, low levels of PROCR mRNA expression were an independent prognostic marker. However, expression levels of TLR4 and L-selectin mRNA were not associated with any prognostic value.
*Conclusion:* Considering that the PROCR is an important anticoagulant receptor, low PROCR mRNA expression levels associated with a poor prognosis in patients with DIC represents an exhaustion of the natural anticoagulant system, and reflects the final decompensate stage of DIC. The leukocyte PROCR may contribute to a dampening of florid activation of coagulation reactions *in vivo*.



## Introduction

The cellular responses that occur during inflammatory processes are tightly regulated [1]. Inflammatory signal such as lipopolysaccharides (LPS) are mediated by toll-like receptors (TLRs) on the surfaces of neutrophils and monocytes. Although 1 study reported that the expression levels of TLRs decreased in patients with sepsis [2], another study demonstrated enhanced expression of TLRs [3]. Therefore, the relationship between TLR expression levels and sepsis is unclear at present.

L-selectin, a member of the leukocyte adhesion molecule family, is expressed on lymphocytes, monocytes, and neutrophils in peripheral blood. Because adhesion molecules are essential to leukocyte-endothelial cell interactions during inflammatory process [4], pro-inflammatory stimuli can promote the synthesis of adhesion molecules and result in neutrophil adhesion to endothelial cells [5]. Furthermore, it has been reported that L-selectin expression on neutrophils decreased in those with systemic inflammatory response syndrome [6,7].

The endothelial protein C receptor (PROCR) augments the activation of protein C, which plays a role in coagulation inhibition through inactivation of activated coagulation factors V and VIII [8]. PROCR is expressed not only in endothelial cells, but also in peripheral monocytes and neutrophils [9,10]. The expression level of PROCR in monocytes may be altered depending on inflammatory status [11].

Inflammation and coagulation are closely linked and interdependent processes [12,13]. Hence, inflammatory stimuli result in coagulation activation, culminating in disseminated intravascular coagulation (DIC). During inflammatory processes, up- or down-regulation of leukocyte receptors occurs, and expression is tightly regulated by physiologic or pathologic signals which control systemic immune or coagulation responses [1].

* Corresponding author at: Department of Laboratory Medicine, Seoul National University College of Medicine, 101, Daehak-ro Jongno-gu, Seoul 110–744, Republic of Korea. Tel.: +82 2 2072 0853; fax: +82 2 747 0359.
*E-mail address:* lukekhk@snu.ac.kr (H.K. Kim).
[1] Authors contributed equally.

http://dx.doi.org/10.1016/j.thromres.2014.08.026
0049-3848/

Recently, we demonstrated down-regulation of the monocyte anticoagulant thrombomodulin in DIC and an association between this down-regulation and poor prognosis [14]. These findings led us to investigate the possible association of changes in PROCR expression levels and poor prognosis in patients with DIC. Moreover, expression of the TLR4 and L-selectin leukocyte receptors can be postulated to show tightly regulated pattern in DIC. Therefore, we measured the mRNA expression levels of 3 leukocyte receptors, PROCR, TLR4 and L-selectin, in peripheral blood using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays in patients suspected of having DIC to explore possible prognostic factors for DIC.

## Materials and methods

### *Study Population*

We studied a total of 114 patients who were considered to be clinically suggestive of having DIC and underwent DIC screening tests. Medical records were reviewed and demographic data, clinical information, and laboratory results were obtained. Based on the International Society on Thrombosis and Haemostasis (ISTH) subcommittee scoring system [15,16], patients with cumulative scores <5 and >5 were defined as having "no overt-DIC" and "overt-DIC", respectively. Patients with survival days exceeding 28 days (survivors) were compared with those with 28 days or less (non-survivors).

### *Blood samples*

Peripheral blood was collected in sodium citrate tubes (Becton Dickinson, San Jose, CA, USA) and centrifuged for 15 min at 1550 x g. The plasma was used directly for coagulation assays and the buffy coat layer was stored at −70 °C.

### *Coagulation assays*

Prothrombin time (PT) and fibrinogen were measured using standard clotting assays. An immunoturbidimetric assay was used to measure D-dimers, and chromogenic assays using an ACL 3000 coagulation analyzer (Beckman Coulter, Fullerton, CA, USA) were employed to measure protein C and antithrombin.

### *Quantitative RT-PCR of PROCR, TLR4 and L-selectin*

RNA from the buffy coat layer of the peripheral blood was extracted using TRIzol (Life Technologies, Gaithersberg, MD, USA) reagent according to the manufacturer's instructions. Subsequently, RNA was reverse transcribed using a reverse transcription kit (Invitrogen, Grand Island, NY, USA) with 1 μg of RNA in a final volume of 20 μl. RT-PCR was performed with the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Lincoln, CA, USA). Gene expression was quantified using the TaqMan Universal PCR Master Mix, a PROCR-specific primer (Hs00941182_m1), a TLR4-specific primer (Hs00152939_m1), a SELL-specific primer (Hs00174151_m1, for L-selectin) and FAM-labeled probe sets (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; VIC MGB probe, primer limited) was used as an internal control gene in quantifying the mRNA expression levels of the PROCR, TLR4, and L-selectin genes. The mRNA levels were scaled in relative ratio by comparison with GAPDH mRNA.

### *Ex vivo effect of LPS on PROCR mRNA expression*

Peripheral whole blood was collected in EDTA tubes from 3 healthy volunteers (2 man and 1 woman) under informed consent. Whole blood was incubated with vehicle (phosphate buffered saline), 2 ng/ml LPS (Sigma Aldrich) or 1 U/ml thrombin (Sigma Aldrich). After 4 h of incubation at 37 °C, PROCR mRNA was measured by RT-PCR analysis (see above).

### *Statistical analysis*

SPSS 18.0 K for Windows (SPSS; Chicago, IL, USA) was used in all statistical analyses. Continuous data comparisons were performed using the Student t-test. The chi-square test was used to compare categorical variables. Kaplan-Meier analysis by the log-rank method was used to analyze survival. Prognostic parameters were identified by both univariate and multivariable Cox regression analyses. 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 $< 0.05$ was considered statistically significant.

## Results

### *Demographic and laboratory characteristics of patients in relation to DIC diagnosis and mortality*

Overt-DIC was diagnosed in 26 of the 114 patients according to DIC criteria (Table 1). Age and gender were similar between patients with and without overt-DIC. In overt-DIC patients, prolonged PT, and lower levels of platelets, fibrinogen, antithrombin, and protein C were found in comparison to those without overt-DIC. No significant differences in absolute neutrophil or monocyte counts were observed between the 2 groups.

When compared between patients with and without 28-day hospital mortality, platelets, antithrombin, and protein C levels were significantly lower in non-survivors (Table 1).

### *Gene expressions of leukocyte receptors in relation to DIC diagnosis and mortality*

The level of PROCR mRNA was more decreased in those with overt-DIC as compared to those without ($6.62 \pm 44.78$ vs. $1.37 \pm 3.68$), although this difference did not reach the level of statistical significance. In contrast, levels of TLR4 and L-selectin mRNAs did not differ between the 2 groups (Table 1). Similarly, the PROCR mRNA level was also lower in non-survivors than in survivors ($6.88 \pm 44.79$ vs. $0.46 \pm 0.76$), although this difference did not reach the level of statistical significance.

Using dichotomy values that promoted ROC curves with optimal prognostic power, patients with low levels of PROCR mRNA ($\leq 0.55$) exhibited poor survival compared to patients with higher levels of PROCR mRNA (Fig. 1). However, no significant differences in survival rates were evident for TLR2 and L-selectin mRNAs.

Univariate Cox-regression analysis revealed not only antithrombin and protein C, but also PROCR mRNA expression level (hazard ratio = 3.01, $P = 0.043$) as significant prognostic factors (Table 2). Mortality risk was still increased in patients with low levels of PROCR mRNA as compared to patients with high levels in the multivariable Cox-regression analysis, indicating that the low PROCR mRNA expression was an independent prognostic marker. The hazard ratio of PROCR in the multivariable Cox-regression analysis was comparable to that of antithrombin.

The level of PROCR mRNA was not significantly different according to the diagnosis. When we compared the level of PROCR mRNA in overt DIC patients with malignancy ($n = 4$) and infection ($n = 8$), PROCR mRNA level tended to be decreased in patients with infection ($0.30 \pm 0.44$) compared with patients with malignancy ($2.02 \pm 3.65$), although there was no statistical significance ($p = 0.570$).