이학박사 학위논문

Diagnostic and prognostic usefulness of neutrophil extracellular traps in disseminated intravascular coagulation: Histone-induced pro-coagulative phenotype of endothelial cells

파종성혈관내응고에서 호중구세포외기질의 진단 및 예후 지표 유용성: 히스톤에 의한 내피세포 응고표현형 항진

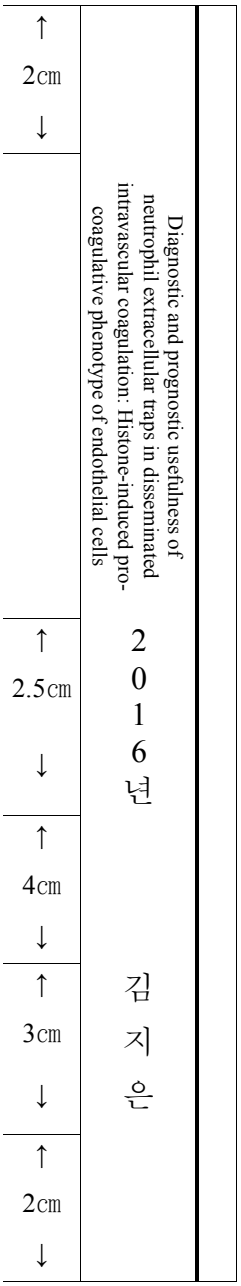
2016년 2 월

서울대학교 대학원

협동과정 종양생물학 전공

김 지 은

서식 1(외표지) : 책등(측면)


↑
2㎝
↓
Diagnostic and prognostic usefulness of neutrophil extracellular traps in disseminated intravascular coagulation: Histone-induced pro-coagulative phenotype of endothelial cells
2016년
김지인
↑
2.5㎝
↓
↑
4㎝
↓
↑
3㎝
↓
↑
2㎝
↓

이학박사 학위논문

Diagnostic and prognostic usefulness of neutrophil extracellular traps in disseminated intravascular coagulation: Histone-induced pro-coagulative phenotype of endothelial cells

파종성혈관내응고에서 호중구세포외기질의 진단 및 예후 지표 유용성: 히스톤에 의한 내피세포 응고표현형 항진

2016년 2 월

서울대학교 대학원

협동과정 종양생물학 전공

김 지 은

파종성혈관내응고에서 호중구세포외기질의 진단 및 예후 지표 유용성: 히스톤에 의한 내피세포 응고표현형 항진

지도교수 김 현 경

이 논문을 이학박사 학위논문으로 제출함.
2015년 10월

서울대학교 대학원
협동과정 종양생물학 전공
김 지 은

김지은의 이학박사 학위논문을 인준함.
2016년 1월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

Diagnostic and prognostic usefulness of neutrophil extracellular traps in disseminated intravascular coagulation: Histone-induced pro-coagulative phenotype of endothelial cells

by

Ji-Eun Kim

A thesis submitted to the Interdisciplinary Graduate Program in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Tumor Biology at Seoul National University College of Medicine

January 2016

Approved by Thesis Committee:

Professor _____ Chairperson

Professor _____ Vice Chairperson

Professor _____

Professor _____

Professor _____

ABSTRACT

Ji-Eun Kim

Interdisciplinary Program in Tumor Biology

The Graduate School

Seoul National University

Introduction: Neutrophils can be induced to release DNA combined with histones. The resulting neutrophil extracellular trap (NET) provides a scaffold for growing hemostatic plug. NET might be actively formed in clinical conditions that are characterized by formation of vascular thrombi. Endothelium usually provides anticoagulant surface, but the thrombotic event may change the anticoagulant endothelium to procoagulant phenotype. Resting endothelial cells express anticoagulant thrombomodulin (TM), but not procoagulant tissue factor (TF). Stress condition, however, can induce TF. This study hypothesized that active NET formation may occur in overt-disseminated intravascular coagulation (DIC) and that the circulating levels of NET may have clinical diagnostic and prognostic implication. In addition, the elevated histones may alter endothelial TF and TM expression levels.

Methods: The plasma levels of DNA-histone complexes and double-stranded DNA (dsDNA), considered to be in vivo markers of NET, were measured in 199 patients suspected of having DIC and 20 healthy controls. Surface antigens, soluble forms, and mRNA levels of TF and TM were measured by flow cytometry, ELISA, and real-time RT-PCR, respectively. TF and TM activity were measured using procoagulant activity, thrombin generation, or

chromogenic assays. Involvement of NF-κB pathway and toll-like receptors (TLR) were assessed.

Result: The circulating levels of DNA-histone complexes and dsDNA were significantly elevated in overt-DIC. The increased levels of these two markers correlated with the severity of coagulopathy including DIC score and D-dimer. Multivariable Cox regression analysis, adjusted for the conventional DIC markers, revealed that elevated DNA-histone complexes and dsDNA are poor independent prognostic markers. Histones dose-dependently induced surface antigens, activity and mRNA levels of endothelial TF. Histone-treated endothelial cells significantly shortened the lag time and enhanced the endogenous thrombin potential of normal plasma, which was normalized by a TF neutralizing antibody. Histones induced phosphatidylserine and protein-disulfide isomerase expression in endothelial cells. Histones also reduced the surface antigen, activity, and mRNA levels of endothelial TM. TLR9 inhibitor significantly blocked the TF up-regulation and TM down-regulation, whereas TLR2, and TLR4 inhibitors partly blocked the TF up-regulation. Polysialic acid and heparin reversed the histone-induced TF up-regulation and TM down-regulation. Activated protein C did not affect the TF up-regulation, but interrupted TM down-regulation.

Conclusion: The circulating levels of NET release reflect the coagulation activation and adverse clinical outcomes in patients with DIC, thereby providing potential clinical relevance for mortality prediction in DIC. Histones induced the endothelial procoagulant phenotype through TF up-regulation and TM down-regulation. The effects of histones were partly mediated by TLR2, TLR4, and TLR9. Strategies to inhibit the harmful effects of histones in endothelial cells may be required in order to prevent a

thrombotic environment.

Keywords: Neutrophil extracellular trap (NET), DIC, Cancer, DNA-histone complex, dsDNA, Histones, Tissue factor (TF), Thrombomodulin (TM), Endothelial cells

Student Number: 2009-30591

CONTENTS

LIST OF TABLES AND FIGURES

ABBREVIATIONS

Abbreviations	Full name
NET	Neutrophil extracellular trap
DIC	Disseminated intravascular coagulation
dsDNA	double-stranded DNA
PT	prothrombin time
aPTT	activated partial thromboplastin time
ROC	Receiver operating characteristic
TF	Tissue factor
TM	Thrombomodulin
TLR	Toll-like receptor
PDI	Protein-disulfide isomerase
EGF domain	Epidermal growth factor
PSA	Polysialic acid
APC	Activated protein C
MMP	Metalloproteinase
ETP	Endogenous thrombin potential

INTRODUCTION

In recent studies, neutrophils have been reported to play an essential role in thrombosis [1]. When pathogens such as bacteria invade the circulation, neutrophils defend against the pathogens, through the release of neutrophil extracellular trap (NET). NET was firstly designated by Arturo Zychlinsky in 2004. It is called NET that activated neutrophil generate extracellular fibers, which are structured as net composed with nuclear constituents, such as double-strand DNA (dsDNA), histones as the most abundant proteins, and other granular proteins. NET is triggered not only by infectious pathogens, but also by reactive oxygen species, antibodies, and activated platelets [2, 3]. As a result, an increase of NET formation has been reported in various clinical conditions including sepsis, trauma, autoimmune diseases, deep vein thrombosis, atherosclerosis, and thrombotic microangiopathy [3-10]. Meshwork of DNA strands around histones from the main skeleton of NET. NET not only binds to the pathogens to kill, it also entraps the platelets and red blood cells that are the main components of vascular thrombi. [11, 12].

Disseminated intravascular coagulation (DIC) is characterized by systemic activation of coagulation and anticoagulation pathways leading to florid fibrin deposition and formation of microthrombi. DIC induces the consumption of platelets and coagulation factors which caused the bleeding. It is resulting the increase of multi-organ failure and mortality [9, 13, 14]. The underlying conditions of DIC such as sepsis and trauma are known to promote NET formation [8, 9, 11, 15].

Histones exist predominantly in a form of nucleosome associated with DNA within the cell nucleus. The increased levels of circulating histones are induced by NET formation. NET is actively formed in patients with inflammatory, autoimmune, and thrombotic diseases [4-7, 16, 17]. Therefore, the contribution of histones to the process of coagulation has received recent attention [1]. In the previous reports, histones induce platelet activation [17-20], induce endothelial cell damage, and enhance plasma thrombin formation *via* binding to protein C and thrombomodulin (TM) [2, 15, 21, 22].

Endothelial cells form the lumen of all blood vessels. Its function is to regulate systemic blood flow and tissue perfusion rates, selective barrier controlling the paracellular exchange of fluid and molecules. Endothelial cells demonstrate constitutive anticoagulant properties that serve to suppress coagulation activation. These anticoagulant properties are mediated by TM. TM, also known as CD141, is a 74 kDa transmembrane protein. The mature TM is consisted of five distinct domains; an amino-terminal region homologous to that of C-type lectins, a 6-tandem repeated epidermal growth factor (EGF)-like domain, a serine/threonine-rich sequence, a transmembrane domain, and a cytoplasmic tail. The EGF-like domain 5-6 is critical site to bind thrombin [23]. When thrombin binds to TM on the endothelial surface, it activates protein C, which can extinguish coagulation amplification through inactivation of factors V and VIII [24, 25].

Tissue factor (TF), also known as CD142 and thromboplastin, is a 47 kDa transmembrane glycoprotein. TF is the initiator of coagulation by binding coagulation factor VII [26, 27]. TF is constitutively expressed by perivascular cells, such as pericytes and fibroblasts, whereas it is not expressed by resting endothelial cells. In certain pathologic environments, TF can be induced in

endothelial cells and contribute to local fibrin formation [28, 29]. TF is normally encrypted on the cell surface, but can be fully activated (decrypted) by certain stimuli [30, 31]. Phosphatidylserine, which normally exists on the inner layer of the plasma membrane, could enhance the procoagulant activity of TF. The increase of transferring phosphatidylserine to the outer layer provides the negatively charged phospholipid surface that assists TF to bind Factor VIIa [32-34]. In addition, the results from recent reports have suggested that protein-disulfide isomerase (PDI) involves the regulation of TF activity. PDI is mostly existed in endoplasmic reticulum and assist in protein folding. In the cell surface, PDI regulates the formation of a disulfide bond within the extracellular domain of TF that resulted the encrypted or decrypted forms of TF [35, 36].

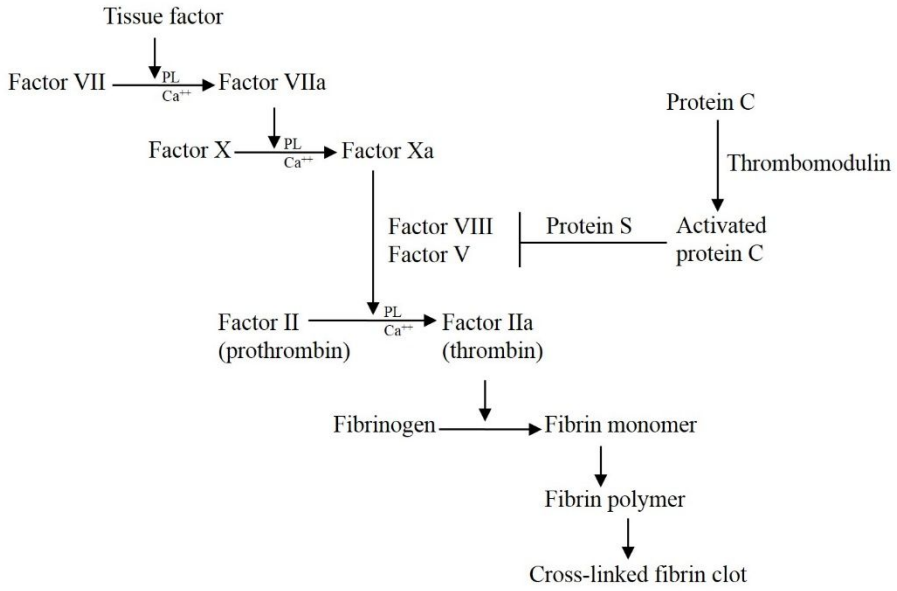


Figure 1. Extrinsic pathway of coagulation. Tissue factor initiate extrinsic pathway of coagulation to bind and activate factor VII. It results to promote thrombin generation and the formation of fibrin clot.

Firstly since NET contributes to thrombus formation, we presumed that DIC process is closely related with increased NET formation and the resulting NET may in turn promote hypercoagulability. The present study investigated circulating levels of NET in patients suspected of having DIC and analyzed their potential values to assess coagulation severity and to predict clinical outcome. Second, as high levels of circulating histones caused from increased NET formation may compromise the anticoagulant barrier of endothelial cells. This study investigated how histones affected procoagulant TF and anticoagulant TM expression in endothelial cells. Finally, the involvement of the NF-κB pathway and toll-like receptors (TLRs) in mediating the effects of histones was examined.

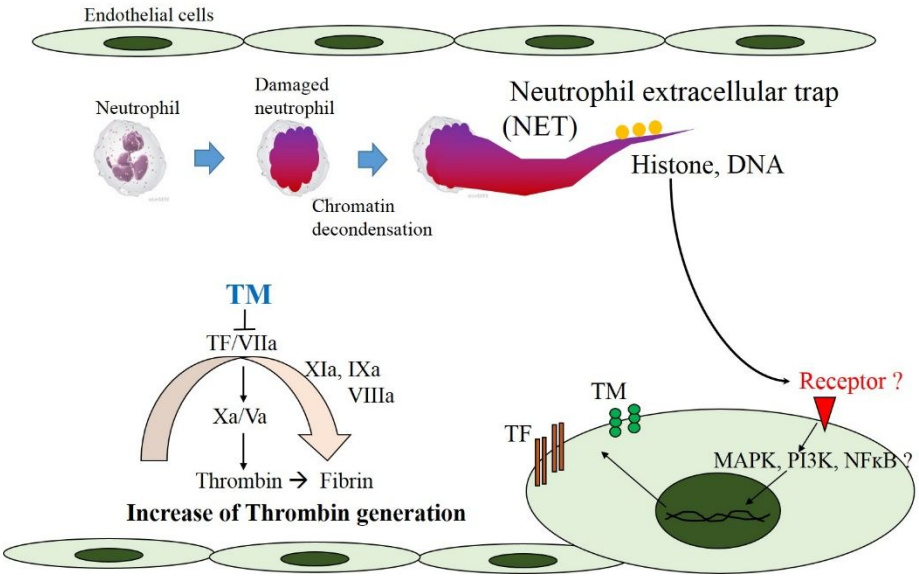


Figure 2. Hypothesis. This study hypothesizes that DIC increases the damaged neutrophil, and it promotes the formation of neutrophil extracellular trap (NET). The circulating histone from NET may compromise the anticoagulant barrier of endothelial cells. Histones may affect procoagulant TF and anticoagulant TM expression in endothelial cells. NF-κB pathway and toll-like receptors (TLRs) should be mediated the effects of histones.

MATERIALS AND METHODS

Study population

Study population was calculated using following formula:

$$n_c = \frac{2(Z_a+Z_b)^2\rho^2(\lambda+1)/\lambda}{(\mu_c-\mu_t)^2}$$

Z is a constant set by convention according to the accepted 5% α error and whether it is a two-sided effect. Therefore, Z_a is 1.96, and Z_b is 0.842 as a constant set by convention according to in 80% power of the study. In the above-mentioned formula ρ is the estimated standard deviation and μ is the mean of each group. In this study, ρ was 10, μ_c was 11, μ_t was 16. This study assorted the patients into two groups, thus λ was 2. Fallowing the formula, n_c, the required number of patients, was 188 [37].

A total of 199 patients who were clinically suspected of having DIC and who underwent DIC screening battery tests were enrolled in the study. Written consent from patient was exempted, since all data were acquired anonymously and retrospectively from laboratory information system without any additional blood sampling. Exclusion criteria were thrombotic or bleeding disorders, use of warfarin or heparin medications within 3 days of blood collection, or pediatric patients. In addition, 20 healthy adults were enrolled under written informed consent in order to determine the reference range of circulating NET levels. The Institutional Review Board of Seoul National University Hospital approved this study.

The patients' characteristics are described in Table 1. Patients were

diagnosed as having overt-DIC, which was defined as a cumulative score of > 5 by the International Society of Thrombosis and Haemostasis (ISTH) Sub-Committee scoring system [13]. It was arbitrarily classified the patients who did not meet the criteria of overt-DIC as no overt-DIC.

Blood samples and assays

Peripheral blood was collected in sodium citrate tubes (Becton Dickinson, San Jose, CA, USA). The whole blood samples were centrifuged for 15 minutes at 1550 x g within 2 h after sample withdrawal. The prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen were assayed on an ACL 3000 automated coagulation analyzer (Instrumentation Laboratory, Milan, Italy) using HemosIL RecombiPlasTin and SynthASil reagents (Instrumentation Laboratory). D-dimer was tested by an immunoturbidimetric assay (Instrumentation Laboratory). Antithrombin and protein C activity were measured by a chromogenic assay (HemosIL Antithrombin and Protein C; Instrumentation Laboratory).

The circulating levels of NET in plasma were quantified using two commercial kits; The DNA-histone complex was quantified using an ELISA kit (Cell Death Detection ELISA, Roche Diagnostics, IN, USA); the double-stranded DNA (dsDNA) was measured using Quant-iT PicoGreen dsDNA reagent (Molecular Probes, Eugene, OR, USA) and a microplate fluorometer (Fluoroskan Ascent, Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's guidelines.

Cell culture

Human endothelial cell line, EA.hy926 was purchased from ATCC. Cells were maintained in DMEM medium (WelGENE, Seoul, South Korea) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Clonetics™; Basel, Switzerland) and maintained in EGM-2 BulletKit™ medium (Lonza). The cells were starved without serum for 1 h, then stimulated with calf thymus histone (Roche Diagnostics, IN). After 4 h, the cells and supernatant were harvested.

Flow cytometric analysis

The cells were starved without serum for 1 h, then stimulated with histone. After 4 h, the cells and supernatant were harvested. The cells were stained with rabbit anti-CD142 (TF) polyclonal antibody conjugated with alexa fluor 647 (Bioss Inc., Woburn, MA, USA). Phosphatidylserine was detected with PE conjugated annexin V (BD Biosciences, Franklin Lakes, NJ, USA). Rabbit IgG conjugated with alexa fluor 647 (Bioss Inc.) was used as isotype control. DyLight™ 488 conjugated mouse anti-PDI antibody (clone 1D3; Enzo Life Sciences, Farmingdale, NY, USA), PE conjugated mouse anti-CD141 (TM) antibody (BD Biosciences) and PE conjugated mouse anti-CD201 (EPCR) antibody (BD Biosciences) was used. Mouse anti-TM (clone PBS-01; Abcam, Cambridge, UK) against EGF domain 5 of TM was stained, then FITC conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used for second antibody.

Human histone H3 and H4 (Millipore, Temecula, CA, USA) was

used to confirm that the similar reactions were induced on the cells. To neutralize histone, Polysialic acid (PSA; Sigma-Aldrich, St Louis, MO, USA) and heparin (Greencross, South Korea) were mixed with histones and incubated before the addition to cells. To inhibit the TLR pathway, anti-human TLR2 antibody (eBioscience, San Diego, CA, USA), anti-human TLR4 antibody (eBioscience), and TLR9 antagonist (ODN TTAGGG; InvivoGen, San Diego, CA, USA) were preincubated for 1 h, then cells were stimulated with histones. Mouse IgG2a, K antibody (eBioscience) was used as isotype control. GM6001 (Enzo Life Sciences) was used to suppress the MMP activity.

Detection of soluble proteins by ELISA

The soluble TF was measured by human Coagulation Factor III/Tissue Factor DuoSet (R&D Systems, Minneapolis, MN, USA). The assay was performed according to the instructions provided by the manufacturer. The soluble TM was measure by Thrombomodulin Human ELISA Kit (Abcam) fallowing the protocol from the manufacturer.

Detection of mRNA expression by quantitative real-time PCR

Total RNA was extracted from EA.hy926 using TRIzol (Life Technologies, Gaithersburg, MD, USA) reagent according to instructions of the manufacturer. Synthesis of cDNA was performed with 1 μg total RNA by using GoScript reverse transcription system (Promega, Fitchburg, WI, USA). Real-time PCR amplification was carried out using the ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA, USA),

TaqMan Universal PCR Master Mix, and TF, PDI, TFPI and thrombomodulin-specific primer and 6-carboxyfluorescene (FAM)-labeled probe sets (Applied Biosystems) for quantitative gene expression. Expression levels of the genes were normalized to internal glyceraldehyde 3-phosphate dehydrogenase primer/probe pair levels (VIC MGB probe, primer limited, Applied Biosystems). These expression levels were presented as relative expression.

Measurement of Procoagulant activity

After stimulation with histones for 4 h, cells were harvested and washed with phosphate buffered saline (PBS) twice. Cells were resuspended to 5 x 10^6 / mL in PBS and 20 μL of cell suspension was added in a cuvette. 80 μL of Normal plasma (Pool Norm; Diagnostica Stago, France) was mixed with cells suspension and the formation of clot was induced with addition of 100 μL of pre-warmed 0.025 M $CaCl_2$. The clotting time was measured by ST Art (Diagnostica Stago). The standard curve of TF activity was produced using HemosIL RecombiPlasTin 2G (Instrumentation Laboratory).

Thrombin generation assay

Thrombin generation was measured in a Fluoroskan Ascent Fluorometer (Thermo Lab systems, Helsinki, Finland) as described by Hemker et al. (17). Briefly, 80 μL plasma was mixed with 20 μL of 1 x 10^4 cells stimulated with histones for 4 h. Then, 20 μL of a fluorogenic substrate mixed with $CaCl_2$ was added. The thrombin generation amount was measured

by using Thrombinoscope software (Diagnostica Stago). Lag time is the starting point of thrombin generation, and endogenous thrombin potential (ETP) is the area under the thrombin generation curve.

Murine mab against human TF (clone VD8; American Diagnostica Inc.) and recombinant annexin V (BD Biosciences) was used to block the surface TF and PS on the cells. To inhibit surface PDI, anti-PDI antibody (clone RL90; Abcam), glutathione (Sigma-Aldrich), quercetin (Sigma-Aldrich) were used.

TM activity assay

Twenty µl of 1 x 10^4 cells was mixed with 80 µL reaction buffer (Tris 0.05M, NaCl 0.25 M, $CaCl_2$ 4 mM, pH 8.0) added 4 µg/mL human protein C (Haematologic technologies Inc., River Road, VT) and 0.03 U/mL human thrombin (Sigma-Aldrich). Then 20 µL of the substrate, S-2366 (Chromogenix, Milano, Italy) was supplied as final concentration 200 µM. The absorbance from 405 nm was measured at 37°C by a Multiscan GO (Thermo Fisher Scientific Inc.) for 90 minutes. The standard curve was generated with rabbit TM (Haematologic technologies Inc.).

Measurement of NF-κB activation

The histone-treated EA.hy926 cells were fixed in 4% paraformaldehyde in PBS. The cells were stained with mouse anti-human NF-κB p65 antibody (Santa Cruz Biotechnology, Inc.). The second antibody used was FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.).

The nuclei were then stained with DAPI (ImmunoBioscience Corp., Mukilteo, WA, USA). All images were visualized by confocal microscopy (FV1000: OLYMPUS, Tokyo, Japan)

Nuclear contents from the histone-treated EA.hy926 cells were extracted before the NF-κB p65 was measured using a TransAM NFκB Family Transcription Factor Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using SPSS 21.0 for Windows (IBM Corporation, Armonk, NY, USA). Data comparisons were carried out using the student's *t*-test or chi-square test. Receiver operating characteristic (ROC) curve analysis was carried out for mortality prediction of each parameter. ROC curve analysis using MedCalc (Mariakerke, Belgium) was used to determine the optimal cutoff value. Kaplan–Meier survival analysis was used to analyze the cumulative survival curves. Univariable and multivariable Cox regression analyses were carried out to identify significant prognostic markers. Two-sided P-values of <0.05 were considered to be statistically significant.

RESULTS

Circulating levels of DNA-histone complex and dsDNA in relation to coagulation severity

According to the DIC scoring system criteria, 53 out of the total 199 patients were found to be with overt-DIC (Table 1). There were no differences seen with respect to age and sex, between overt-DIC group and no overt-DIC group. The well-known DIC parameters including platelets, PT, aPTT, D-dimer, and fibrinogen showed significant changes in overt-DIC. The decrease in protein C and antithrombin levels was found to be more in overt-DIC than in no overt-DIC. Overt-DIC patients showed significantly high levels of DNA-histone complexes and dsDNA as compared to the levels in no overt-DIC. The reference range of DNA-histone complex and dsDNA established by testing 20 normal healthy controls (mean age 46; 12 males and 8 females) were 0-56 AU and 1.71-1.99 ng/mL, respectively. Even no overt-DIC patients were found to have markedly higher levels of DNA-histone complexes (146±246 AU) and dsDNA (2.99±2.51 ng/mL) than those in healthy controls. Statistically, the DNA-histone complex levels strongly correlated with dsDNA levels ($r = 0.544$, $P < 0.001$).

	According to DIC diagnosis [a]		According to prognosis [b]	
	No overt-DIC (n=146)	Overt-DIC (n=53)	Survivors (n=166)	Non survivors (n=33)
Age, yrs,	57.1 ± 18.4	53.0 ± 17.8	55.2 ± 18.4	59.8 ± 17.6
Male/Female (%)	88 (60.3)/ 58 (39.7)	34 (64.2)/19 (35.8)	104 (62.7)/62 (37.3)	18 (54.5)/15 (45.5)
Clinical diagnosis, n (%)				
Sepsis/severe infection	23 (15.8)	8 (15.1)	23 (13.9)	8 (24.2)
Solid malignancies	63 (43.2)	20 (37.7)	72 (43.4)	11 (33.3)
Hematologic malignancies	25 (17.1)	13 (24.5)	25 (15.1)	13 (39.4)
Organ destruction	6 (4.1)	2 (3.8)	8 (4.8)	0 (0.0)
Trauma	4 (2.7)	0 (0.0)	4 (2.4)	0 (0.0)
Severe hepatic failure	4 (2.7)	8 (15.1)	11 (6.6)	1 (3.0)
Severe toxic or immunologic reaction	9 (6.2)	0 (0.0)	9 (5.4)	0 (0.0)
Vascular abnormalities	6 (4.1)	2 (3.8)	8 (4.8)	0 (0.0)
Obstetrical calamities	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Other†	6 (4.1)	0 (0.0)	6 (3.6)	0 (0.0)
Neutrophil ($x10^9$/L)	7.20 ± 8.76	6.22 ± 6.42	6.95 ± 8.49	6.90 ± 6.57
Platelets ($x10^9$/L)	160 ± 116**	60.3 ± 67.9	142 ± 115*	88.4 ± 97.0
PT (sec)	14.1 ± 9.37*	18.4 ± 5.08	15.1 ± 9.18	16.0 ± 5.25
aPTT (sec)	33.8 ± 8.72**	39.1 ± 10.9	34.7 ± 9.40	37.8 ± 10.4
D-dimer (μg/mL)	5.11 ± 11.2*	10.7 ± 16.4	6.16 ± 13.0	8.87 ± 13.1
Fibrinogen (mg/dL)	360 ± 119**	269 ± 164	343 ± 135	302 ± 152
Protein C (%)	74.9 ± 30.7**	43.0 ± 28.9	69.2 ± 33.2*	52.3 ± 30.5
Antithrombin (%)	75.7 ± 21.9**	52.6 ± 23.5	72.1 ± 24.2*	56.7 ± 22.2
DNA-histone complex (AU)	146 ± 246*	232 ± 286	131 ± 198**	356 ± 412
dsDNA (ng/ml)	2.99 ± 2.51**	4.62 ± 4.13	2.94 ± 2.29**	5.89 ± 4.98

Table 1. The characteristics of the study population

Values are presented as the mean ± standard deviation. †Other clinical diagnoses included organ destruction (n = 1), diabetes mellitus (n = 3), and chronic kidney disease (n = 2). a Total population was classified into two groups in terms of overt-DIC criteria. b Total population was divided into survivor and non-survivor according to 150-day mortality. Data comparisons were carried out using the student's *t*-test. * $P < 0.05$, ** $P < 0.001$.

Abbreviations: DIC, disseminated intravascular coagulation; PT, prothrombin time; aPTT, activated partial thromboplastin time, dsDNA; double stranded DNA

The circulating levels of DNA-histone complexes and dsDNA were found to increase gradually in relation to the DIC score (Figure 3). D-dimer is another maker which has related with the severity of DIC. When divided into 3 tertile groups according to the D-dimer levels, the mean values of DNA-histone complex and dsDNA correlated with D-dimer levels (Figure 4).

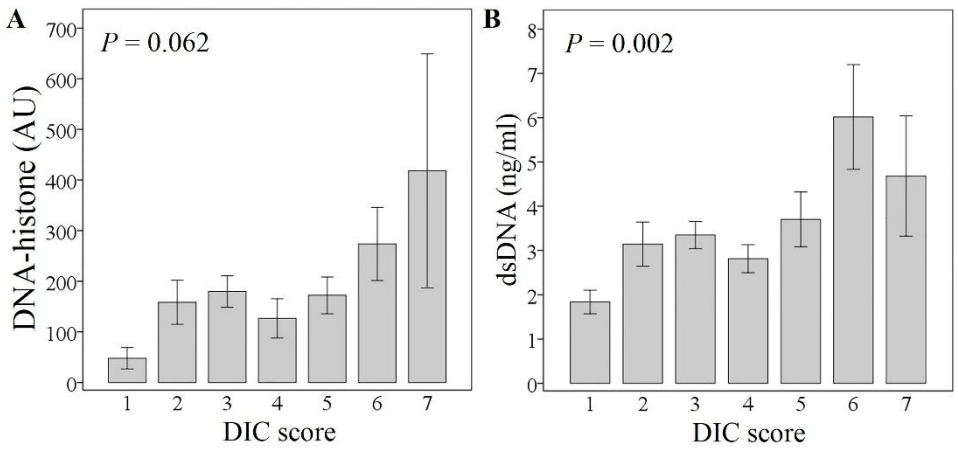


Figure. 3. Trend of circulating levels of DNA-histone complex (A) and dsDNA (B) based on disseminated intravascular coagulation (DIC) score in all patients. Data comparisons were carried out using the one-way ANOVA.

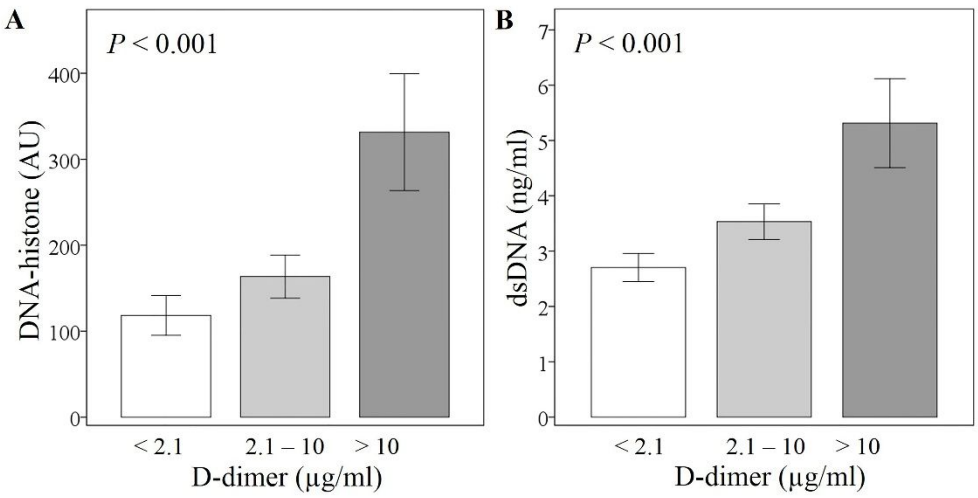


Figure 4. Association of circulating levels of DNA-histone complex (A) and dsDNA (B) with the D-dimer levels in all patients. Data comparisons were carried out using the one-way ANOVA.

Prognostic values of circulating DNA-histone complex and dsDNA levels

Non-survivors were arbitrarily defined as patients who were dead within 28-hospital days. Out of the total 199 patients, 33 were categorized as non-survivors (Table 1). There were no significant differences in age and sex between survivors and non-survivors. Platelet counts were decreased in non-survivors whereas PT, D-dimer, and fibrinogen levels were not significantly different between survivors and non-survivors. Plasma levels of protein C and antithrombin were significantly decreased in non-survivors as compared to that in survivors. The increase in the circulating levels of DNA-histone complexes and dsDNA was significantly higher in non-survivors than in survivors.

The prognostic values of DNA-histone complexes and dsDNA were evaluated using ROC curves. The AUC of DNA-histone complexes and dsDNA were slightly higher than that of platelet and D-dimer (Figure 5).

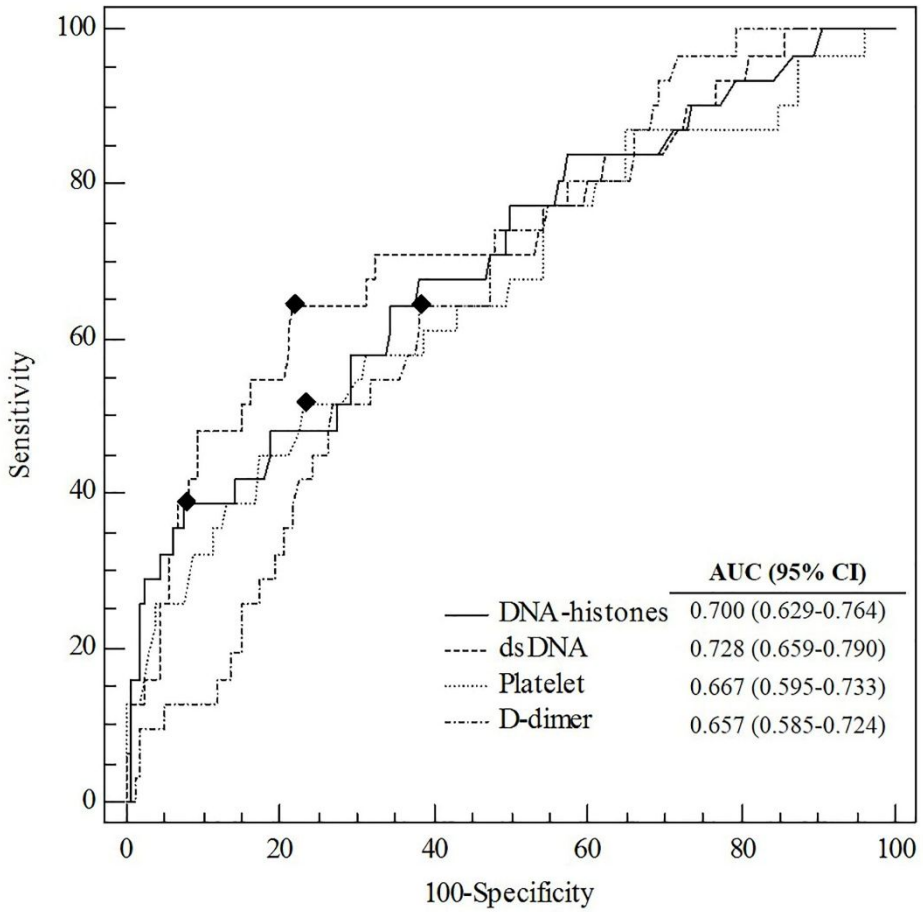


Figure 5. Receiver operating characteristic (ROC) curves and the area under the ROC curves (AUC) for DNA-histone complexes, dsDNA, platelets and D-dimer for mortality prediction in disseminated intravascular coagulation. Diamond points (◆) represent the optimal cutoff point corresponding to Youden index.

For Kaplan-Meier analysis, the total population was divided into two groups according to the cutoff values that provided the best prognostic power in the ROC analysis. Among all patients, the group with high DNA-histone (>357 AU) or dsDNA (>3.23 ng/mL) exhibited poor survival rates compared to the group with low DNA-histone or dsDNA. Similarly, among cancer patients, the group with high levels of DNA-histone (>357 AU) or dsDNA (>5.13 ng/mL) showed poorer survival than the group with low levels of DNA-histone or dsDNA. Likewise, in non-cancer patients, the group with high levels of DNA-histone or dsDNA showed poor prognosis, although the statistical significance of non-cancer patients was slightly less than that of cancer patients (Figure 6).

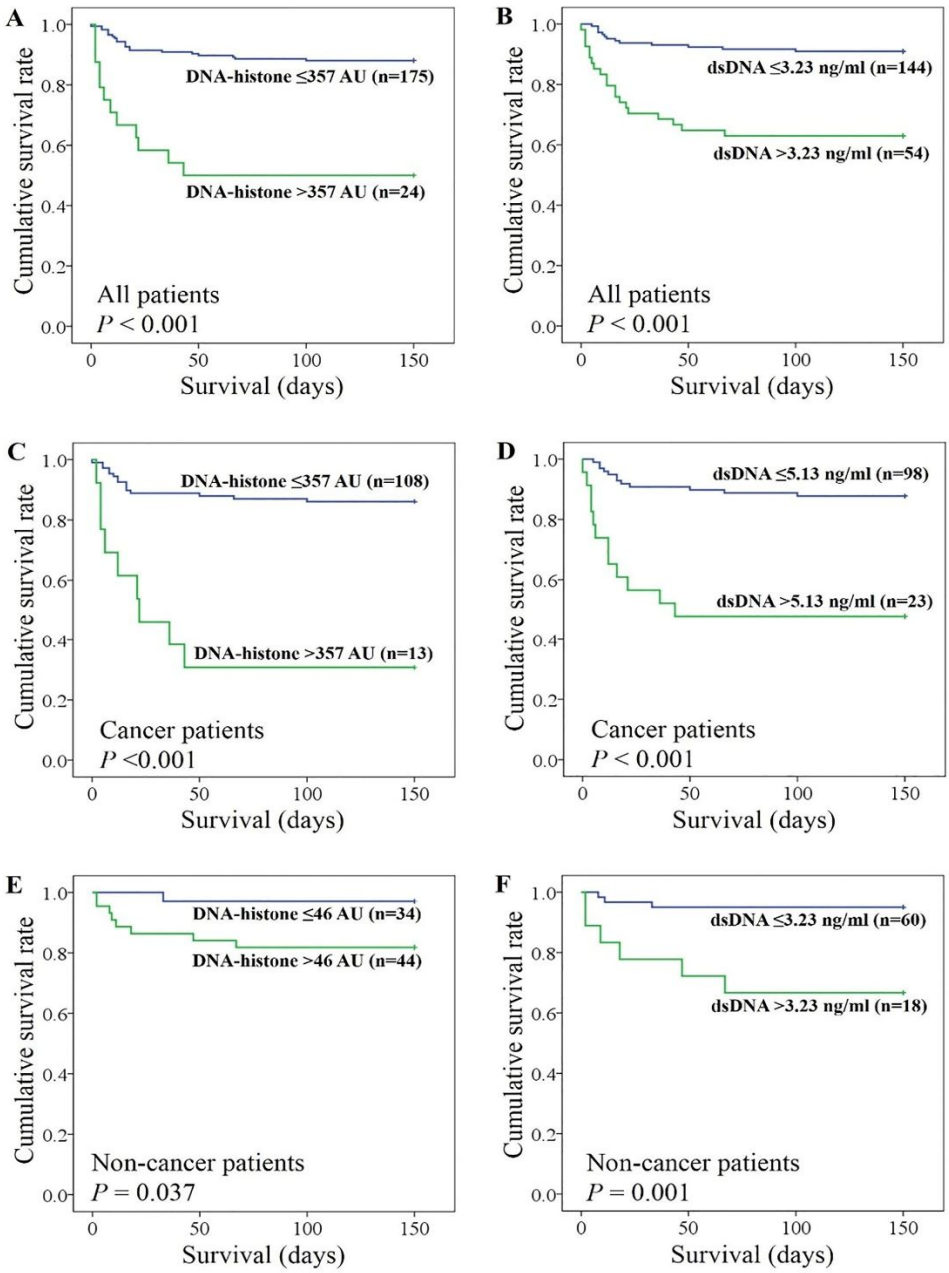


Figure 6. Kaplan-Meier survival analysis stratified for DNA-histones complex and dsDNA levels in all patients (A, B), cancer patients (C, D), and non-cancer patients (E, F). Values with the highest prognostic power to predict the 28-day mortality were set as the cut-off values.

Univariable Cox regression analysis showed significant hazard ratios for platelets, PT, D-dimer, fibrinogen, antithrombin, and protein C. Notably, the hazard ratios of DNA-histone (5.66) and dsDNA (4.93) were higher than that of D-dimer (2.41). In multivariable Cox regression analysis, the conventional DIC markers except platelet count lost their prognostic significance of mortality. Interestingly, DNA-histone and dsDNA were revealed as independent prognostic factors (Table 2).

Table 2. Univariable and multivariable Cox regression analysis for mortality prediction in all patients

	Univariable			Multivariable		
Variables	HR	95% CI	*P* value	HR	95% CI	*P* value
Platelet ($\leq 55 \times 10^9$ vs. $>55 \times 10^9$/L)	3.15	1.57 – 6.32	0.001	2.24	1.10 – 4.59	0.027
PT (>13.2 vs. ≤13.2 sec)	2.96	1.40 – 6.22	0.004	1.96	0.84 – 4.58	0.119
D-dimer (>3.69 vs. ≤3.69 mg/mL)	2.41	1.20 – 4.85	0.014	1.31	0.60 – 2.85	0.493
Fibrinogen (≤ 274 vs. >274 mg/dL)	0.47	0.24 – 0.93	0.031	0.75	0.36 – 1.56	0.439
Antithrombin (≤62 vs. >62 μg/L)	2.53	1.18 – 5.44	0.018	1.08	0.45 – 2.62	0.862
Protein C (≤72 vs. >72 pg/mL)	3.99	1.54 – 10.35	0.004	2.51	0.78 – 8.14	0.124
DNA-histone (>357 vs. ≤357 AU)	5.66	2.78 – 11.52	0.000	3.89	1.65 – 9.15	0.002
dsDNA (>3.23 vs. ≤3.23 ng/ml)	4.93	2.45 – 9.91	0.000	2.41	1.04 – 5.55	0.039

The cut-off values were determined as the values which produced the best prognostic value.

Abbreviation: HR, hazard ratio; CI, confidence interval; PT, prothrombin time; dsDNA, double stranded DNA.

Histones induce TF expression in endothelial cells

Histones dose-dependently induced TF expression in endothelial cells. Corresponding to the TF antigen expression, the TF activity, as measured by procoagulant activity, was dose-dependently increased. Histones also up-regulated the TF mRNA level in a dose-dependent manner. The histone-treated endothelial cells significantly shortened the lag time and enhanced the ETP, as compared with the endothelial cells without histone treatment. The neutralizing antibody against TF normalized the shortened lag time and enhanced ETP in histone-treated endothelial cells to control levels. To investigate the relative effect of individual histones on TF expression of endothelial cells, they were treated with each subtype of histones. H3 and H4 were more potent than H1 or H2A/2B in accomplishing histone-induced TF up-regulation (Figure 7). The amounts of soluble TF in the culture supernatants were not increased in histone-treated cells (data not shown).

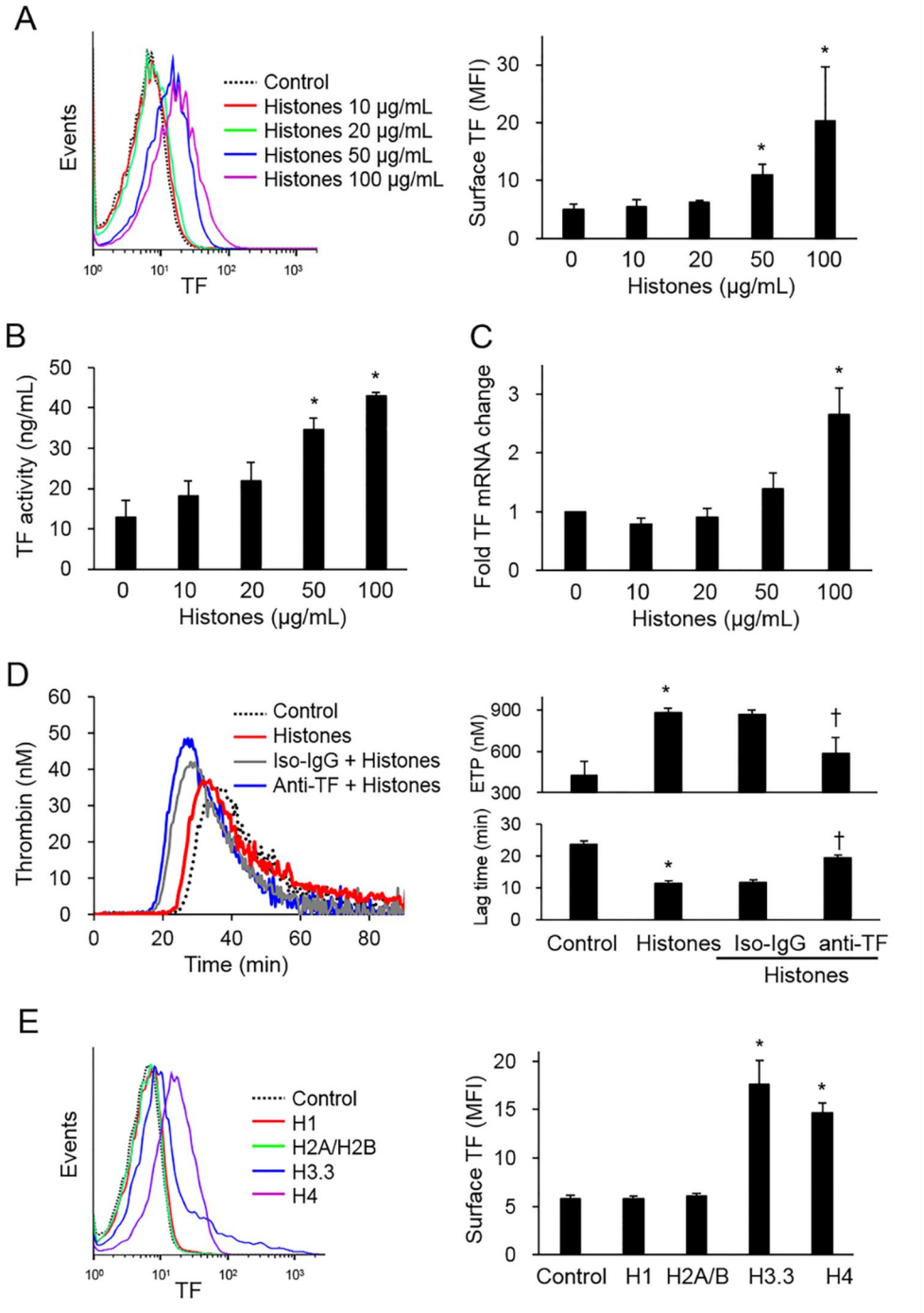


Figure 7. Histones induced TF expression in endothelial cells. (A) After EA.hy926 cells were stimulated with various levels of histones for 4 h, the surface expression of TF antigens was determined by flow cytometry. A

summary of the TF surface expression stimulated by histones is shown in the right panel. (B) The procoagulant activity of TF was measured in EA.hy926 cells incubated with various levels of histones for 4 h. (C) The expression of TF mRNA was quantified using real-time RT-PCR in EA.hy926 cells incubated with various levels of histones for 3 h. (D) EA.hy926 cells were either stimulated with or without 50 μg/mL histones for 4 h, and incubated with PBS (control), mouse isotype IgG (Iso-IgG, 30 μg/mL), or inhibitory TF antibody (anti-TF, 30 μg/mL) for 10 minutes. The ETP and lag time were analyzed using a thrombin generation assay. (E) EA.hy926 cells were stimulated with individual human recombinant histone (20 μg/mL H1, H2, H3, H4) for 4 h, and the expression of surface TF antigens was determined by flow cytometry (representative of three independent experiments). Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated).

Histones increase phosphatidylserine and PDI expression in endothelial cells

Histones increased surface phosphatidylserine in a dose-dependent manner. Annexin V was used to cover the exposed phosphatidylserine. The addition of annexin V to histone-treated cells did not modify the ETP or lag time in TGA (Figure 8). TF activity, as measured by procoagulant activity, was similarly unaffected by the addition of annexin V (Figure 9)

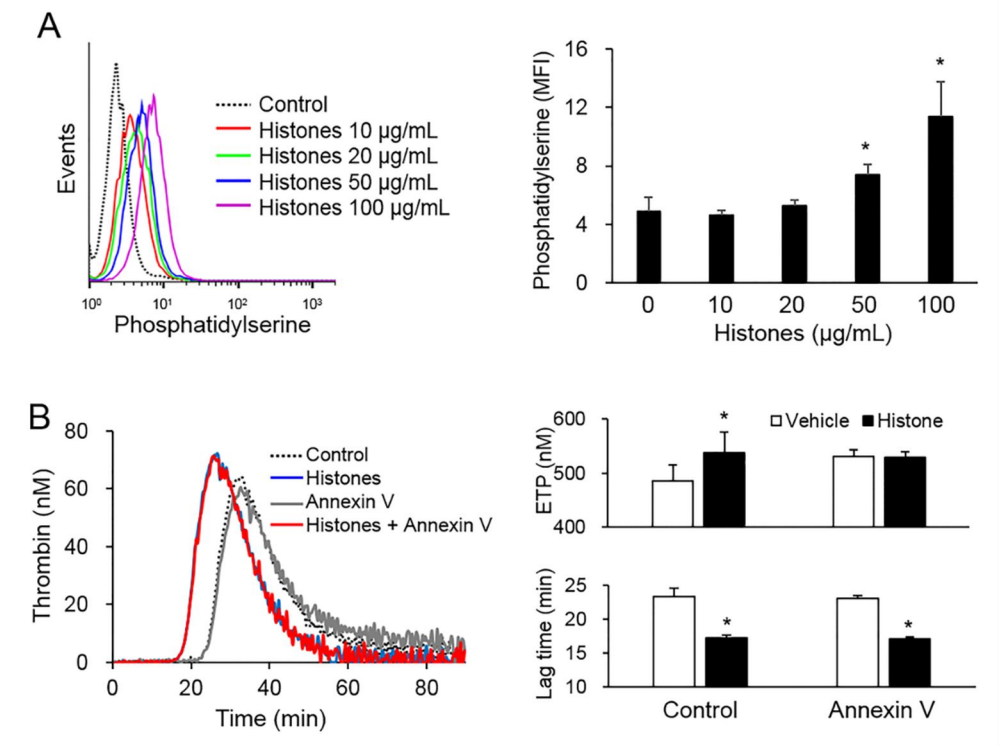


Figure 8. Histones induced phosphatidylserine exposure in endothelial cells. (A) EA.hy926 cells were stimulated with various levels of histones for 4 h, and the surface phosphatidylserine level was determined by flow cytometer. (B) EA.hy926 cells were stimulated with or without 50 μg/mL histones for 4 h, and incubated with PBS (vehicle) or 10 μg/mL annexin V for 15 min. The ETP and lag time were analyzed using a thrombin generation assay. Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated).

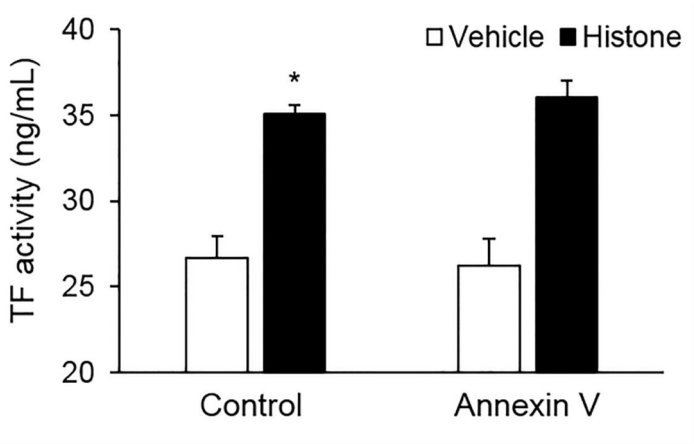


Figure 9. Annexin V did not affect the induced TF activity of histone-stimulated cells. EA.hy926 cells were stimulated with or without 50 µg/mL histones for 4 h, then incubated with PBS or 10 mg/mL annexin V for 15 min. The TF activity was analyzed using a procoagulant assay. Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated).

Histones increased the surface PDI expression in a dose-dependent manner. Several PDI inhibitors were used to block the regulation of TF decryption by PDI. PDI neutralizing antibody (RL90) shortened the lag time in histone-treated cells. Both glutathione and quercetin significantly shortened the lag time and increased the ETP in histone-treated cells (Figure 10). TF activity, as measured by procoagulant activity, was also increased by the three PDI inhibitors (Figure 11).

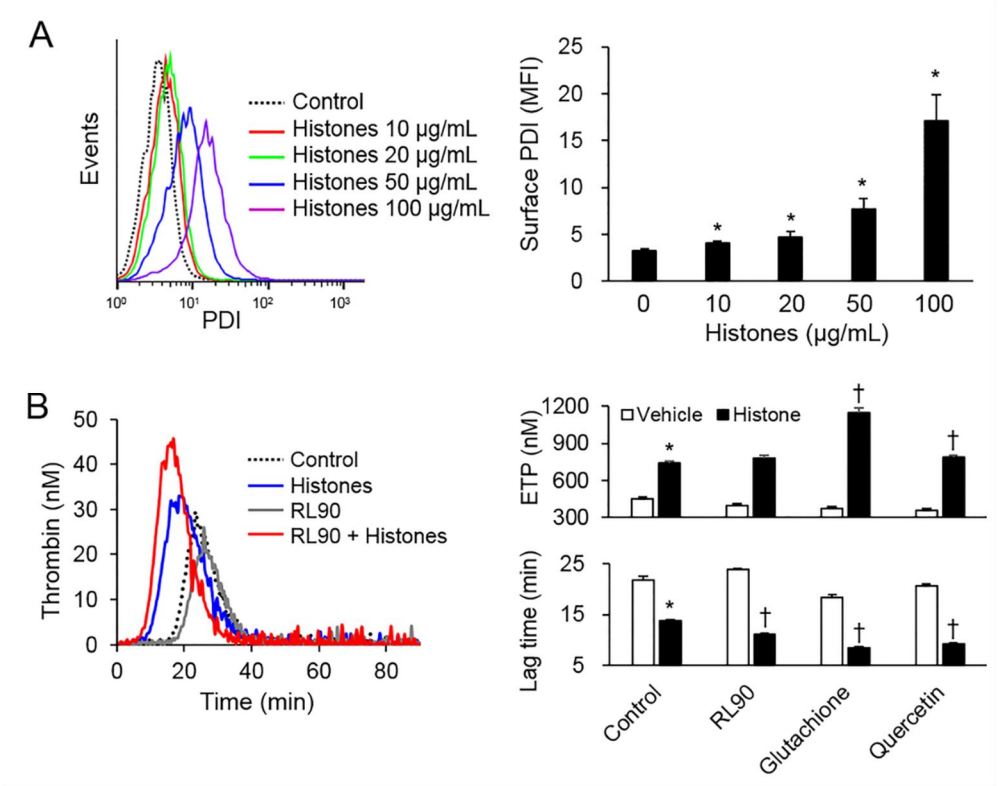


Figure 10. Histones increased PDI expression in endothelial cells. (A) EA.hy926 cells were stimulated with various levels of histones for 4 h, and the levels of surface PDI expression were determined by flow cytometry. (B) EA.hy926 cells were pre-incubated with PBS (control) or inhibitors, such as anti-PDI antibody (RL90, 10 μg/mL), glutathione (7.5 mM), and quercetin (200 μM) for 1 h. The cells were then stimulated with or without 50 μg/mL histones for 4 h. The ETP and lag time were analyzed using a thrombin generation assay. Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated), † $P<0.05$ vs. histone-treated cells.

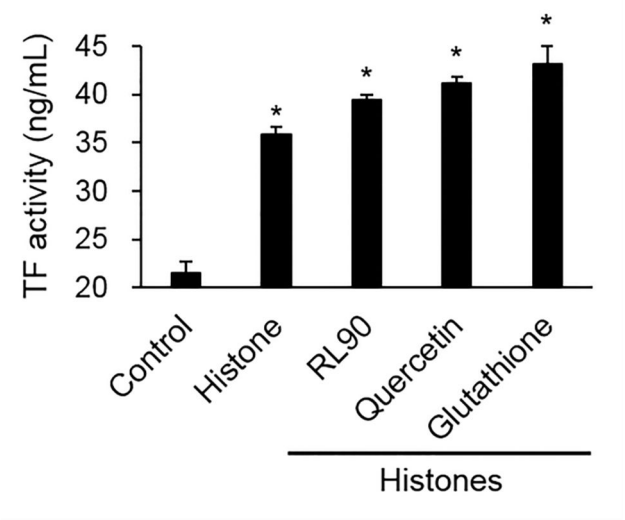


Figure 11. TF activity of histone-stimulated cells with protein-disulfide isomerase (PDI) inhibitors. EA.hy926 cells were pre-incubated with inhibitors, such as an anti-PDI antibody (RL90, 10 μg/mL), glutathione (7.5 mM), and quercetin (200 μM) for 1 h, prior to stimulation with 50 μg/mL histones for 4 h. The TF activity was analyzed using a procoagulant assay. Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated)

Histones reduce TM expression in endothelial cells

Histones reduced the expression of endothelial TM in a dose-dependent manner. The TM mRNA expression was markedly decreased in low concentrations of histone-treated cells, and remained significantly decreased in high concentrations of histone-treated cells. TM activity was significantly decreased in histone-treated cells as well. Because some stimuli can induce the lectin-like domain shedding of surface TM by MMPs [38], it was investigated whether an MMP inhibitor could block the histone-induced TM reduction. The MMP inhibitor, GM6001 did not modify the histone-induced TM reduction. Likewise, the histone-induced TM reduction was still observable through the use of a TM antibody against the EGF domain 5 of TM. The soluble TM levels were not increased in the culture supernatants of histone-treated cells. Among the individual histones, H4 was most potent than other subtype of histones in endothelial TM reduction (Figure 12).

Similar to results obtained from EA.hy926 cells, the primary HUVECS also showed a procoagulant phenotype induced by histones (Figure 13)

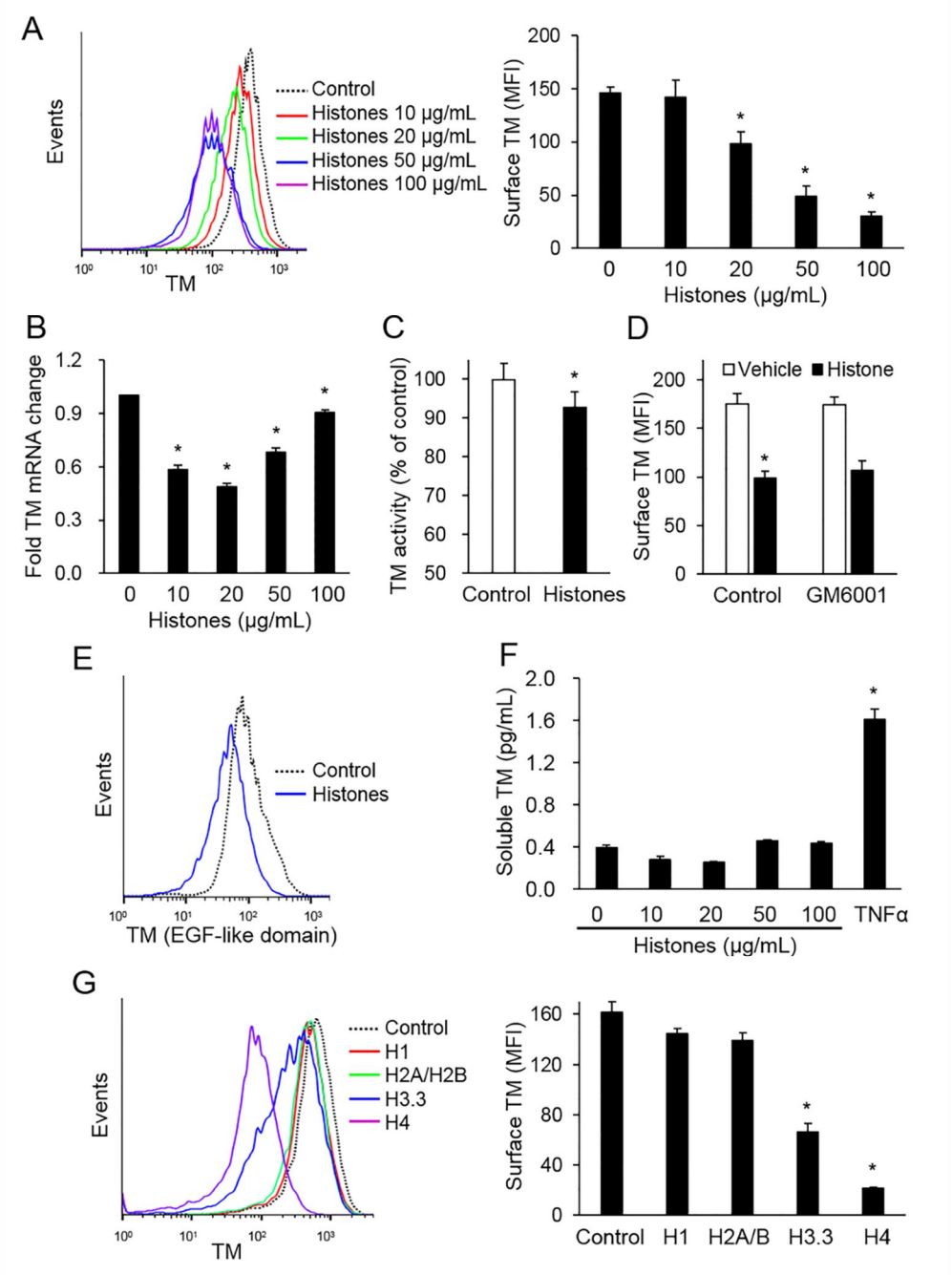


Figure 12. Histones reduced the endothelial surface expression of TM. (A) EA.hy926 cells were treated with various levels of histones for 4 h, and the expression levels of surface TM was determined by flow cytometry with an anti-TM antibody (epitope: lectin-like domain). (B) TM mRNA expression

levels were quantified by real-time RT-PCR in EA.hy926 cells incubated with various levels of histones for 3 h. (C) EA.hy926 cells were treated with 50 μg/mL histones for 4 h, and TM activity was measured using the chromogenic assay described in the Materials and Methods section. (D) EA.hy926 cells were pre-incubated with an MMP inhibitor (10 μM GM6001) for 30 min, and then stimulated with or without 50 μg/mL histones, for 4 h. The level of surface TM was determined by flow cytometry with the aforementioned anti-TM antibody. (E) EA.hy926 cells were treated with or without 50 μg/mL histones for 4 h and the level of surface TM expression was measured using the anti-EGF-like domain of the TM. (F) EA.hy926 cells were treated with various levels of histones or 2 ng/mL TNF-α (as a positive control) for 4 h, and the soluble TM levels in the culture supernatants were measured by ELISA. (G) EA.hy926 cells were stimulated with individual human recombinant histones (20 μg/mL H1, H2, H3, or H4) for 4 h, and the level of surface expression of TF antigens was determined by flow cytometry (representative of 3 independent experiments). Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated).

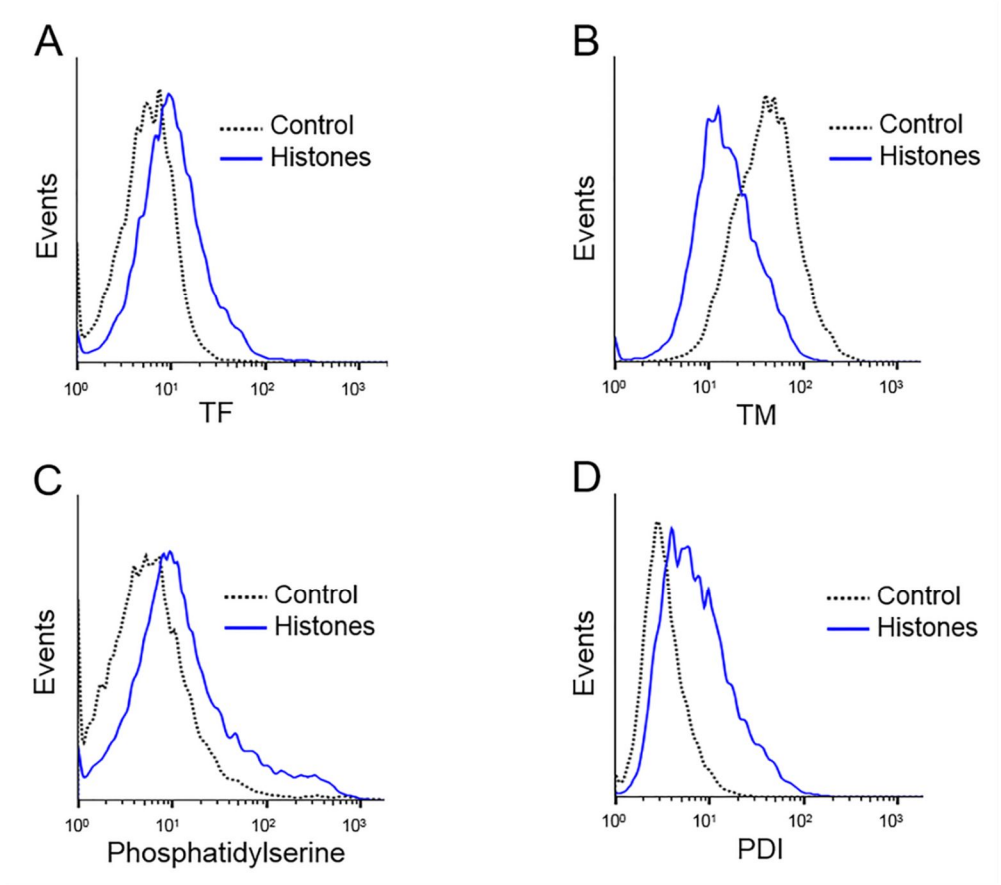


Figure 13. Histones influenced the expression of several proteins on HUVECs. After HUVECs were stimulated with 50 μg/mL histones for 4 h, surface expression levels of tissue factor (TF; A), thrombomodulin (TM; B), phosphatidylserine (C), and protein-disulfide isomerase (PDI; D) antigen were determined using flow cytometry.

PSA and heparin neutralize the histone effect in the endothelial phenotype

PSA and heparin are known to bind to histones due to their negatively charged properties [39, 40]. It was assessed whether these molecules could neutralize the histone effect on the endothelial procoagulant phenotype. Pretreatment of PSA or heparin in the histone reagent significantly blocked the histone-induced TF up-regulation and TM down-regulation. APC, which can cleave histones [11], could not inhibit the histone-induced TF up-regulation, but completely abolished the histone-induced TM down-regulation (Figure 14).

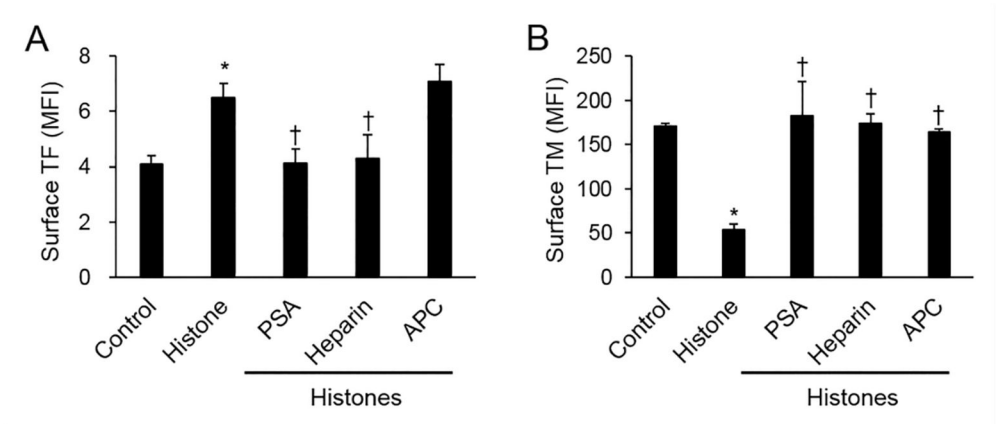


Figure 14. Neutralization of the histone effect by PSA, heparin, and APC. Histones were pretreated with or without 62.5 μM PSA, 100 IU/mL heparin, and 100 nM APC for 15 min. The pretreated histones (50 μg/mL) were added to the EA.hy926 cells for 4 h. The surface expression levels of TF (A) and TM (B) were measured by flow cytometry. Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated), † $P<0.05$ vs. histone-treated.

Inhibition of TLR2, TLR4, and TLR9 suppresses the histone effect on the endothelial phenotype

The neutralizing antibodies against potential histone receptors (TLR2 or TLR4) or the antagonist for TLR9, were pre-incubated with the cells prior to histone stimulation. Anti-TLR2 and anti-TLR4 partially blocked the histone-induced TF up-regulation. Interestingly, the TLR9 antagonist significantly inhibited the histone-induced TF up-regulation. Anti-TLR2 and anti-TLR4 did not inhibit the histone-induced TM down-regulation. However, the TLR9 antagonist completely blocked the histone-induced TM down-regulation (Figure 15). Similarly, HUVECs also showed that the TLR9 antagonist blocked the changes of TF and TM expression by histones (Figure 16).

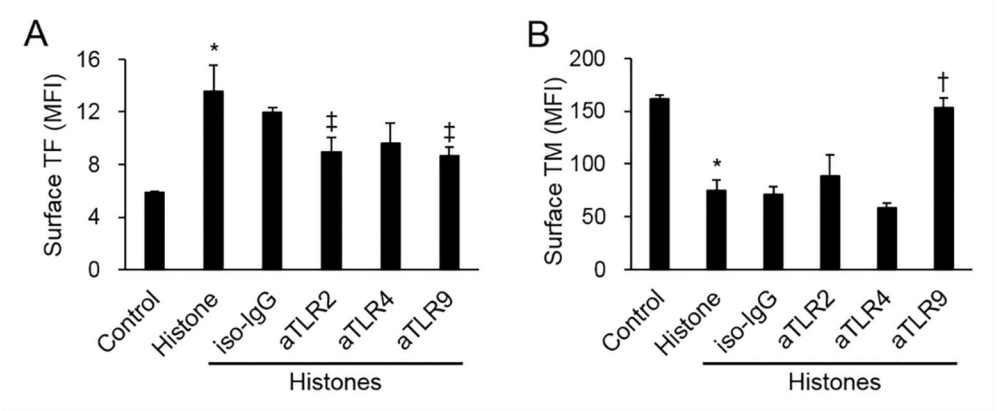


Figure 15. Inhibition of TLR in the histone-induced procoagulant phenotype. EA.hy926 cells were pre-incubated with mouse isotype IgG (Iso-IgG, 50 μg/mL), mouse anti-human TLR2 (aTLR2, 50 μg/mL), or anti-human TLR4 (aTLR4, 50 μg/mL) or TLR9 antagonists (aTLR9, 5 μM) for 30 min, and then stimulated with or without 50 μg/mL histones for 4 h. The surface expression levels of TF and TM were determined by flow cytometry. Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated), † $P<0.05$ vs. histone-treated, ‡ $P<0.1$ vs. histone-treated.

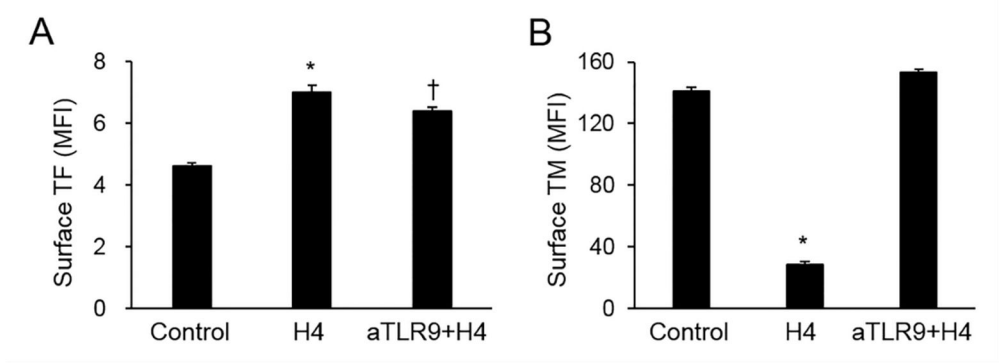


Figure 16. Inhibition of TLR in the histone-induced procoagulant phenotype of HUVECs. HUVECs were pre-incubated with mouse isotype IgG (Iso-IgG, 50 μg/mL), mouse anti-human TLR2 (aTLR2, 50 μg/mL), or anti-human TLR4 (aTLR4, 50 μg/mL) or TLR9 antagonists (aTLR9, 5 μM) for 30 min, and then stimulated with or without 50 μg/mL histones for 4 h. The surface expression levels of TF and TM were determined by flow cytometry. Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. control (histones not treated), † $P<0.05$ vs. histone-treated.

Because the NF-κB pathway is an important downstream target of TLR [41], it was investigated the effect of histones on the nuclear translocation of p65 in EA.hy926 cells using immunofluorescent staining with anti-NF-κB p65. In control and histone-treated cells most of the p65 was located in the cytoplasm, whereas Tumor necrosis factor-α (TNF α), which has been shown to activate the NF-κB pathway [19], stimulation induced some nuclear translocation of p65. Results from the NF-κB ELISA showed that histones did not increase NF-κB signals. SB203580 as a MAPK inhibitor, and LY294002 as a PI3K inhibitor were partially inhibited the histones-induced TF up-regulation. The inhibitors had no effect on the histones-induced TM down-regulation (Figure 17).

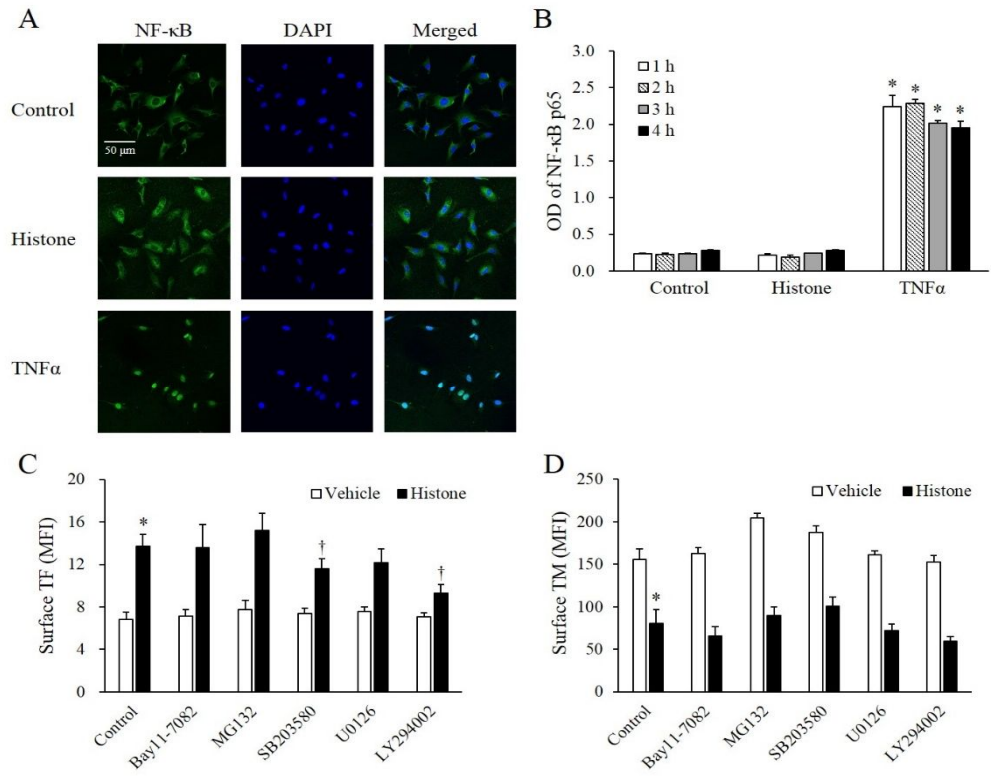


Figure 17. Effect of histones on NF-κB activation in endothelial cells. (A) EA.hy926 cells were stimulated with vehicle, 50 μg/mL of histones or 2 ng/mL of TNF-α for 1 h. NF-κB p65 was stained with FITC (green: left panels), and the nuclei were marked with DAPI (blue: middle panels). The merged images are shown in the right panels. All images were visualized by confocal microscopy. (B) Nuclear contents were extracted from the aforementioned cells. The optical density from the antibody against NF-κB p65 was measured according to the manufacturer's guideline. (C, D) After EA.hy926 cells were pre-incubated with Bay11-7082 (10 μM), MG132 (10 μM), SB203580 (5 μM), U0126 (20 μM)), LY294002 (10 μM) or not (control), 50 μg/mL of histones were applied prior to the assessment of the surface expression levels of TF and TM by flow cytometry. Data comparisons were carried out using the student's *t*-test. * $P<0.05$ vs. negative control.

DISCUSSTION

Neutrophils can be induced by pathogens or other stimuli to release DNA combined with histones and cytoplasmic components such as neutrophil elastase, myeloperoxidase, and cathepsin G [14]. The resulting NET provides a scaffold to grow a hemostatic plug. Therefore, the NET formation may be inevitable in clinical conditions that are characterized by formation of vascular thrombi. Even though NET is known to be an important scaffold for vascular thrombi, there has been no data on the clinical significance of NET in DIC. This study demonstrated that the levels of circulating DNA-histone complexes and dsDNA gradually increased in accordance with DIC severity. In addition, the elevated levels of circulating DNA-histone complexes and dsDNA showed independent prognostic significance.

These data provide an interesting insight for the association of NET with coagulation severity. There have been several reports that explain how NET contributes to coagulation activation [1, 5, 15]. Nucleic acids can bind coagulation factor XII and XI, thereby activating the intrinsic pathway [42]. The histones can increase thrombin formation by impairment of thrombomodulin-dependent protein C activation [21]. Histones can also activate platelets and endothelial cells [11, 15]. In animal models, histone infusion has been shown to increase platelet-rich microthrombi [20]. Considering these effects of NET on coagulation activation in the previous reports, it is plausible that the increased levels of DNA-histone complex and dsDNA play a role in the amplification of coagulation activation in DIC processes.

One of the intriguing findings of this study was that the increased levels of DNA-histone complexes and dsDNA showed powerful prognostic values that were independent of the already known prognostic DIC markers. This suggests that the continuous formation of NET in patients with DIC could not only amplify the coagulation activation and but also affect other detrimental damage to the host [19]. As activated protein C is known to cleave histones [11], the decreased level of activated protein C in patients with advanced DIC may aggravate histone removal and result in high mortality. In other words, NET formation is thought to play a role in a vicious cycle of hypercoagulability.

Several reports have indicated the presence of extracellular nuclear proteins that are released from dying cells during sepsis [19, 22]. Elevated levels of circulating nucleosome, high mobility group box-1, and plasma DNA were observed in critically ill patients [19, 25, 27]. Furthermore, high nucleosome levels have also been reported in advanced cancer patients [29]. This increase may originate from enhanced cell death or from impaired degradation of nucleosomes in serum by deoxyribonuclease (DNase) [7, 31]. In view of various underlying clinical conditions in the study population, it is not easy to clearly identify the kind of factor(s) that increased the NET formation. In the present study, the main underlying conditions of DIC were sepsis, severe infections, and malignancies; therefore, the main contributor of NET formation is likely to be cell death induced by various stimuli.

The neutrophil elastase has been found to digest cross-linked fibrin in DIC, finally increasing plasma level of cross-linked fibrin degradation product by elastase (e-XDP) [33]. Although this study didn't measure the e-XDP level, it is likely to correlate the NET markers with e-XDP, because NET

formation induces a large amount of neutrophil elastase which increases the e-XDP level.

Circulating levels of DNA-histone complexes and dsDNA may be dependent on neutrophil counts partly, because they are released from neutrophil. However in this study the DNA-histone complexes and dsDNA levels were not different among subtype of hematologic malignancies (data not shown). Future study may be required to investigate the effect of peripheral neutrophil count on DNA-histone complexes and dsDNA levels in resting status.

Cancer is considered to be a significant contributor to the formation of circulating NET [29]. Therefore, the test population was divided into cancer and non-cancer groups to exclude the effect of cancer on circulating NET formation. Interestingly, circulating NET levels showed significant prognostic values in both cancer patients as well as non-cancer patients (Fig 3). In addition, the association of circulating NET levels with coagulation severity remained unchanged, when analyzed in each cancer and non-cancer patient (data not shown). These findings suggest that the circulating NET levels generated in DIC processes provide prognostic significance regardless of the presence of cancer.

Specimen type is important when measuring circulating DNA levels because in vitro clot formation in serum results in an increase of artificially generated DNA increment in specimen tube [36]. Hence, using plasma instead of serum can assess more accurate values reflecting in vivo circulating levels of DNA. This study measured the circulating levels of DNA-histone complexes and dsDNA in plasma, thus representing true in vivo condition.

The current study has a few limitations. First, although the circulating DNA-histone complexes and dsDNA were measured, but it could not be visualized the real NET formation due to methodological limitations. Second, the study could not explain the detailed changes associated with circulating DNA-histone complexes and dsDNA because this was designed to be a cross-sectional study representing one specific point in time. Third, the study did not evaluate the NET levels with respect to cancer stage. The cancer patients in this study were of various types including stomach, lung, pancreas, breast and prostate cancers, which made it unfeasible to analyze the relationship for each cancer type. However, the significant correlation of NET levels with coagulation severity was observed, suggesting it to be a good representative of DIC severity regardless of cancer stage. Fourth, the assay kits for DNA-histone complexes and dsDNA testing could not be used for clinical use so far. Future studies are required to evaluate the test performance and to validate the usefulness.

Recent reports have described a pathogenic role for histones as prothrombotic substances [1, 11, 17, 18, 21]. Histones promote not only platelet activation [17, 18], but also coagulation activation through inhibition of TM-dependent protein C activation [21]. Since histones are cationic proteins, they can bind to anionic TM and inhibit the anticoagulant protein C system. Despite experimental results demonstrating the direct role of histones in the activation of plasma coagulation, there have been no published reports regarding the effects of histones on the endothelial anticoagulant phenotype. This study demonstrated a novel role for histones in inducing the endothelial procoagulant phenotype. Histones induced the procoagulant phenotype of

endothelial cells by up-regulating the procoagulant TF and down-regulating the anticoagulant TM.

The resting endothelium does not express TF, which is an important initiator of the extrinsic coagulation pathway. However, pathologic stimuli, such as interleukin-6 and endotoxin, can transform the anticoagulant endothelium to be procoagulant. In this results, histones enhanced levels of both TF antigen and activity through TF mRNA synthesis in endothelial cells. In this in vitro cell-based TGA, histone-pretreated endothelial cells increased the thrombin generation that was inhibited by the TF neutralizing antibody. Because intravascular TF contributes to the activation of coagulation in various disorders, such as infection, trauma, and malignancy [3], histone-induced endothelial TF expression can be considered to play an essential role in coagulation activation, especially in those clinical conditions associated with high circulating histone levels [5, 16, 43, 44].

TF activity is also affected by phosphatidylserine exposure. These results showed that histones dose-dependently increased phosphatidylserine exposure in endothelial cells. This finding may be related to the cytotoxic effects of histones [11]. To investigate whether the histone-induced phosphatidylserine externalization contributed to TF activity, annexin V was added to the histone-treated endothelial cells, and the TF activity was then measured. Even after the cover of exposed phosphatidylserine in the histone-treated cells with annexin V, the lag time was not changed. Similar to these results, other studies have demonstrated that TF activity was only partially blocked or not blocked at all by annexin V, suggesting additional molecular mechanisms of TF activation [45-47].

PDI is another known modulator of TF decryption that acts by regulating the redox state of the disulfide bond in the extracellular domain of TF [48]. Endothelial cells express PDI mainly in the endoplasmic reticulum, but also partially on the surface. Interestingly, these results showed that histones dose-dependently increased PDI expression on the surface of endothelial cells. PDI inhibitors were used to investigate the involvement of PDI in TF activity. The PDI inhibitors enhanced the TF activity in histone-treated endothelial cells. Although there have been several reports indicating that PDI inhibitors increased TF activity and thrombosis [32, 49], the expression of PDI in endothelial cells has been suggested to be a negative regulator of coagulation[48]. Consistent with the results of the aforementioned report [48], these results demonstrated that PDI inhibition increased TF activity and indicated that endothelial PDI is a negative regulator of TF activity. It is assumed that the histone-induced PDI increment may be a physiological defense mechanism against the histone-induced procoagulant endothelial phenotype.

TM is a transmembrane glycoprotein expressed in endothelial cells [50]. TM is composed of 5 domains, and the EGF-like domain has catalytic site [38]. Inflammatory stimuli enhance soluble TM shedding from endothelial cells using several proteases including elastase, proteinase, and cathepsin G [51]. Previous experimental results have indicated that these processes were considerably blocked by MMP inhibitors [52]. In these experiments, however, an MMP inhibitor did not change the histone effect, suggesting that histones did not induce the soluble TM shedding from endothelial cells, but rather decreased the presence of surface TM through reduced mRNA synthesis. Inflammatory stimuli have also been shown to

suppress endothelial TM expression [50]. Similar to the aforementioned result, these results support the notion that endothelial TM down-regulation may be a causal factor of a hypercoagulability in the clinical conditions associated with high circulating histone levels.

PSA is a highly negatively charged glycan that can bind to histones [39]. The finding that PSA abolished the histone-induced endothelial procoagulant phenotype suggested a potential role for this agent as an anticoagulant. Heparin can also block the histone effect via binding to histones. Additionally, APC that degrades histones [11] could not block the histone-induced TF up-regulation, but completely blocked the histone-induced TM down-regulation. This finding suggested that even the histones degraded by APC may act on the downstream signal for TF transcription. Regarding TM down-regulation, however, the degraded histones were considered to have lost these effects. Because APC is one of the therapeutic candidates for dampening the hypercoagulability of DIC [53], the finding that APC could not block the histone-induced endothelial procoagulant phenotype should be considered as an important limitation of APC therapy.

It has been shown that histones bind to TLR2 and TLR4 [52]. Recently, TLR9 was reported as a histone receptor in a model of histone-induced inflammatory liver injury [52, 53]. This study evaluated the possible role of these receptors in the histone-induced endothelial procoagulant phenotype. TLR2 and TLR4 were partially involved in histone-induced TF up-regulation, whereas they were not involved in histone-induced TM down-regulation, suggesting that additional activation mechanisms may be required for histone-induced TF up-regulation. Interestingly, the results showed that the blockade of TLR9 significantly inhibited the histone-induced TF up-

regulation, and completely abolished the histone-induced TM down-regulation. Although TLR9 has been shown to recognize bacterial DNA, the data suggest that TLR9 may bind to histones and induce downstream proinflammatory signals. This study also demonstrated that histones had not involved to activate the NF-κB pathway. This result is contrast to the results from previous reports that histones activated the NF-κB pathway through TLRs [54, 55]. But the inhibitors of MAPK, and PI3K were partially inhibited histones-induced TF up-regulation. Because there are many downstream pathways of TLRs, NF-κB inhibition alone may be insufficient to block the histone effect.

In conclusions, it demonstrated that circulating DNA-histone complexes and dsDNA correlated well with DIC severity. In addition, the high levels of circulating DNA-histone complexes and dsDNA showed significantly independent prognostic values in DIC. This study demonstrated that histones induced the endothelial procoagulant phenotype through TF up-regulation and TM down-regulation. The histone-induced TF up-regulation was partially mediated by TLR2, TLR4, and TLR9, and the histone-induced TM down-regulation was mediated by TLR9. These findings provide a new insight into the potential clinical relevance of circulating DNA-histone complex and dsDNA, which serve as powerful markers for mortality prediction of DIC in future. They also suggested that histones play a role in damaging the normal physiologic anticoagulant endothelial surface, resulting in a pathologic procoagulant endothelial surface. Because circulating histone levels are

increased in various diseases including sepsis, DIC, cancer, and autoimmune disorders that are associated with thrombosis [4-6, 16], the findings of the histone-induced endothelial procoagulant phenotype may help to elucidate the pathogenesis of the hypercoagulable status in those diseases. Strategies to inhibit the harmful effects of histones in endothelial cells may be required in order to prevent a thrombotic environment.

REFERENCES

[1] Martinod K, Wagner DD. Thrombosis: tangled up in NETs. Blood. 2014;123:2768-76.
[2] Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? The Journal of cell biology. 2012;198:773-83.
[3] Arai Y, Yamashita K, Mizugishi K, Watanabe T, Sakamoto S, Kitano T, et al. Serum neutrophil extracellular trap levels predict thrombotic microangiopathy after allogeneic stem cell transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2013;19:1683-9.
[4] Borissoff JI, Joosen IA, Versteylen MO, Brill A, Fuchs TA, Savchenko AS, et al. Elevated levels of circulating DNA and chromatin are independently associated with severe coronary atherosclerosis and a prothrombotic state. Arteriosclerosis, thrombosis, and vascular biology. 2013;33:2032-40.
[5] van Montfoort ML, Stephan F, Lauw MN, Hutten BA, Van Mierlo GJ, Solati S, et al. Circulating nucleosomes and neutrophil activation as risk factors for deep vein thrombosis. Arteriosclerosis, thrombosis, and vascular biology. 2013;33:147-51.
[6] Chen Q, Ye L, Jin Y, Zhang N, Lou T, Qiu Z, et al. Circulating nucleosomes as a predictor of sepsis and organ dysfunction in critically ill patients. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases. 2012;16:e558-64.
[7] Hakkim A, Furnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proceedings of the National Academy of Sciences of the United States of America. 2010;107:9813-8.
[8] Abrams ST, Zhang N, Manson J, Liu T, Dart C, Baluwa F, et al. Circulating histones are mediators of trauma-associated lung injury. American journal of respiratory and critical care medicine. 2013;187:160-9.
[9] Levi M, Toh CH, Thachil J, Watson HG. Guidelines for the diagnosis and management of disseminated intravascular coagulation. British Committee for Standards in Haematology. British journal of haematology. 2009;145:24-33.
[10] Zeerleder S, Stephan F, Emonts M, de Kleijn ED, Esmon CT, Varadi K, et al. Circulating nucleosomes and severity of illness in children suffering from meningococcal sepsis treated with protein C. Critical care medicine. 2012;40:3224-9.

[11] Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, et al. Extracellular histones are major mediators of death in sepsis. Nature medicine. 2009;15:1318-21.
[12] Martinod K, Wagner DD. Thrombosis: tangled up in NETs. Blood. 2013.
[13] Taylor FB, Jr., Toh CH, Hoots WK, Wada H, Levi M, Scientific Subcommittee on Disseminated Intravascular Coagulation of the International Society on T, et al. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. Thrombosis and haemostasis. 2001;86:1327-30.
[14] Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303:1532-5.
[15] Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Jr., et al. Extracellular DNA traps promote thrombosis. Proceedings of the National Academy of Sciences of the United States of America. 2010;107:15880-5.
[16] Kim JE, Lee N, Gu JY, Yoo HJ, Kim HK. Circulating levels of DNA-histone complex and dsDNA are independent prognostic factors of disseminated intravascular coagulation. Thrombosis research. 2015;135:1064-9.
[17] Semeraro F, Ammollo CT, Morrissey JH, Dale GL, Friese P, Esmon NL, et al. Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. Blood. 2011;118:1952-61.
[18] Carestia A, Rivadeneyra L, Romaniuk MA, Fondevila C, Negrotto S, Schattner M. Functional responses and molecular mechanisms involved in histone-mediated platelet activation. Thrombosis and haemostasis. 2013;110:1035-45.
[19] Zeerleder S, Zwart B, Wuillemin WA, Aarden LA, Groeneveld AB, Caliezi C, et al. Elevated nucleosome levels in systemic inflammation and sepsis. Critical care medicine. 2003;31:1947-51.
[20] Fuchs TA, Bhandari AA, Wagner DD. Histones induce rapid and profound thrombocytopenia in mice. Blood. 2011;118:3708-14.
[21] Ammollo CT, Semeraro F, Xu J, Esmon NL, Esmon CT. Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. Journal of thrombosis and haemostasis : JTH. 2011;9:1795-803.
[22] Semeraro N, Ammollo CT, Semeraro F, Colucci M. Sepsis, thrombosis and organ dysfunction. Thrombosis research. 2012;129:290-5.
[23] Anastasiou G, Gialeraki A, Merkouri E, Politou M, Travlou A. Thrombomodulin as a regulator of the anticoagulant pathway: implication in

the development of thrombosis. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis. 2012;23:1-10.
[24] Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proceedings of the National Academy of Sciences of the United States of America. 1996;93:10212-6.
[25] Rhodes A, Wort SJ, Thomas H, Collinson P, Bennett ED. Plasma DNA concentration as a predictor of mortality and sepsis in critically ill patients. Critical care. 2006;10:R60.
[26] Mackman N. Role of tissue factor in hemostasis, thrombosis, and vascular development. Arteriosclerosis, thrombosis, and vascular biology. 2004;24:1015-22.
[27] Hatada T, Wada H, Nobori T, Okabayashi K, Maruyama K, Abe Y, et al. Plasma concentrations and importance of High Mobility Group Box protein in the prognosis of organ failure in patients with disseminated intravascular coagulation. Thrombosis and haemostasis. 2005;94:975-9.
[28] Bode M, Mackman N. Regulation of tissue factor gene expression in monocytes and endothelial cells: Thromboxane A2 as a new player. Vascular pharmacology. 2014;62:57-62.
[29] Wittwer C, Boeck S, Heinemann V, Haas M, Stieber P, Nagel D, et al. Circulating nucleosomes and immunogenic cell death markers HMGB1, sRAGE and DNAse in patients with advanced pancreatic cancer undergoing chemotherapy. International journal of cancer Journal international du cancer. 2013;133:2619-30.
[30] Henriksson CE, Klingenberg O, Ovstebo R, Joo GB, Westvik AB, Kierulf P. Discrepancy between tissue factor activity and tissue factor expression in endotoxin-induced monocytes is associated with apoptosis and necrosis. Thrombosis and haemostasis. 2005;94:1236-44.
[31] Peitsch MC, Hesterkamp T, Polzar B, Mannherz HG, Tschopp J. Functional characterisation of serum DNase I in MRL-lpr/lpr mice. Biochemical and biophysical research communications. 1992;186:739-45.
[32] Rao LV, Pendurthi UR. Regulation of tissue factor coagulant activity on cell surfaces. Journal of thrombosis and haemostasis : JTH. 2012;10:2242-53.
[33] Madoiwa S, Tanaka H, Nagahama Y, Dokai M, Kashiwakura Y, Ishiwata A, et al. Degradation of cross-linked fibrin by leukocyte elastase as alternative pathway for plasmin-mediated fibrinolysis in sepsis-induced disseminated intravascular coagulation. Thrombosis research. 2011;127:349-55.
[34] Eilertsen KE, Osterud B. Tissue factor: (patho)physiology and cellular biology. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis. 2004;15:521-38.
[35] Chen VM. Tissue factor de-encryption, thrombus formation, and thiol-

disulfide exchange. Seminars in thrombosis and hemostasis. 2013;39:40-7.
[36] Lee TH, Montalvo L, Chrebtow V, Busch MP. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. Transfusion. 2001;41:276-82.
[37] Skaik YA. Sample size calculation. International journal of Ayurveda research. 2010;1:132.
[38] Wu HL, Lin CI, Huang YL, Chen PS, Kuo CH, Chen MS, et al. Lysophosphatidic acid stimulates thrombomodulin lectin-like domain shedding in human endothelial cells. Biochemical and biophysical research communications. 2008;367:162-8.
[39] Mishra B, von der Ohe M, Schulze C, Bian S, Makhina T, Loers G, et al. Functional role of the interaction between polysialic acid and extracellular histone H1. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010;30:12400-13.
[40] Pal PK, Starr T, Gertler MM. Neutralization of heparin by histone and its subfractions. Thrombosis research. 1983;31:69-79.
[41] Levi M, van der Poll T, Schultz M. Infection and inflammation as risk factors for thrombosis and atherosclerosis. Seminars in thrombosis and hemostasis. 2012;38:506-14.
[42] Altincicek B, Stotzel S, Wygrecka M, Preissner KT, Vilcinskas A. Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. Journal of immunology. 2008;181:2705-12.
[43] Wolberg AS, Kon RH, Monroe DM, Ezban M, Roberts HR, Hoffman M. Deencryption of cellular tissue factor is independent of its cytoplasmic domain. Biochemical and biophysical research communications. 2000;272:332-6.
[44] Le DT, Rapaport SI, Rao LV. Studies of the mechanism for enhanced cell surface factor VIIa/tissue factor activation of factor X on fibroblast monolayers after their exposure to N-ethylmaleimide. Thrombosis and haemostasis. 1994;72:848-55.
[45] Cho J, Furie BC, Coughlin SR, Furie B. A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. The Journal of clinical investigation. 2008;118:1123-31.
[46] Popescu NI, Lupu C, Lupu F. Extracellular protein disulfide isomerase regulates coagulation on endothelial cells through modulation of phosphatidylserine exposure. Blood. 2010;116:993-1001.
[47] Langer F, Ruf W. Synergies of phosphatidylserine and protein disulfide isomerase in tissue factor activation. Thrombosis and haemostasis. 2014;111:590-7.
[48] Van de Wouwer M, Collen D, Conway EM. Thrombomodulin-protein C-

EPCR system: integrated to regulate coagulation and inflammation. Arteriosclerosis, thrombosis, and vascular biology. 2004;24:1374-83.
[49] Lohi O, Urban S, Freeman M. Diverse substrate recognition mechanisms for rhomboids; thrombomodulin is cleaved by Mammalian rhomboids. Current biology : CB. 2004;14:236-41.
[50] Wang L, Bastarache JA, Wickersham N, Fang X, Matthay MA, Ware LB. Novel role of the human alveolar epithelium in regulating intra-alveolar coagulation. American journal of respiratory cell and molecular biology. 2007;36:497-503.
[51] Dhainaut JF, Yan SB, Joyce DE, Pettila V, Basson B, Brandt JT, et al. Treatment effects of drotrecogin alfa (activated) in patients with severe sepsis with or without overt disseminated intravascular coagulation. Journal of thrombosis and haemostasis : JTH. 2004;2:1924-33.
[52] Allam R, Kumar SV, Darisipudi MN, Anders HJ. Extracellular histones in tissue injury and inflammation. Journal of molecular medicine. 2014;92:465-72.
[53] Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, et al. Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. Hepatology. 2011;54:999-1008.
[54] Sohn RH, Deming CB, Johns DC, Champion HC, Bian C, Gardner K, et al. Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated by activation of nuclear factor-kappa B. Blood. 2005;105:3910-7.
[55] Levi M, van der Poll T. The role of natural anticoagulants in the pathogenesis and management of systemic activation of coagulation and inflammation in critically ill patients. Seminars in thrombosis and hemostasis. 2008;34:459-68.

논 문 초 록

김 지 은

협동과정 종양생물학

서울대학교 의과대학

서론: 호중구는 히스톤과 결합한 DNA가닥의 분비를 일으킬 수 있다. 호중구세포외기질은 지혈마개가 형성될 수 있는 뼈대를 제공한다. 호중구세포외기질의 형성은 아마도 혈관내응고 발생을 나타내는 필연적인 임상조건일 것이다. 혈관내벽은 주로 항응고성 표면을 유지하지만, 응고가 일어날 때는 이러한 항응고성 혈관내벽의 표현형이 응고촉진형으로 전환된다. 휴지기의 내피세포는 항응고성 thrombomodulin (TM)은 발현하고 응고촉진형 조직인자 (tissue factor, TF)는 발현하지 않는다. 그러나 스트레스를 받으면 TF 발현이 유도될 수 있다. 이 연구는 NET형성이 명확한 파종성혈관내응고(DIC)에서 활성화되고, 혈중 NET의 농도는 환자를 진단 및 예후를 예측하는지 관찰하였다. 또한 증가한 혈중 히스톤이 내피세포의 TF와 TM의 발현을 조절하는데 관여하는지 확인하였다.

방법: 199명의 파종성혈관내응고로 의심되는 환자와 20명의 정상인의 혈장내 DNA-histone complexes와 double-stranded DNA (dsDNA) 의 농도를 측정해서 체내에서 생성되는 호중구세포외기질의 지표로 사용하였다. 내피세포에서 히스톤에 의한 TF와 TM의 표면항원 및 수용성 항원, mRNA 발현 정도를 flow

cytometry, ELISA, and real-time RT-PCR로 측정하였다. TF와 TM의 기능을 procoagulant activity, thrombin generation, or chromogenic assays로 측정하였다. NF-κB pathway와 toll-like receptors (TLR)의 관여를 확인하였다.

결과: 혈중 DNA-histone complexes와 dsDNA 농도는 중증 파종성혈관내응고에서 유의하게 증가하였다. 이 두 마커의 증가는 DIC score 와 D-dimer가 나타내는 응혈이상과 관련이 있는 것으로 나타났다. 다변수 회기분석을 통해서 기존 파종성혈관내응고 마커들과 비교했을 때 이 두 마커들은 독립적인 불량예후인자로써 유용한 것으로 나타났다. 히스톤은 농도 비례하게 내피세포의 TF 표면항원을 증가시키고 TF의 기능 및 mRNA 발현도 같은 양상을 보였다. 히스톤은 thrombin generation assay의 반응시간을 단축시키고 트롬빈 생성량을 증가시킨다. 히스톤에 의한 TF 표면항원을 항체를 사용해서 억제했을 때 반응시간과 트롬빈 생성량은 대조군과 같은 수준으로 낮아지는 것을 확인했다. 히스톤은 세포표면의 phosphatidylserine과 protein-disulfide isomerase (PDI)를 증가시켰다. 또한 히스톤은 내피세포의 TM 표면항원 및 그 기능, mRNA 발현을 감소시켰다. NF-κB pathway는 히스톤이 내피세포에 미치는 영향을 나타내는데 관여하지 않았다. 각각 TLR2와 TLR4의 억제제는 히스톤에 의한 TF 증가를 부분적으로 저해하는 반면, TLR9 억제제는 통계적으로 유의하게 히스톤에 의한 TF 증가와 TM 감소를 저해하였다. Polysialic acid와 heparin을 사용해서 히스톤에 의한 TF 증가와 TM 감소를 반전시켰다. Activated protein C는

히스톤에 의한 TF증가에는 영향을 미치치 못하고 TM 감소만 억제하는 효과를 보였다.

결론: 혈중 호중구세포외기질의 농도는 응고항진 정도를 반영하고 파종성혈관내응고 환자의 불량한 예후를 나타낸다. 또한 이는 파종성혈관내응고의 사망률과 연관 지을 수 있다. 히스톤은 내피세포의 TF 증가과 TM 감소를 유도함으로 인해서 응고항진성 표현형을 유도한다. 이러한 효과를 나타내는데 NF-κB pathway는 관여하지 않고 TLR2, TLR4, TLR9이 연관되어있다. 내피세포의 응고항상성을 손상시키는 히스톤 효과를 억제하는 치료법을 개발하는 것은 혈전형성을 조절하는데 도움이 될 것으로 기대한다.

Keywords: 호중구세포외기질, 파종성혈관내응고, 종양, DNA-histone complex, dsDNA, 히스톤, 조직인자 (tissue factor; TF), Thrombomodulin (TM), 내피세포

Student Number: 2009-30591