이학석사 학위논문

Extracellular histone released from leukemic cells increases their adhesion to endothelium and protects them from spontaneous and chemotherapy-induced leukemic cell death

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ABSTRACT

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Introduction: When leukocytes are stimulated by reactive oxygen species (ROS), they release nuclear contents into the extracellular milieu, called by extracellular traps (ET). The nuclear contents are mainly composed of the histone–DNA complex and neutrophil elastase. This study investigated whether leukemic cells could release ET and the released histone could induce endothelial activation, eventually resulting in leukemic progression.

Methods: The circulating ET biomarkers (histone–DNA complex, cell-free double-stranded DNA (dsDNA), and neutrophil elastase) were measured by ELISA in 80 patients with hematologic diseases and 40 healthy controls. ET formation and ROS levels were investigated during leukemic cell proliferation in vitro. Histone-induced surface expression of endothelial adhesion molecules and cell survival were measured by flow cytometry.

Results: Acute leukemia patients had high levels of ET biomarkers, which correlated with peripheral blast count. Leukemic cells produced high ROS levels and released extracellular histone, which was significantly blocked by antioxidants. Histone significantly induced the surface expression of 3 endothelial adhesion molecules and promoted leukemic cell adhesion to endothelial cells, which was inhibited by histone inhibitors (heparin, polysialic acid, and activated protein C), neutralizing antibodies against these adhesion molecules, and a Toll like receptor(TLR)9 antagonist. When leukemic cells were co-cultured with endothelial cells, adherent leukemic cells showed better survival than the non-adherent ones, demonstrating that histone-treated endothelial cells protected leukemic cells from both spontaneous and

chemotherapy-induced death.

Conclusion: Our data demonstrate for the first time that extracellular histone can be released from leukemic cells through a ROS-dependent mechanism. The released histone promotes leukemic cell adhesion by inducting the surface expression of endothelial adhesion molecules and eventually protects leukemic cells from cell death.

Keywords: Extracellular traps, Histone, Reactive oxygen species, Leukemia, Endothelial adhesion molecule

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ABBREVIATIONS

Abbreviations	Full name
ROS	Reactive oxygen species
ET	Extracellular trap
dsDNA	double-stranded DNA
TLR	Toll-like receptor
E-selectin	Endothelial cell selectin
ICAM-1	Intercellular adhesion molecule-1
VCAM-1	Vascular cell adhesion molecule-1
MPN	Myeloproliferative neoplasms
ANC	Absolute neutrophil count
hEC	Human endothelial cell line, EA.hy926
HUVEC	Human umbilical vein endothelial cells
ATA	Aurintricarboxylic acid
PSA	Polysialic acid
NAC	N-acetyl-L-cysteine
APDC	4-amino-2,4-pyrrolidinedicarboxylic acid
APC	Activated protein C
Ara-C	Cytosine D–arabinofuranoside

INTRODUCTION

When leukocytes are stimulated by microorganisms or reactive oxygen species (ROS), they release their nuclear contents into the extracellular milieu, which results in the formation of so-called extracellular traps (ET) [1]. The released nuclear contents are mainly composed of the histone–DNA complex and soluble enzymes including neutrophil elastase and cathepsin G. In the innate immune response, the ET are responsible for microorganism entrapment and killing [2]. A recent report has shown that leukemic cell lines release ET upon chemical stimulation [3]. Since leukemic cells are counterparts of normal leukocytes and produce excessive ROS [4], it is likely that ROS overproduction by these cells induces ET formation.

Histone, the main component of ET, induces inflammation and activates platelets through Toll-like receptor (TLR) activation, thereby exhibiting detrimental effects on host [5]. Recent reports have shown the increased circulating histone levels in inflammatory, autoimmune, and thrombotic disorders [6,7]. Research into the mechanisms of detrimental effects of ET on the host has primarily focused on inflammatory disorders, while the potential effect of ET in hematologic malignancies remains underappreciated.

The normal endothelium physiologically provides anti-adhesive surface [8]. However, various inflammatory stimuli induce expression of endothelial adhesion molecules such as endothelial cell selectin (E-selectin), intercellular

adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), resulting in leukocyte or leukemic cell adhesion to the activated endothelium. Leukemic cell adhesion to endothelium contributes to leukemic progression and chemotherapy resistance [9-12].

In this study, we hypothesized that circulating histone released from leukemic cells induces endothelial activation, which might protect leukemic cells from spontaneous and chemotherapy-induced death. To prove this hypothesis, we measured the circulating levels of 3 ET biomarkers (histone–DNA complex, cell-free double-stranded DNA, and neutrophil elastase) in patients with several hematologic diseases, and demonstrated a significant correlation between the histone–DNA complex and peripheral blast count. We also demonstrated that several leukemic cell lines released the histone–DNA complex, and this release was inhibited by ROS blockers. Furthermore, histone induced surface expression of endothelial adhesion molecules and thus increased leukemic cell adhesion to endothelium, which was inhibited by neutralizing antibodies against adhesion molecules and by a TLR-9 antagonist. Finally, the adhesion of leukemic cells to histone-activated endothelial cells prevented their spontaneous and chemotherapy-induced death.

MATERIALS AND METHODS

Study population

A total of 80 patients with hematologic diseases and 40 healthy normal controls were enrolled. Final diagnosis of patients was acute leukemia (n=21), myeloproliferative neoplasms (MPN, n=45), and aplastic anemia (n=14). The acute leukemia group was composed of acute myeloid leukemia (n=14), acute lymphoblastic leukemia (n=6), and mixed phenotype acute leukemia (n=1). MPN patients were subdivided into 2 groups based on absolute neutrophil count (ANC): MPN with neutrophilia (ANC $\geq 7.5\times10^9$/L; n=13) and MPN without neutrophilia (ANC $< 7.5\times10^9$/L; n=32). The study was approved by the Seoul National University Hospital Institutional Review Board, and written informed consent was obtained from all subjects.

Peripheral whole blood was collected in sodium citrate tubes (Becton Dickinson, San Jose, CA) and centrifuged for 15 min at 2000 ×*g* within 1.5 h of sampling. Plasma aliquots were stored at −70°C for measurements of histone−DNA complex, cell-free dsDNA, and neutrophil elastase.

Cell culture

Human monocytic cell lines (U937 and THP-1) and a promyelocytic leukemia cell line (HL-60) were cultured in RPMI 1640 (Welgene, Daegu, Korea)

containing 10% fetal calf serum (FBS; Thermo Fisher Scientific, Waltham, MA) at 37°C with 5% CO_2. Human endothelial cell line EA.hy926 (hEC) was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Welgene) with 10% FBS at 37°C with 5% CO_2. Human umbilical vein endothelial cells (HUVEC) were maintained in EGM-2 BulletKit (Lonza, Basel, Switzerland).

For autonomous proliferation, U937, THP-1, and HL-60 cells at a final concentration of 1×10^6 cells/mL in RPMI 1640 with 10% FBS were cultured without media exchange for 5 days. Cell numbers were counted at 0 h, 6 h, 1 day, and 5 days using a hemocytometer, and the culture supernatants were collected for measurement of ET biomarkers. The ET formation in U937 cells (fresh or cultured for 5 days) was visualized by confocal microscopy.

For experiments with inhibitors, Cl-amidine (Calbiochem, San Diego, CA), aurintricarboxylic acid (ATA; Sigma-Aldrich), quercetin (Sigma-Aldrich), N-acetyl-L-cysteine (NAC; Sigma-Aldrich), or 4-amino-2,4-pyrrolidinedicarboxylic acid (APDC; Sigma-Aldrich) was added into U937 cell suspensions and the cells were then cultured for 5 days at 37°C.

Quantitation of ET biomarkers and ROS activity

The levels ET biomarkers were measured by commercial ELISA kits (Cell Death Detection, Roche Diagnostics; Quant-iT Picogreen dsDNA assay kit, Thermo Fisher Scientific; Human PMN Elastase Platinum, eBioscience, San Diego, CA). Total ROS activity in cell lysates was measured by ELISA with an OxiSelect *In Vitro* ROS/RNS assay kit (Cell Biolabs, San Diego, CA).

Imaging of extracellular traps by confocal microscopy

U937 cells (fresh or cultured for 5 days) were plated on a slide using a cytospin centrifuge. The cells were fixed with 4% paraformaldehyde, stained with SYTOX green (Thermo Fisher Scientific) and mounted with Fluoroshield containing DAPI (ImmunoBioScience Corporation, Mukilteo, WA). Images were acquired on a Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) with a 100× objective and processed by Olympus FluoView (Olympus).

Measurement of surface adhesion molecule expression in endothelial cells

hEC or HUVEC were starved in DMEM with 1% FBS for 1 h, washed and treated with or without calf thymus histone (Roche Diagnostics) for 5 h. The cells were stained with phycoerythrin (PE)-conjugated monoclonal antibody (mAb) against E-selectin, PE-conjugated mAb against ICAM-1, or allophycocyanin-conjugated mAb against VCAM-1 (all from BD Biosciences, Franklin Lakes, NJ). Cells were analyzed using a FACS LSRII flow cytometer (BD Biosciences) and FLOWJO software (TreeStar, San Carlos, CA). In some experiments, four individual human recombinant histones (H1, H2A/H2B, H3.3, H4; New England BioLabs, Ipswich, MA) were used instead of calf thymus histone.

Cell adhesion assay and its inhibition experiments

hEC were starved for 1 h and then incubated with or without 50 μg/mL histone for 5 h. Then, U937 cells (1×10^6 cells/mL) were added onto the hEC layer for 30 min. The non-adherent cells were collected and washed three times. The adherent round U937 cells were enumerated under a light microscope (Olympus).

For neutralizing histone, histone was pre-mixed with 62.5 μg/mL polysialic acid (Sigma-Aldrich), 100 U/mL heparin (Sigma-Aldrich), and 100 nM activated protein C (APC; Haematologic Technologies Inc., Essex Junction, VA) were each pre-mixed with 50 μg/mL histone for 1 h, 10 min, and 30 min, respectively, and the mixtures were then added to hEC. In another experiment, 50 μg/mL anti-E-selectin antibody, 10 μg/mL anti-ICAM-1 antibody, or 30 μg/mL anti-VCAM-1 antibody (all from R&D Systems, Minneapolis, MN) was incubated with 50 μg/mL histone–treated hEC for 10 min and then U937 cells were added. In other experiments, hEC were pre-treated for 1 h with 50 μg/mL isotype-IgG_{2a} control antibody, 50 μg/mL anti-human TLR2, or 50 μg/mL anti-TLR4 antibody (all from eBioscience), or 5 μM TLR9 antagonist (ODN TTAGGG; InvivoGen, San Diego, CA), and then stimulated with histone.

Measurement of leukemic cell survival

U937 cells were suspended at a final concentration of 1×10^6 cells/mL in RPMI 1640 containing 10% FBS and plated on hEC layers pre-treated with 50 μg/mL histone for 1 h. The co-cultured cells were treated with cytosine D–

arabinofuranoside (Ara-C; Hospira Australia Pty Ltd., Mulgrave, Australia) for 24 h or without Ara-C for 48 h. Then adherent and non-adherent U937 cells were collected separately and stained with fluorescein isothiocyanate (FITC)-conjugated mAb against CD45 (BD Biosciences), PE-conjugated mAb against CD105 (BD Biosciences), 7-aminoactinomycin D (*7-AAD;* Beckman Coulter, Fullerton, CA), or isotype control. The cells were analyzed using a FACS LSRII flow cytometer and FLOWJO software.

Statistical analysis

Statistical analyses were conducted using the SPSS statistical package (version 22.0, SPSS Inc., Chicago, IL). The comparisons between two groups were made using *t* test or Chi-square test. Multiple linear regression analyses were used to analyze factors contributing to ET biomarkers and the results were expressed as regression coefficient (β) and standard error (SE). Spearman correlation analyses were used to investigate the correlation of ET biomarkers with peripheral blast count. A $P < 0.05$ was regarded as statistically significant.

RESULTS

Circulating levels of ET markers in patients with hematologic diseases

The baseline characteristics of the study population are shown in Table 1. Three ET markers (histone–DNA complex, cell-free dsDNA, and neutrophil elastase) were measured. The level of the histone–DNA complex was significantly higher in the acute leukemia group (311±402) than in the MPN groups either with or without neutrophilia (118±117, P=0.049 and 53±41, P=0.008, respectively). No significant increase in the histone–DNA complex level was observed in patients with aplastic anemia compared with normal control. The circulating levels of cell-free dsDNA and neutrophil elastase were also highest in the acute leukemia group (Table 1). Among patients with MPN, those with neutrophilia exhibited a higher level of neutrophil elastase than those without neutrophilia. Based on the cut-off values (95 percentile of normal control values), positivity for the histone–DNA complex and cell free dsDNA was highest in the acute leukemia group (81.0% and 71.4%, respectively).

To investigate the factor(s) contributing to the circulating levels of the histone–DNA complex, cell-free dsDNA, and neutrophil elastase, we performed multiple linear regression analysis (Table 2). Peripheral blast count (β=0.495, SE=0.001) was the most significant factor contributing to the histone–DNA complex level; the ANC contribution was also significant

(β=0.313, SE=0.002). Likewise, peripheral blast count (β=0.731, SE<0.001) and ANC (β=0.228, SE=0.001) significantly contributed to the cell-free dsDNA level. ANC (β=0.860, SE=0.002) was the only significant contributing factor for neutrophil elastase. In simple correlation analyses, peripheral blast count was significantly correlated with the levels of the histone–DNA complex and cell-free dsDNA, but not with that of neutrophil elastase (Figure 1B, 1C & 1D). There was a significant correlation between neutrophil elastase and ANC (r=0.510, *P*=0.018).

Table 1. The baseline characteristics and laboratory results of the study populations

	Normal control (n=40)	Acute leukemia (n=21)	MPN with neutrophilia (n=13)	MPN without neutrophilia (n=32)	Aplastic anemia (n=14)
Age (years)	41±10	46±16	60±15*	55±18*	41±22
Male/Female	19/21	15/6	5/8	16/16	7/7
Hemoglobin (g/dL)		8.6±2.1	14.1±4.4	12.9±2.4	8.5±2.3
WBC ($x10^9$/L)		43.16±51.85	16.32±3.79	7.48±2.4	2.62±0.98
ANC ($x10^9$/L)		5.11±10.44	12.39±3.27	4.82±1.66	0.98±0.53
Platelets ($x10^9$/L)		47±37	731±270	706±364	24±15
PT (sec)		13.5±2.0	13.2±1.9	12±1.3	11.1±1.3
aPTT (sec)	33.8±2.3	32±4.4	38.7±5.1	35.9±3.8	31.8±5.0
Antithrombin (%)	96±21	87±17	80±18	86±14	107±19
Fibrinogen (mg/dL)	226±51	309±125	244±68	250±82	280±132
PB blast count ($x10^9$/L)	0±0	28.02±40.54	0±0	0±0	0±0
Histone-DNA complex					
Mean±SD (AU)	30±20	311±402**	118±117*	53±41*	18±18
Positivity[a] (n)	2 (5.0%)	17 (81.0%)***	9 (69.2%)**	9 (28.1%)*	0 (0%)
Cell free dsDNA					
Mean±SD (ng/mL)	62.7±11.7	121.5±62.1***	86.2± 25.5**	75.0± 14.4**	79.1±19.5**
Positivity[a] (n)	2 (5.0%)	15 (71.4%)***	7 (53.8%)**	6 (18.8%)	6 (42.9%)**
Neutrophil elastase					
Mean±SD (ng/mL)	26.1±13.9	126.4±275.9	100.6± 118.0*	45.0± 27.3*	20.2±10.2
Positivity[a] (n)	2 (5.0%)	6 (28.6%)*	4 (30.8%)*	4 (12.5%)	0 (0%)

Acute leukemia groups (n=21) includes acute myeloid leukemia (n=14), acute lymphoblastic leukemia (n=6) and mixed phenotype acute leukemia (n=1)

Values are presented as the mean ± standard deviation or number of subjects. [a]Positivity was defined when the circulating levels of histone-DNA complex, cell free dsDNA and neutrophil elastase were above the upper normal cutoff of 69 AU, 84.2 ng/mL and 62.7 ng/mL, respectively. $^{*}P<0.05$, $^{**}P<0.001$ vs normal control (*t* test for comparisons of mean values and Chi-square test for comparisons of positivity).

Abbreviations: MPN, myeloproliferative neoplasms; ANC; absolute neutrophil count; PT, prothrombin time; aPTT, activated partial prothrombin time; PB, peripheral blood.

Table 2. Multiple regression analyses for determination of contributing factors to histone-DNA complex, cell free dsDNA and neutrophil elastase levels in patients

	Histone-DNA complex	Cell free dsDNA	Neutrophil elastase
Modified R^2	0.711	0.495	0.677
Age	0.005 (0.836)	-0.001 (0.185)	-0.082 (0.566)
Hemoglobin	-0.120 (5.306)	-0.104 (1.175)	-0.120 (3.595)
ANC	0.313 (0.002)**	0.228 (0.001)*	0.860 (0.002)**
Platelets	0.049 (0.045)	-0.075 (0.010)	-0.153 (0.031)
PT	-0.003 (10.33)	0.116 (2.287)	0.015 (7.000)
Antithrombin	0.122 (0.965)	0.144 (0.214)	0.046 (0.654)
PB blast count	0.495 (0.001)**	0.731 (<0.001)**	-0.091 (0.001)

Values are expressed in regression coefficients β (standard error). *P <0.05, **P <0.001.
Abbreviations: ANC; absolute neutrophil count; PT, prothrombin time; PB, peripheral blood; dsDNA, double stranded DNA.

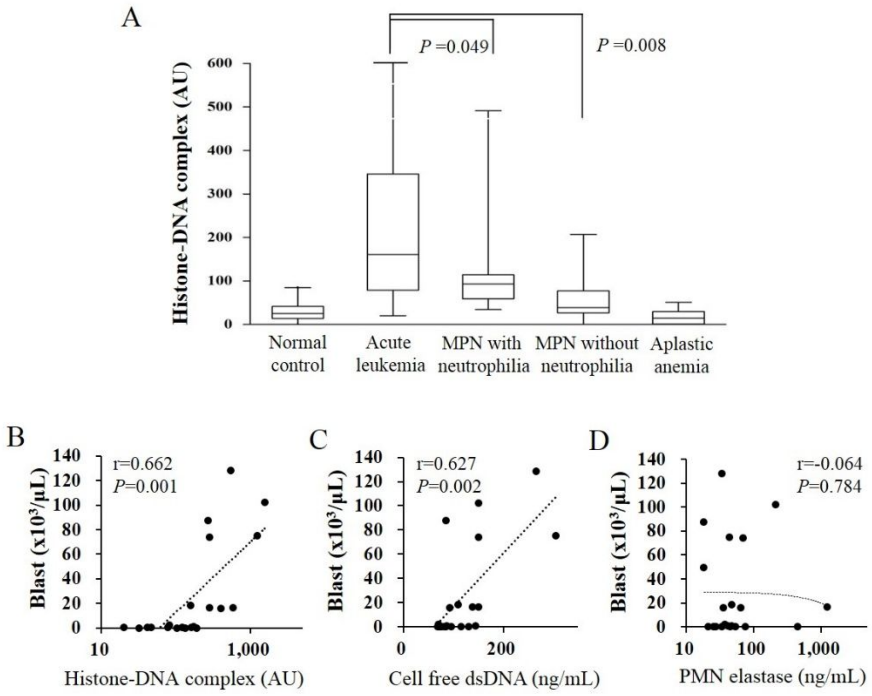


Figure. 1. Circulating histone levels are increased in patients with acute leukemia and correlate with peripheral blast counts. (A) Circulating levels of the histone–DNA complex in normal controls (n=40) and in patients with acute leukemia (n=21), myeloproliferative neoplasms (MPN) with neutrophilia (n=13), MPN without neutrophilia (n=32), and aplastic anemia (n=14). (B, C, D) Correlation of peripheral blast count with circulating levels of histone–DNA complex, cell-free double-stranded DNA (dsDNA), and neutrophil elastase in acute leukemia patients.

Extracellular histone is released from leukemic cells

Since peripheral blast count was the most significant contributor to the histone–DNA complex level, it seemed plausible that leukemic blast cells may release the histone–DNA complex into the circulation. To test whether these cells release the histone–DNA complex *in vitro*, we cultured three leukemic cell lines (U937, THP-1, and HL-60) for 5 days without media exchange. The cell number gradually increased (Figure 2A) and the levels of the histone–DNA complex and cell-free dsDNA in the culture supernatant also gradually increased (Figure 2B & 2C). Neutrophil elastase levels in all culture supernatants were less than 0.1 ng/mL (data not shown). To visualize the ET formation, we stained fresh U937 cells and U937 cells cultured for 5 days with SYTOX green and DAPI (Figure 2D). Fresh cells had normal intact round nuclei, whereas those cultured for 5 days showed irregular extracellular dsDNA structures stained with both SYTOX green and DAPI.

We investigated whether ROS production in leukemic cells may induce the release of the histone–DNA complex. As expected, U937 cells cultured for 5 days showed a significantly higher intracellular ROS production than the fresh ones (Figure 2E). Cl-amidine, an inhibitor of peptidylarginine deiminase that induces nuclear chromatin decondensation during ET formation, significantly blocked the histone–DNA complex release (Figure 2F). In addition, four antioxidants (ATA, quercetin, NAC, and APDC) also significantly blocked it to various degrees.

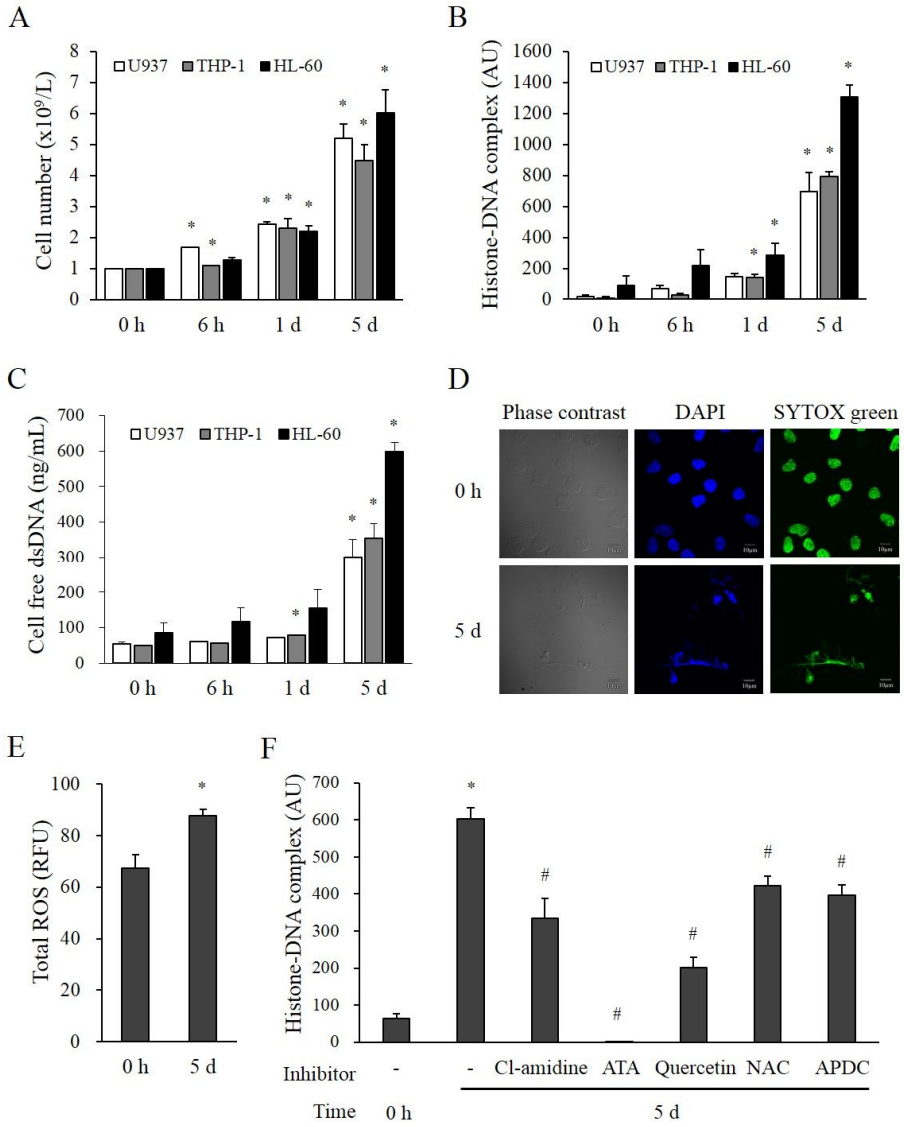


Figure 2. Extracellular traps are released from leukemic cells. Three leukemic cell lines (U937, THP-1, HL-60) were cultured in DMEM with 10% fetal calf serum without medium exchange for 5 days. (A) The numbers of autonomously proliferated cells are expressed as mean ± SEM of 4 experiments. (B, C) The levels of the histone–DNA complex and cell-free dsDNA were measured in the corresponding culture supernatants. (D) U937 cells (fresh or cultured for 5 days) were stained with SYTOX green and DAPI and observed using a confocal laser microscope. Phase contrast images (left),

DAPI images (middle), and SYTOX green images (right) are shown are (representative of 3 independent experiments). (E) Total ROS activity was measured in lysates of U937 cells prepared before and after culturing for 5 days (mean±SEM of 3 experiments). (F) U937 cells were pretreated with an inhibitor of extracellular trap formation (200 μM Cl-amidine) or with antioxidants (ATA 100 μM, quercetin 100 μM, NAC 50 μM, or APDC 100 μM) and the histone–DNA complex levels were measured in the supernatants after 5 day-culture. $^{*}P < 0.05$ versus the same leukemic cell line at 0 h. $^{\#}P < 0.05$ versus no inhibitor.

Histone induces the expression of adhesion molecules on endothelial cell surface

To explore whether circulating histones activate endothelial cells, we measured the surface expression of endothelial adhesion molecules using flow cytometry. Histone increased the surface expression of all 3 adhesion molecules examined (E-selectin, ICAM-1, and VCAM-1) in a dose-dependent manner (Figure 3A, 3D & 3G, respectively). Among individual histones, H3.3 and H4 significantly increased adhesion molecule expression, whereas H1 and H2A/H2B did not (Figure 3C, 3F & 3I). Histone also increased the expression of these adhesion molecules on the surface of HUVEC (Figure 4).

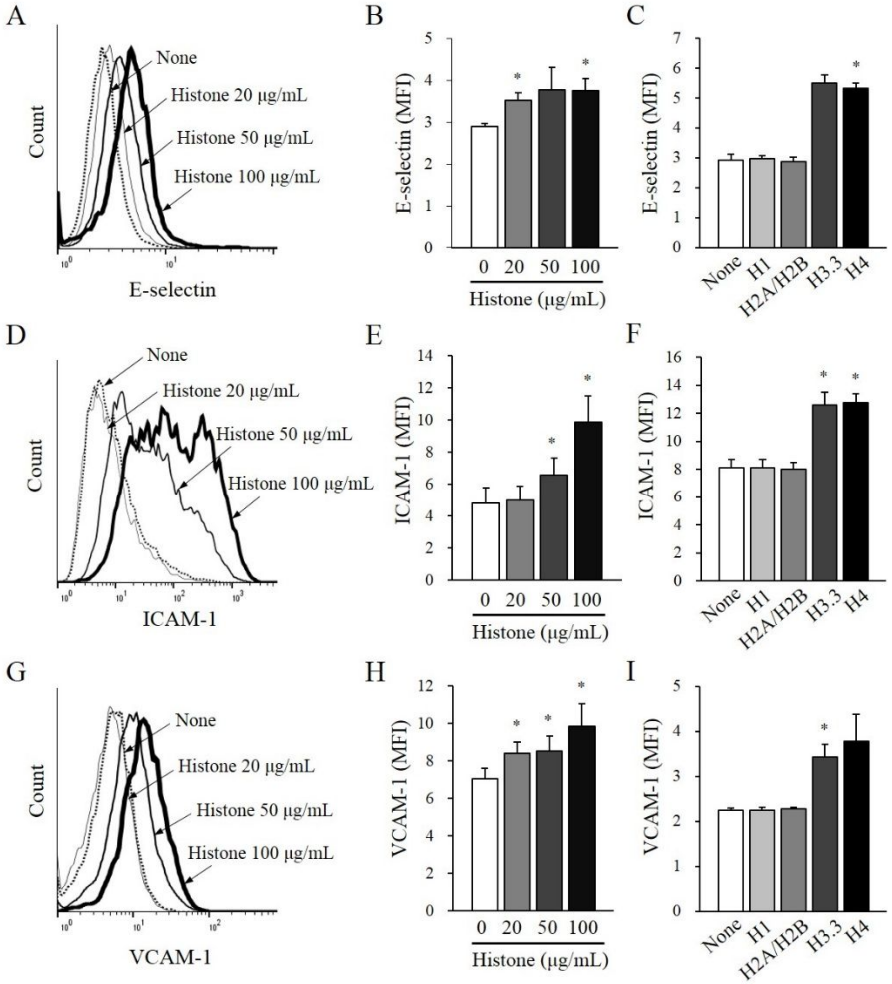


Figure 3. Histone induces surface expression of endothelial adhesion molecules. Endothelial cells (EA.hy926) were cultured with various concentrations of calf thymus histone or 20 μg/mL individual human recombinant histones (H1, H2A/H2B, H3.3, or H4) for 5 h and then the surface expressions of (A, B, C) E-selectin, (D, E, F) ICAM-1, and (G, H, I) VCAM-1 were determined by flow cytometry. Histograms are representative of four independent experiments. The bar graphs indicate mean fluorescence intensity (MFI) of each adhesion molecule expression ± SEM of 4 experiments. $^{*}P < 0.05$ versus no histone addition.

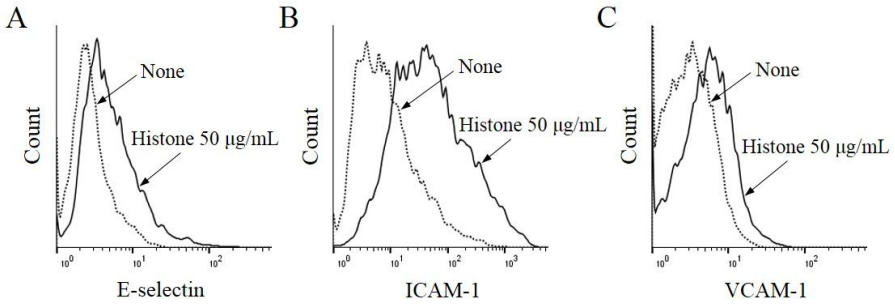


Figure 4. Histone induces adhesion molecules on human umbilical vein endothelial cells (HUVEC). HUVEC were treated with 50 µg/mL calf thymus histone for 5 h and then the surface expression of E-selectin, ICAM-1, and VCAM-1 was determined by flow cytometry. Histograms are representative of three independent experiments.

Histone increases leukemic cell adhesion to endothelial cells

Since histones enhanced the expression of adhesion molecules on endothelial cell surface, we investigated whether leukemic cells are more likely to adhere to histone-treated endothelial cells than to the untreated ones. As expected, the adhesion of U937 cells was significantly enhanced by histone treatment of endothelial cells (Figure 5A & 5B).

Since polysialic acid and heparin bind tightly to histones and APC cleaves histones into fragments [13-15], we investigated whether these histone inhibitors would reverse the histone effect. Histone pre-incubated with polysialic acid, heparin, or APC failed to increase leukemic cell adhesion when used to treat endothelial cells (Figure 5A & 5B). Accordingly, polysialic acid, heparin, and APC inhibited (albeit partially) adhesion molecule expression on endothelial cells (Figure 6A). Neutralizing antibodies against the 3 adhesion molecules (anti-E-selectin, anti-ICAM-1, and anti-VCAM-1) significantly inhibited the leukemic cell adhesion to histone-treated endothelial cells (Figure 7A).

Endothelial cells expressed variable amounts of TLR2, TLR4, and TLR9 on their surface (Figure 8). To investigate which receptor is involved in the histone-induced increase in endothelial adhesion molecule expression, we pre-incubated endothelial cells with neutralizing antibodies against TLR2, TLR4, or TLR9 before histone stimulation. Anti-TLR2 and anti-TLR4 failed to block the histone-induced increase in endothelial adhesion, whereas anti-TLR9 almost completely blocked it (Figure 7B). Accordingly, the histone-induced

expression of adhesion molecules was inhibited by anti-TLR9, but not by anti-TLR2 or anti-TLR4 (Figure 6B), indicating that TLR9 is involved in the histone-induced induction of endothelial adhesion molecules.

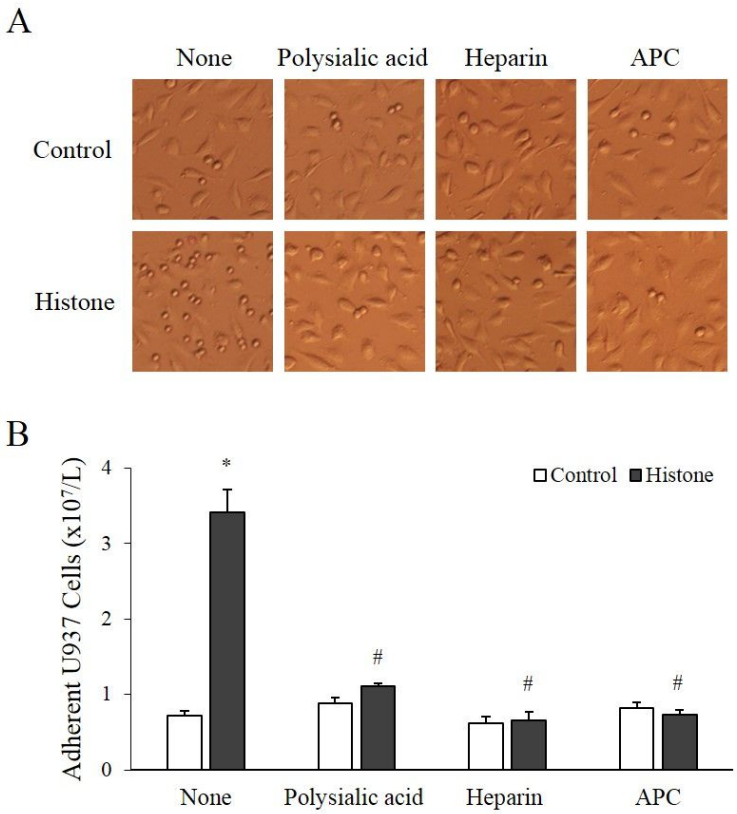


Figure 5. Histone increases leukemic cell adhesion to endothelial cells (EA.hy926; hEC). (A) Calf thymus histone (50 μg/mL) was pre-mixed with 62.5 μg/mL polysialic acid, 100 U/mL heparin, or 100 nM activated protein C (APC) for 1 h, 10 min, and 30 min, respectively, and then added to hEC for 5 h. Then, U937 cells were added onto histone-treated or untreated hEC layers for 30 min. After washing, the adherent small round U937 cells on hEC layers were counted under a microscope (per ×100 field). (B) The numbers of adherent U937 cells are shown as mean ± SEM of 4 experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus histone-treated.

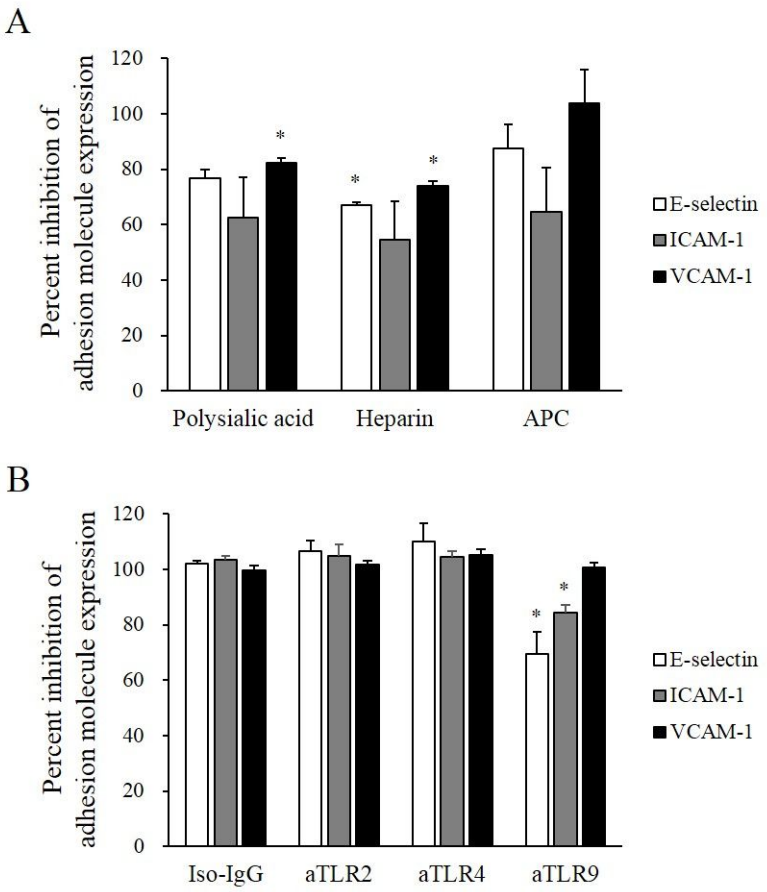


Figure 6. Effects of histone inhibitors and antibodies against Toll-like receptor (TLR)2, TLR4, and TLR9 on histone-induced endothelial adhesion molecule expression. (A) Calf thymus histone (50 μg/mL) was pre-mixed with polysialic acid, heparin, or activated protein C (APC) for 1 h, 10 min and 30 min, respectively. The mixtures were then added to endothelial cells (EA.hy926; hEC) for 5 h and the surface expression of E-selectin, ICAM-1, and VCAM-1 was determined by flow cytometry. The percent inhibition of mean fluorescence intensity of each adhesion molecule compared to no inhibitor addition is shown as mean ± SEM of 4 experiments. (B) Antibodies against Toll-like receptor (TLR)2, TLR4, and TLR9 were

incubated with hEC before histone stimulation. The above surface staining was then performed. $^{*}P < 0.05$ versus no inhibitor addition (A) or isotype control (B).

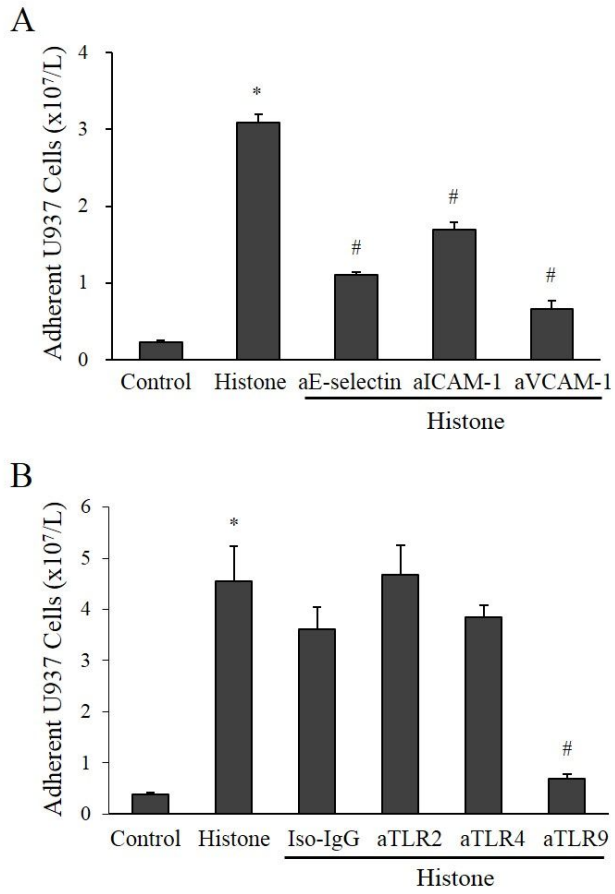


Figure 7. Neutralizing antibodies against adhesion molecules and a Toll-like receptor (TLR)9 antagonist inhibit leukemic cell adhesion to histone-treated endothelial cells (EA.hy926; hEC). (A) Histone-treated hEC were treated with neutralizing antibodies against adhesion molecules (50 μg/mL anti-E-selectin, 10 μg/mL anti-ICAM-1, or 30 μg/mL anti-VCAM-1) for 10 min. Then U937 cells were added and incubated for 30 min, the adherent U937 cells were counted. (B) Antagonists of TLR2 (50 μg/mL), TLR4 (50 μg/mL) and TLR9 (50 μM) were pre-incubated with hEC before histone stimulation and the adhesion assays were then performed. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus histone-treated.

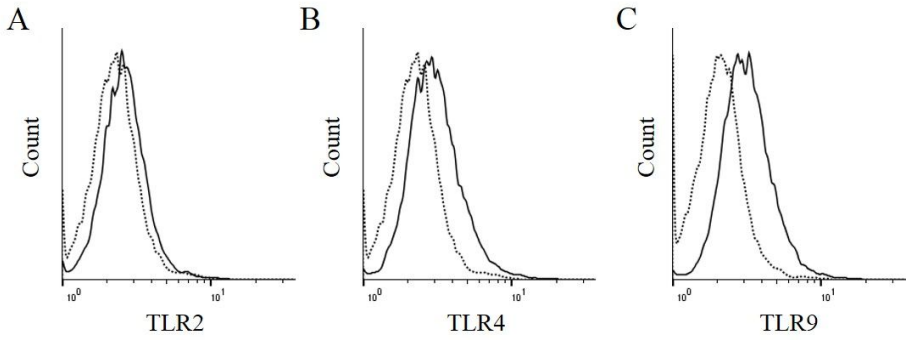


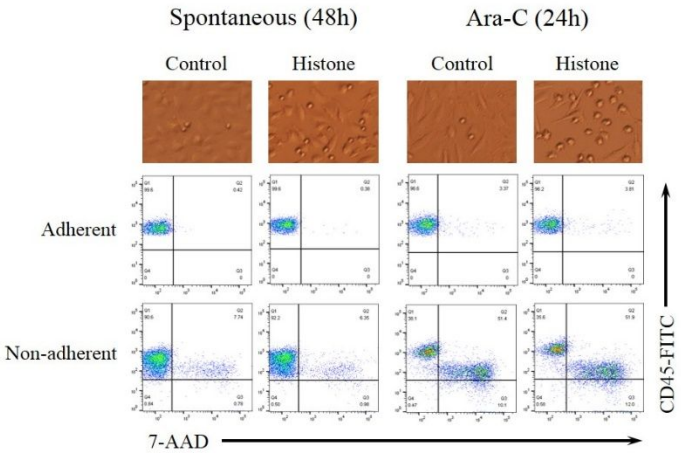
Figure 8. Surface expression of Toll-like receptor (TLR)2, TLR4, and TLR9 on endothelial cells (EA.hy926). The expression of TLRs measured by flow cytometry. Histograms are representative of three independent experiments.

Leukemic cells are protected from cell death by their adhesion to histone-treated endothelial cells

To investigate whether spontaneous cell death differed between adherent and non-adherent U937 cells, U937 cells were plated on histone-treated endothelial cells and cultured under serum-deprived conditions for 48 h. Then the adherent and non-adherent U937 cells were collected separately and stained with antibodies against CD45 and CD105, and with 7-AAD. After removal of CD105-positive endothelial cells, both CD45-positive and 7-AAD-negative cells (surviving U937 cells) were counted. When U937 cells were layered onto untreated endothelial cells, 92.1±0.8% of adherent cells survived, which was significantly higher than 86.8±1.5% for non-adherent cells (Figure 6). Likewise, the proportion of surviving U937 cells layered onto histone-treated endothelial cells was significantly higher among the adherent cells (91.9±1.1%) than among non-adherent cells (89.3±1.1%). Since the number of adherent U937 cells was higher when they were layered on histone-treated than on untreated endothelial cells, the absolute number of total surviving U937 cells (adherent+non-adherent) was also higher when histone-treated endothelial cells were used (87.6±7.4 × 10^6/L) than in the case of untreated endothelial cells (36.0±5.3 × 10^6/L).

For evaluation of chemotherapy-induced cell death, Ara-C, a commonly used chemotherapeutic agent, was added for 24 h after U937 adhesion to histone-treated or untreated endothelial cells. Similar to the results for spontaneous cell death, the number of surviving U937 cells was significantly

higher among adherent cells than among non-adherent cells, and the number of adherent cells was higher when histone-treated endothelial cells were used than in the case of untreated ones (Figure 9). As a result, the total number of surviving U937 cells was significantly higher when they were layered on histone-treated than on untreated endothelial cells.



Total adherent cell count (x10^6/L)	0.367±0.058	0.924±0.080	0.054±0.003	0.437±0.011
7AAD negative adherent cells (%)	92.1±0.8	91.9±1.1	67.7±2.0	65.5±2.8
Total surviving adherent cells (x10^6/L)	33.8±5.3	84.8*±7.2	3.6±0.2	28.7*±1.7
Total non-adherent cell count (x10^6/L)	2.5±0.3	3.1±0.3	1.2±0.2	1.1±0.1
7AAD negative non-adherent cells (%)	86.8±1.5	89.3±1.1	25.8±1.3	24.2±0.8
Total surviving non-adherent cells (x10^6/L)	2.2±0.3	2.8±0.3	0.256±0.008	0.262±0.010
Total surviving cells (x10^6/L)	**36.0±5.3**	**87.6*±7.4**	**3.9±0.2**	**29.0*±1.7**

Figure 9. Survival of leukemic cells is increased by their adhesion to histone-treated endothelial cells (EA.hy926). Representative adhesion assay images and flow cytometry plots of CD45 and 7-AAD staining of adherent and non-adherent U937 cells in experiments on spontaneous cell death (48 h) and Ara-C-induced cell death (24 h. Total surviving cells are the sum of total surviving adherent cells and total surviving non-adherent cells. $^*P < 0.05$ versus histone-treated.

DISCUSSTION

We demonstrated that the circulating histone level was elevated in patients with acute leukemia, that leukemic cells released extracellular histone *in vitro*, and that this release was blocked by antioxidants. Extracellular histone induced the surface expression of endothelial adhesion molecules and thus promoted the endothelial adhesion of leukemic cells, which protected them from spontaneous or chemotherapy-induced cell death.

Elevated circulating histone levels were reported in various inflammatory, autoimmune, and thrombotic disorders [6,7], but not for leukemia. Our study found a high histone level in acute leukemia. Interestingly, the circulating histone level was strongly correlated with the peripheral leukemic blast count. In multiple linear regression analysis, peripheral blast count was the strongest contributor to the histone–DNA complex level. These findings suggest that blast cells are a major source of circulating histone in acute leukemia.

We further investigated whether leukemic cells release the ET *in vitro*. Three leukemic cell lines produced considerable amounts of the histone–DNA complex during culture. Similar to our data, other reports also showed that cultured leukemic cell lines formed ET under certain conditions [3,16]. However, the detailed mechanism of ET formation is not known. Since it has been reported that ROS are a potent ET inducer and leukemic cells over-produce ROS during proliferation [4,16,17], ROS may potentially cause ET

formation by leukemic cells. As expected, our results showed that U937 cells cultured for 5 days produced high levels of intracellular ROS and that the ET formation was significantly inhibited by ROS inhibitors, suggesting that ROS production during leukemic cell proliferation is an important cause of ET formation.

In our study, patients with MPN and neutrophilia had higher levels of the ET markers than patients with MPN without neutrophilia, and ANC was a significant contributor to the ET markers, suggesting that neutrophils are also a source of circulating histone. Among the ET markers, neutrophil elastase, which is secreted from neutrophils, was not correlated with leukemic blast count and was not found in leukemic cell culture supernatants. This finding suggests that neutrophil elastase originated from neutrophils, not from leukemic cells.

Endothelial cells establish a vascular niche that facilitates leukemic cell growth and drug resistance [18]. Several lines of evidence indicate that endothelial activation is essential for leukemia progression [9-11]. Hence, this study focused on the effect of histone on endothelial activation. Histone dose-dependently induced the surface expression of 3 endothelial adhesion molecules, which facilitated leukemic cell adhesion to endothelial cells. The adhesion molecules are usually induced by inflammatory stimuli such as endotoxin [8]. Histone is also known as a strong inflammatory stimulus and its potential receptors have been suggested to be TLR2, TLR4, and TLR9 [5,19]. In our study, TLR2 and TLR4 antagonists did not block the effect of

histone, but a TLR9 antagonist did, suggesting that histone induces endothelial activation mainly via TLR9.

To verify whether leukemic cells adhere to endothelial cells through adhesion molecules, we treated histone-induced endothelial cells with neutralizing antibodies against E-selectin, ICAM-1, and VCAM-1. As expected, the 3 antibodies significantly blocked the adhesion, indicating that it is mediated by adhesion molecules. Histone-neutralizing (polysialic acid and heparin) and a histone-degrading (APC) substances almost completely blocked the effect of histone, confirming that the histone used was pure and contained no endotoxin. Especially, percent inhibition of VCAM-1 expression nearly was not differences between using neutralizing antibodies against TLR2, TLR4, and TLR9 antagonist, it is supposed to VCAM-1 related to extravasation not adhesion.

When cultured *in vitro*, leukemic cells gradually become apoptotic [20]. Our study demonstrated that leukemic cells adhered to endothelial cells survived better than non-adherent cells; this was true for both spontaneous and chemotherapy-induced cell death, indicating that adhesion is an important survival factor. Our data therefore suggest that the histone-induced adhesion molecules on endothelial cells and increased leukemic cell adhesion protect leukemic cells from spontaneous and chemotherapy-induced death.

Although other stimuli such as direct contact of leukemic cells with soluble angiogenic cytokines have been reported to induce leukemic cell adhesion to endothelial cells [9-11], our data demonstrate for the first time

that another autocrine stimulus, histone, could be released from leukemic cells and promote their adhesion to endothelial cells by inducing endothelial adhesion molecules. These effects would eventually promote leukemia progression through the rescue of leukemic cells from spontaneous and chemotherapy-induced cell death.

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논 문 초 록

유 현 주

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서론: 백혈구는 활성산소 (Reactive oxygen species, ROS)에 의해 자극될 때 핵구성물이 세포외 공간으로 나와 세포외기질 (Extracellular trap, ET)을 형성한다. 핵구성물의 주요성분은 히스톤-DNA 복합체와 엘라스타제 효소이다. 본 연구는 백혈병 세포에서 ET 분비여부, 분비된 히스톤이 내피세포 활성과 백혈병의 진행에 미치는 영향을 탐색하였다.

방법: 80명의 혈액질환 환자와 40명의 정상인에서 혈중 ET 지표들 (히스톤-DNA 복합체, cell-free dsDNA 및 호중구 엘라스타제)을 효소면역측정법으로 측정하였다. ET 형성은 동일초점현미경으로 조명하였고, 백혈병세포에서 생성되는 ROS 농도는 효소면역측정법으로 측정하였다. 히스톤 처리 내피세포의 부착분자 (adhesion molecule)와 생존율은 유세포분석법으로 측정하였다.

결과: 급성백혈병에서 3가지 ET 지표가 모두 높았고, 모세포 (blast) 비율과 상관관계가 있었다. 백혈병세포가 배양되면 ROS 생성이 증가되고, ROS 억제제에 의해서 히스톤 분비가 억제되었다. 또한 히스톤은 내피세포 3개의 부착분자 발현을 증가시켰고, 백혈병세포의 내피세포로 부착도 증가되었다. 히스톤 억제제

(헤파린, polysialic acid 및 activated protein C), 부착분자에 대한 중화항체, 그리고 Toll like receptor9 억제제가 상기 부착과정을 억제하였다. 히스톤 처리 내피세포는 백혈병세포와 함께 배양했을 때, 히스톤 처리 하지 않은 내피세포보다 백혈병세포를 약 2배이상 많이 생존시켰고, arabinofuranoside (Ara-C)를 처리한 상태에서도 약 7배이상 생존을 증가시켰다.

결론: 본 연구는 세포외히스톤이 ROS 의존적 기작을 통해 백혈병세포로부터 분비된다는 것을 처음으로 증명하였다. 분비된 히스톤은 내피세포 부착분자의 발현을 유도하여 백혈병세포와 내피세포의 부착을 촉진하고, 결과적으로 백혈병세포의 사멸을 억제한다.

주요어: 세포외기질, 히스톤, 활성산소, 백혈병세포, 내피세포 부착분자

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