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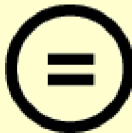
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Cancer cell–induced neutrophil extracellular traps promote both hypercoagulability and cancer progression

암세포 유도 호중구세포외기질에 의한 응고항진성과 암진행성의 촉진

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ABSTRACT

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Introduction: Neutrophils can generate net-like structures of their DNA-histones complex, which is called neutrophil extracellular traps (NET), under various stimuli. Neutrophils and NET formation are abundant in tumor tissue. This study investigated how cancer cell induced NET formation and whether the resulting NET formation promoted plasma thrombin generation and cancer progression.

Methods: Pancreatic cancer cell line (AsPC-1) induced NET formation was measured by the histone-DNA complex level. The endogenous thrombin potential (ETP) was measured by thrombin generation assay. *In vitro* migration and invasion assays and tubule formation were performed. The circulating levels of NET markers and hypercoagulability markers were assessed in 62 patients with pancreatobiliary malignancy and 30 healthy controls.

Result: AsPC-1 significantly induced NET formation in a dose-dependent manner. Conditioned medium (CM) from AsPC-1 also induced NET. Interestingly the NET-formation was abolished by heat-inactivated CM, not by lipid-extracted CM, suggesting important role of protein components. Reactive oxygen species (ROS) inhibitor did not inhibit the cancer cell-induced NET formation, but prostaglandin E1 (PGE1, cyclic adenosine

monophosphate inducer) and antithrombin inhibited. NET significantly promoted ETP of normal plasma. Of note NET promoted cancer cell migration and invasion and angiogenesis, which was inhibited by histone-binding agents (heparin, polysialic acid), DNA degrading enzyme and toll-like receptor neutralizing antibodies. In patients with pancreatobiliary malignancy, the elevated NET markers well correlated with the hypercoagulability makers.

Conclusion: Our findings implicate cancer cell-induced NET formation plays an important role in enhancements of both hypercoagulability and cancer progression, suggesting inhibitory agents of NET formation such as PGE1 and antithrombin can be potential therapeutics to reduce both hypercoagulability and cancer progression.

Keywords: Neutrophil extracellular traps (NET), Cancer cell, Hypercoagulability, Cancer progression, Prostaglandin E1, Antithrombin

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CONTENTS

ABSTRACT --i

CONTENTS --iii

LIST OF TABLES AND FIGURES ------------------------------------

i v

ABBREVIATIONS ---v

INTRODUCTION ---1

MATERIALS AND METHODS---3

RESULTS ---8

DISCUSSION ---24

REFERENCES --31

ABSTRACT IN KOREA --37

LIST OF TABLES AND FIGURES

Figure 1. Pancreatic cancer cell AsPC-1 induces neutrophil extracellular traps.

Figure 2. AsPC-1–induced NET formation is not ROS-dependent but cyclic AMP and thrombin-dependent process.

Figure 3. NET increases thrombin generation in normal plasma.

Figure 4. NET promotes migration and invasion of pancreatic cancer cell.

Figure 5. NET promotes *in vitro* endothelial cell angiogenesis.

Figure 6. The circulating levels of NET and hypercoagulability markers is increased in patients with pancreatobiliary malignancy.

Figure 7. The circulating levels of NET and hypercoagulability markers tend to increase in relation to cancer stage of pancreatobiliary malignancy.

Figure 8. Potential mechanism that cancer cell-induced NET formation promotes both hypercoagulability and cancer progression.

ABBREVIATIONS

Abbreviations	Full name
NET	Neutrophil extracellular traps
ETP	Endogenous thrombin potential
CM	Conditioned medium
ROS	Reactive oxygen species
PGE1	Prostaglandin E1
cAMP	Cyclic adenosine monophosphate
PSA	Polysialic acid
TLR	Toll-like receptor
MMP9	Matrix metalloproteinase 9
dsDNA	Double-stranded DNA
FBS	Fetal bovine serum
PMA	Phorbol 12-myristate-13-acetate
DPI	Diphenyleneiodonium
Anti-TF	Monoclonal antibody against human tissue factor
HPF	High power field
AU	Arbitrary unit
HMGB1	High mobility group box 1

INTRODUCTION

In response to various stimuli such as pathogens and inflammatory cytokines, neutrophils release net-like structures of their DNA-histones complex and antimicrobial peptides, which is called neutrophil extracellular traps (NET) [1-4]. Reactive oxygen species (ROS) mediate some forms of NET formation [5]. The NET plays a role in immune protection through killing pathogens, but it can exert detrimental effects on thrombotic and inflammatory diseases [6].

Neutrophils and NET abundantly exit in tumor tissue [1]. It has been reported that malignant neutrophils were prone to NET formation and that cancer cell and cancer cell-primed platelets could also induce NET formation [7, 8]. However, it remains to be investigated how cancer cell induces NET formation.

NET can promote thrombosis in multiple ways [9]. NET binds to platelets, activate coagulation system and inhibits activation of anticoagulant system and fibrinolysis [9]. Since neutrophils and NET are abundant in tumor tissue, the NET has sparked much interest in tumor-associated thrombosis [1]. In mice, tumor injection induced NET formation and lung thrombosis [10] and NET formation occurred concomitant with the thrombosis appearance in tumor-bearing mice [8]. Cancer is often accompanied by hypercoagulability that is an abnormal state of blood coagulation to increase thrombosis risk [11]. Among hypercoagulability markers, circulating microparticle is considered to be a potent procoagulant and biomarker of thrombosis in cancer [12].

Endogenous thrombin potential (ETP) represents total thrombin amount in human plasma stimulated by tissue factor by using thrombin generation assay and is a sensitive marker of hypercoagulability [13, 14]. Until now it is unclear how the NET influence thrombin generation in cancer.

NET has been reported to influence tumor metastasis [1, 15]. NET was associated with poor prognosis of cancer patients and the soluble mediators from NET such as neutrophil elastase and MMP9 promoted tumor cell growth [1, 16]. However, the detail mechanism of NET-induced tumor progression including migration and angiogenesis needs to be clarified.

Pancreatic cancer not only shows high metastasis potential [17], but it also poses a serious risk of cancer thrombosis [18], This study hypothesized that pancreatic cancer cell by itself induces NET formation and the resulting NET induces both hypercoagulability and tumor progression. We investigated how pancreatic cell line induced NET formation and also whether NET promoted plasma thrombin generation. Furthermore, NET-induced cancer cell migration, invasion and angiogenesis were investigated. Finally, the circulating levels of NET markers (histone-DNA complex, cell free dsDNA) and hypercoagulability markers (microparticle, ETP) were measured in patients with pancreatobiliary cancer to assess the relationship of NET with hypercoagulability.

MATERIALS AND METHODS

Cell culture

The human pancreatic carcinoma cell line (AsPC-1) and human endothelial cell line (EA.hy926) were cultured in RPMI 1640 (WelGENE, Seoul, South Korea) and DMEM medium (WelGENE) with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), respectively.

Measurement of AsPC-1-induced NET formation and ROS activity

AsPC-1 cells were incubated with peripheral whole bloods from healthy volunteers under informed consents for 30 min, 1 hr and 2 hr at 37°C and then the supernatants were collected by centrifugation at 3,000×g for 10 min. The histone-DNA complex levels that reflect the extents of NET formation were measured by using Cell Death Detection ELISA kit (Roche Diagnostics, Indianapolis, IN, USA). For investigation of dose-dependency, various concentrations (0, 2.5×10^4 and 10×10^4 cells/μL) of AsPC-1 cells were incubated for 2 hr at 37°C. Phorbol 12-myristate-13-acetate (PMA, 25 nM, Sigma-Aldrich, St. Louis, MO, USA) were used as a positive control of NET formation.

Isolated neutrophils were prepared according to the previous method [19]. Briefly, after erythrocyte aggregation and sedimentation with hydroxyethyl starch (Pentaspan, Jeil Pharmaceutical, Korea) in peripheral whole blood, Ficoll-Paque with density of 1.077 (Sigma-Aldrich) was treated to separate the mononuclear cells from the neutrophils. The purity of the isolated neutrophils was confirmed to be > 85% of total cells through flow

cytometric analysis by using anti-CD33 antibody (Becton Dickinson, Franklin Lakes, NJ).

Conditioned medium (CM) was prepared with the supernatant obtained by centrifugation at 12,000×g for 10 min after AsPC-1 cell was cultured in RPMI 1640 with 1% FBS (1% FBS RPMI) for 36 hr. To eliminate lipid and protein components from CM, the lipid components were extracted by adding 1.5% charcoal (Sigma-Aldrich) at 4℃ for 24 hr and the protein components were destroyed by heat-treatment of CM at 65℃ for 1 hr.

Inhibitors used in experiment, diphenyleneiodonium (DPI, 20 μM; Tocris Bioscience, Bristol, UK), prostaglandin E1 (PGE1, 1 μg/mL; Mitsubishi Tanabe Pharma Korea, Korea), antithrombin (5 IU/mL, SK plasma, Korea) and monoclonal antibody against human tissue factor (anti-TF, 30μg/mL; clone VD8, American Diagnostica Inc., CT, USA) were pre-incubated with peripheral whole bloods before adding AsPC-1 cells.

The ROS activity levels were measured in 45 min by using an OxiSelect *In Vitro* ROS/RNS assay kit (Cell Biolabs, San Diego, CA).

Preparation of NET

The above isolated human neutrophils (2.5×10^5 cells) suspended in 1% FBS RPMI were treated with 25nM PMA for 1 hr at 37°C and were washed through centrifugation (1,800×g, 10 min). NET was prepared through suspension of the pellets with 1% FBS RPMI and was adjusted to 300 mg/dL of the protein level.

Thrombin generation assay

Thrombin generation assay was performed in commercial normal plasma (Pool Norm; Diagnostica Stago, Asnieres, France) with or without NET addition according to the previous report [20]. Briefly, 80 μL normal plasma pre-mixed with 20 μL NET or 1% FBS RPMI (control) were stimulated with 3 different conditions (5 pM, 1 pM and 0.5 pM TF, American Diagnostica). After 20 μL substrate (FluCa-Kit, Thrombinoscope BV, Maastricht, Netherlands) addition, the thrombin generation amounts were measured using Thrombinoscope software (Thrombinoscope BV).

Migration and invasion assay

For migration assay, AsPC-1 cells were loaded onto the upper chamber of cell culture insert with 8 μM pore size (Costar, Corning Incorporated, NY, USA) and the lower chamber were supplemented with intact isolated neutrophil (2.5×10^5 cells) or NET (300 mg/dL of protein). After 22 hr incubation at 37°C, migrated cells were fixed and stained with Diff-Quik kit (Sysmex Co., Kobe, Japan) and were counted under an optical microscope at 400× magnification.

For NET inhibitors, NET were pre-treated with heparin (200 IU/mL, Sigma-Aldrich) for 20 min at room temperature, polysialic acid (PSA, 62.5 μg/mL, Sigma-Aldrich) for 1 h at 37°C or DNase I (50 IU/m; Washington Biochemical Co., NJ, USA) for 20 min at 37°C. Neutralizing antibodies of toll-like receptors (TLRs) were pre-treated with AsPC-1 for 1 h at room temperature by adding anti-TLR2 (aTLR2, 50 μg/mL; eBioscience, San Diego,

CA) and anti-TLR4 antibody (aTLR4, 50 μg/mL, eBioscience) and a monoclonal mouse $IgG_{2a,k}$ antibody (Iso-IgG, 50 μg/mL, eBioscience) was used as an isotype control.

For invasion assay, rehydrated matrigel invasion chamber with 8 μM pore size (Corning Incorporated) was used.

Tubule formation assay

EA.hy926 cells were seeded at 5 x 10^4 cells per well coated with 200 μL/well of matrigel (Becton–Dickinson Labware, Bedford, MA) and incubated for 4 hr at 37°C in DMEM medium with or without calf thymus histones (Roche Diagnostics). Then, the capillary-like structures were visualized by Olympus CKX41SF (Olympus Corporation, Tokyo, Japan), and tubule length was calculated in four-randomly selected fields using Image J (NIH, Maryland, USA).

Measurement of NET and hypercoagulability markers in patients with pancreatobiliary malignancy

A total of 62 adult patients with pancreatobiliary malignancy were included in the present study. Pancreaticobiliary malignancy was diagnosed based on clinical, laboratory, radiologic and pathologic findings. Cancer staging was performed by radiologic and pathological finding. The peripheral bloods were collected in 0.109 mol/L sodium citrate (Becton Dickinson, San Jose, CA, USA). The plasma were separated with centrifugation of whole blood at 1550 g for 15 min. The aliquots of plasma were stored at −80°C.

Control plasma from healthy adults (n=30) were also included. This study was approved by the Institutional Review Board of Seoul National University College of Medicine.

As NET markers, histone-DNA complex and cell free dsDNA (Quant-iT Picogreen dsDNA assay kit, Thermo Fisher Scientific, Massachusetts, MA, USA) were measured in plasma with patients and controls. As hypercoagulability markers, microparticle (Zymuphen MP-Activity kit, HYPHEN BioMed, Neuville-sur-Oise, France) and ETP were measured.

RESULTS

Pancreatic cancer cell induces NET formation

Since the histone-DNA complex level is a known NET formation marker, it was measured in the supernatant of whole blood incubated with pancreatic cancer cells (AsPC-1). The histone-DNA complex level was significantly increased in whole blood incubated with AsPC-1 for 2hr, compared with control (Figure 1A). AsPC-1 induced the histone-DNA complex level in a dose-dependent manner, along with positive control treated by PMA, a known NET inducer (Figure 1B).

To exclude the effect of other blood cells, AsPC-1 were incubated with isolated neutrophils. The histone-DNA complex level was still high in isolated neutrophils incubated with AsPC-1 (Figure 1C).

To investigate whether direct contact of AsPC-1 cell or soluble factors released by AsPC-1 induces NET, CM harvested from AsPC-1 culture were incubated with whole blood for 2 hr. The CM increased the histone-DNA complex level (Figure 1D). When the lipid components were removed from CM through charcoal treatment, the CM still increased the histone-DNA complex level. However, the heat-treated CM in which protein components were destroyed significantly decreased the histone-DNA complex level (Figure 1D).

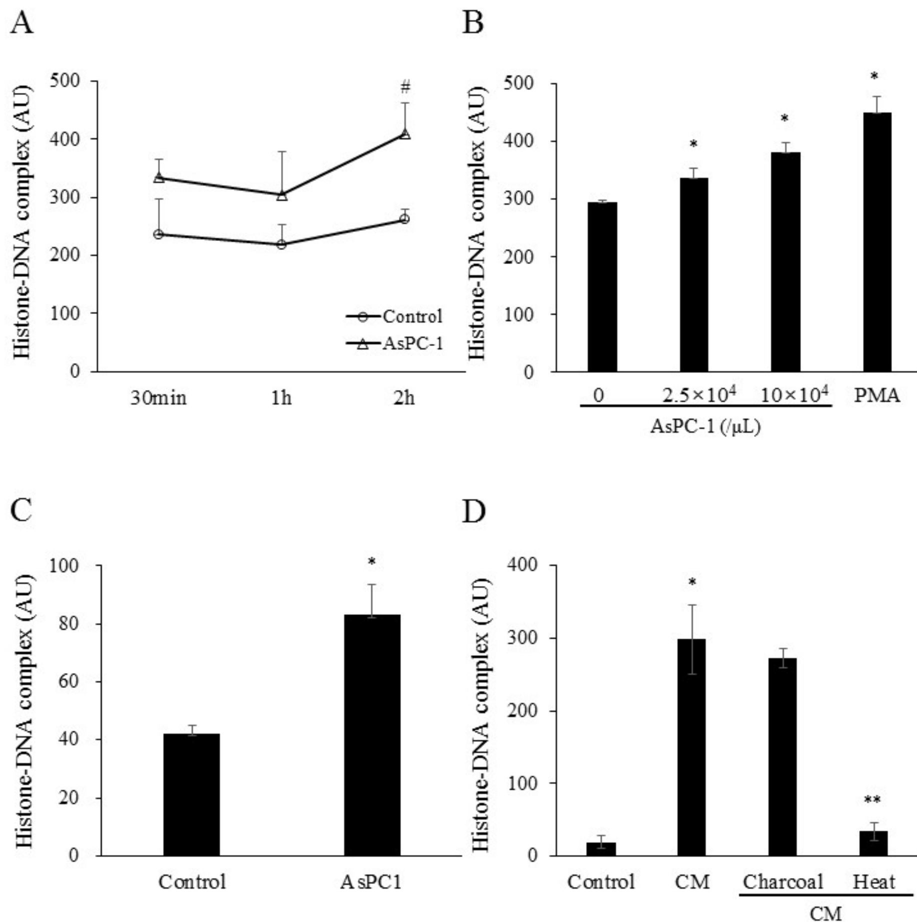

Figure 1. Pancreatic cancer cell AsPC-1 induces neutrophil extracellular traps. (A) Pancreatic cancer cells (AsPC-1) at a concentration of 2.5×10^4 cells/μL were incubated with whole bloods for 30 min, 1 hr and 2 hr at 37°C. The histone-DNA complex levels were measured in the supernatants. (B) AsPC-1 at concentrations of 2.5×10^4 and 10×10^4 and were incubated with whole bloods for 2 hr. Phorbol 12-myristate-13-acetate (PMA, 25nM) was used as a positive control of NET formation. (C) AsPC-1 cells were incubated with isolated neutrophils for 2 hr and the histone-DNA complex level were measured in the supernatants. (D) Conditioned medium (CM) harvested from AsPC-1 culture were incubated with whole bloods for 2hr and the histone-

DNA complex level were measured in the supernatants. Charcoal was added to CM to remove lipid components of CM and heat was treated in CM to destroy protein components. Data are expressed as mean ± SEM of 3 experiments. $^{\#}P < 0.1$ versus control; $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus CM.

Pancreatic cancer-induced NET formation is not ROS-dependent but cyclic AMP and thrombin-dependent.

ROS activities were measured in the supernatants of whole blood incubated with AsPC-1 or PMA. The ROS activities tended to increase as the number of cancer cell added to whole blood increased, but the statistical differences were not significant. (Figure 2A). In the same way, ROS inhibitor, DPI, did not inhibit the AsPC-1-induced NET formation (Figure 2B). Interestingly, PGE1 that inhibits intracellular cyclic AMP production significantly inhibited the AsPC-1-induced NET formation (Figure 2B).

Since thrombin is an essential factor of coagulation and thrombin can enhance platelet-neutrophil interaction [10], we investigated whether thrombin inhibitor inhibits the AsPC-1-induced NET formation. As expectedly, thrombin inhibitor, antithrombin, significantly inhibited the AsPC-1-induced NET formation (Figure 2C). Because AsPC-1 expresses surface TF that can initiate coagulation system and finally produce thrombin [21], we investigated whether TF inhibitor blocks the AsPC-1-induced NET formation. However, anti-TF neutralizing antibody failed to block the AsPC-1-induced NET formation (Figure 2D).

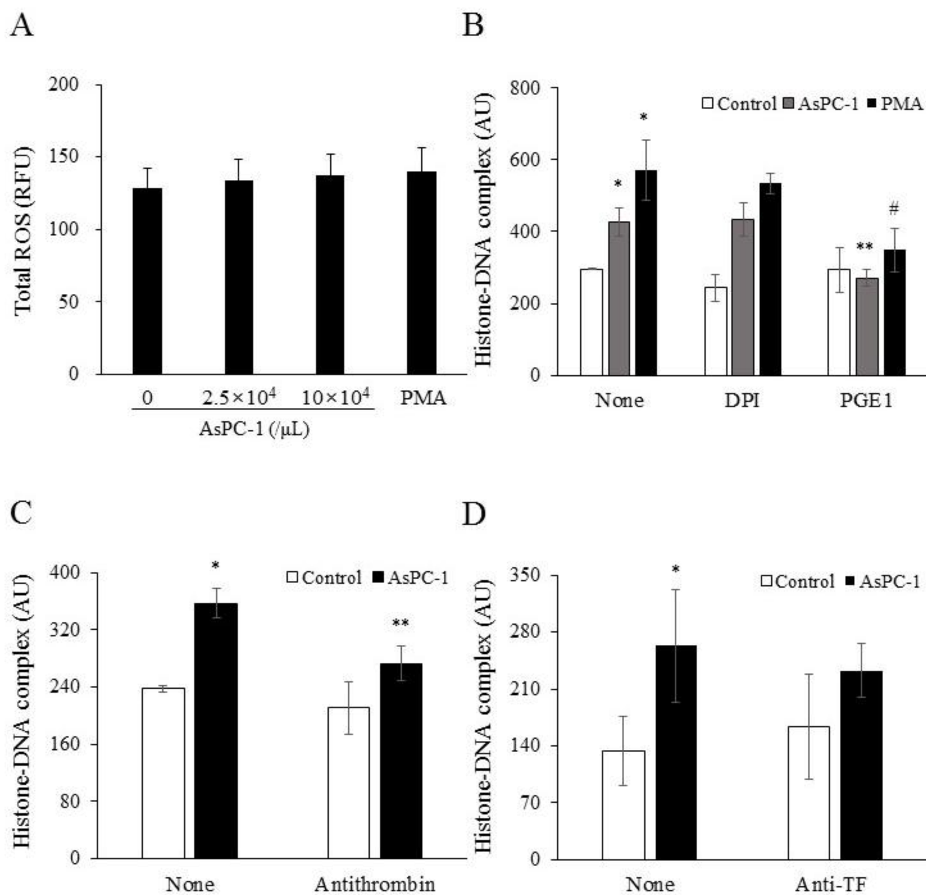

Figure 2. AsPC-1–induced NET formation is not ROS-dependent but cyclic AMP and thrombin-dependent process. (A) AsPC-1 or PMA (positive control) was incubated with whole blood for 2 hr at 37°C. In the supernatants, total ROS activities were measured. (B) diphenyleneiodonium (DPI, 20 μM; ROS inhibitor) or prostaglandin E1 (PGE1, 1 μg/mL; cyclic AMP inducer) was pretreated to the whole blood for 10 min at room temperature and then PMA or AsPC-1 was incubated with the pretreated whole blood for 2 h at 37°C. The histone-DNA complex level was measured in the supernatants. (C) Antithrombin (5 IU/mL) was pre-treated to the whole blood for 10 min at 37°C and the histone-DNA complex level was measured

in the supernatants. (D) Monoclonal antibody against human tissue factor (anti-TF, 30 μg/mL) was pre-treated to the whole blood for 10 min at 37°C and the histone-DNA complex level was measured in the supernatants. Data are expressed as mean ± SEM of 3 experiments. $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus AsPC-1 treated whole blood; $^{\#}P < 0.1$ versus PMA-treated whole blood.

NET promotes thrombin generation

We examined whether the NET by itself increases thrombin generation in normal plasma by using thrombin generation assay. NET significantly increased ETP levels in 3 different stimulating conditions: 5 pM TF (Figure 3A and 3B), 1 pM TF (Figure 3C and 3D), 0.5 pM TF (Figure 3E and 3F).

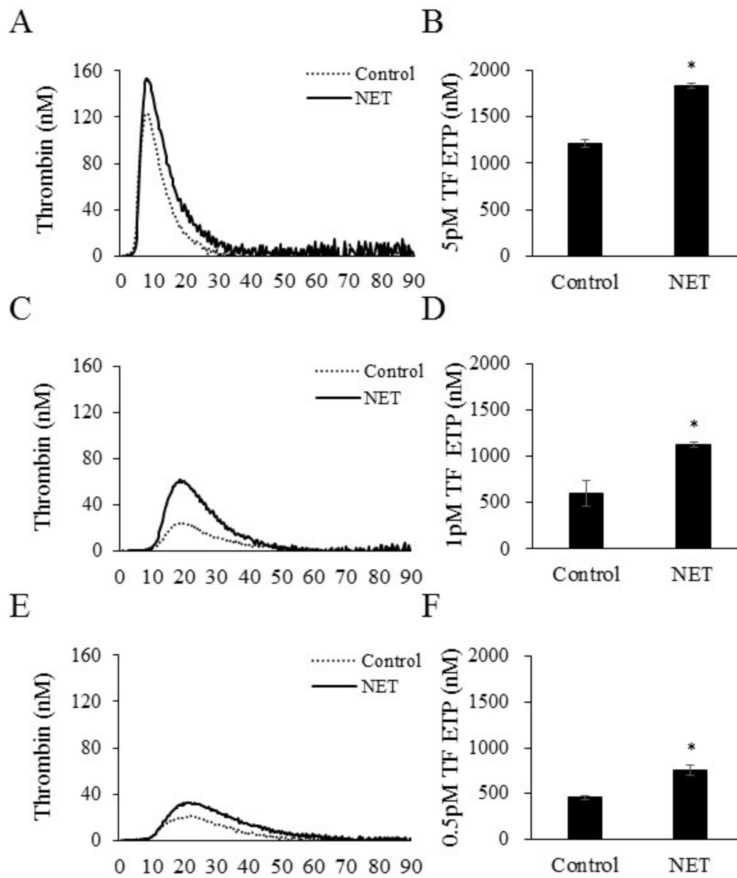

Figure 3. NET increases thrombin generation in normal plasma. NET was made from isolated neutrophil treated with PMA. NET (6.4 mg/dL final protein level) was added to normal plasma and endogenous thrombin potential (ETP) was measured in 3 different conditions of thrombin generation assay (5 pM, 1 pM and 0.5 pM TF-stimulated). All data are presented as mean ± SEM and data were combined from 4 different experiments. $^{*}P < 0.05$ versus control.

NET promotes cancer cell migration and invasion

NET significantly promoted the AsPC-1 cell migration through transwell, compared with vehicle control and intact neutrophil (Figure 4A). Even intact neutrophil promoted the AsPC-1 cell migration, although the migrated cell number was less than NET. In invasion assay, NET also promoted the AsPC-1 cell invasion through matrigel-coated transwell (Figure 4B).

To investigate whether the NET-promoted cancer cell migration and invasion are NET-specific, NET was pre-treated with histone-binding agents (heparin, PSA) or DNA cleavage enzyme (DNase I). All 3 NET inhibitors (heparin, PSA, DNase I) significantly blocked the NET-promoted cancer cell migration (Figure 4C).

Since TLR2 and TLR4 are involved in cell migration and invasion [22, 23], we investigated whether neutralizing antibodies of TLR2 and TLR4 (aTLR2 and aTLR4) block the AsPC-1 cell migration. Both aTLR2 and aTLR4 significantly inhibited the NET-promoted AsPC-1 cell migration (Figure 4D), suggesting that TLR2 and TLR4 are involved in the cancer cell migration.

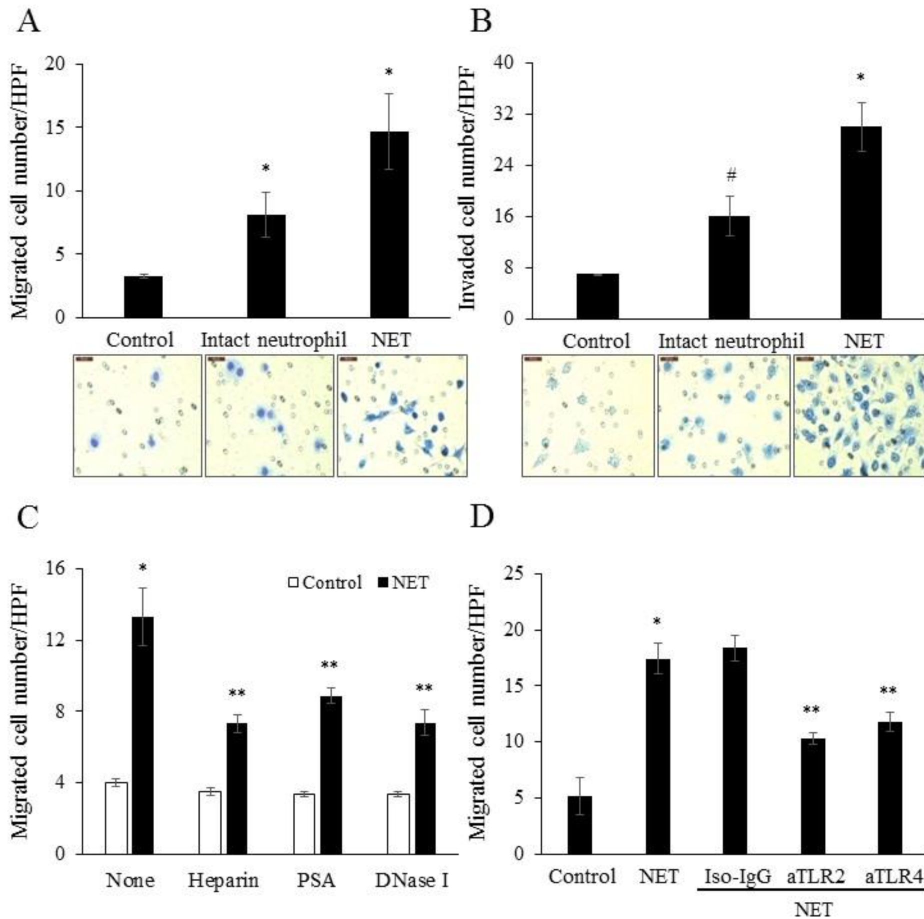

Figure 4. NET promotes migration and invasion of pancreatic cancer cell. As chemoattractants, intact isolated neutrophils (2.5×10^5 cells) and NET (300 mg/dL final protein level) were put to the lower chambers of (A) uncoated transwell and (B) rehydrated matrigel-coated transwell. AsPC-1 (2×10^5 cells/mL) was added to upper chambers. After 22 hr incubation, the migrated and invaded cells were stained with Diff-Quik kit and the results reflect the average of randomly selected six high power field (HPF). The images were taken under an optical microscope per x400 field. (C) Heparin (200 IU/mL), polysialic acid (PSA, 62.5 μg/mL) and DNase I (50 IU/mL) were pre-treated with NET and then the migration assay was performed. (D) Mouse $IgG_{2a,K}$

antibody (Iso-IgG, 50 μg/mL), anti-toll like receptor-2 (aTLR2, 50 μg/mL) and anti-TLR4 (aTLR4, 50 μg/mL) were pre-treated with AsPC-1 cell and then the migration assay was performed. The numbers of migrated or invaded AsPC-1 cell were shown as mean ± SEM of 3 experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.1$ versus control; $^{**}P < 0.05$ versus NET.

NET promotes *in vitro* angiogenesis

Since histone is a major component of NET, we examined whether the histone promotes *in vitro* angiogenesis. Histone significantly increased the tubule formation of endothelial cells in a dose-dependent manner (Figure 5A and 5B). To confirm histone-specific effect on angiogenesis, histone-binding agents (heparin, PSA) were pretreated with histone before histone addition. The heparin and PSA blocked the histone-induced tubule formation (Figure 5C).

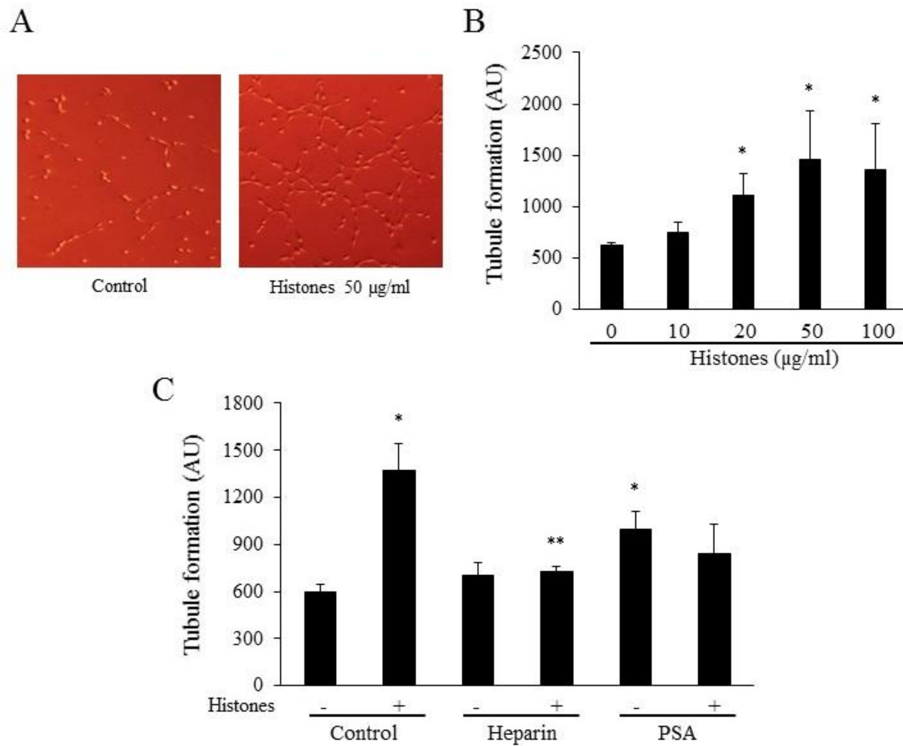

Figure 5. NET promotes *in vitro* endothelial cell angiogenesis. (A) Endothelial cell line (EA.hy926) was incubated with or without histones (50 μg/mL) for 4 hr on matrigel coated-well and the tubule formation of images were taken under an optical microscope per x400 field. (B) Various concentrations of histones were incubated with endothelial cells for 4 hr in matrigel-coated wells. The results reflect the arbitrary unit (AU) of the tubule length that was calculated in four randomly selected fields. (C) As inhibitors of tubule formation, heparin (100 IU/mL) and PSA (62.5 μg/mL) were pre-treated with or without histones for 1 hr. EA.hy926 cells were added to the pre-treated histones for 4 hr. $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus histone-treated.

NET and hypercoagulability markers are increased in patients with pancreatobiliary malignancy

Since cancer cell promoted NET formation, we examined whether the NET formation is increased in cancer patients. As expectedly, in patients with pancreatobiliary malignancy, the circulating levels of NET markers (histone-DNA complex and cell free dsDNA) were significantly increased, compared with healthy controls (Figure 6A and 6B). In the same manner, the circulating levels of hypercoagulable markers (microparticle and ETP) were also increased in patients with pancreatobiliary malignancy (Figure 6C and 6D). Interestingly, the microparticle level significantly correlated with histone-DNA complex and cell free dsDNA levels (r=0.546, r=0.664; $P<0.01$) and ETP also significantly correlated with histone-DNA complex and cell free dsDNA levels (r=0.263, r=0.530; $P<0.05$).

When total patients with pancreatobiliary malignancy (n=62) were subdivided into 3 groups based on cancer stage, patients with stage IV (n = 34) tended to show high levels of NET and hypercoagulability markers compared with patients with cancer stage I/II (n = 8) and stage III (n = 20) (Figure 7).

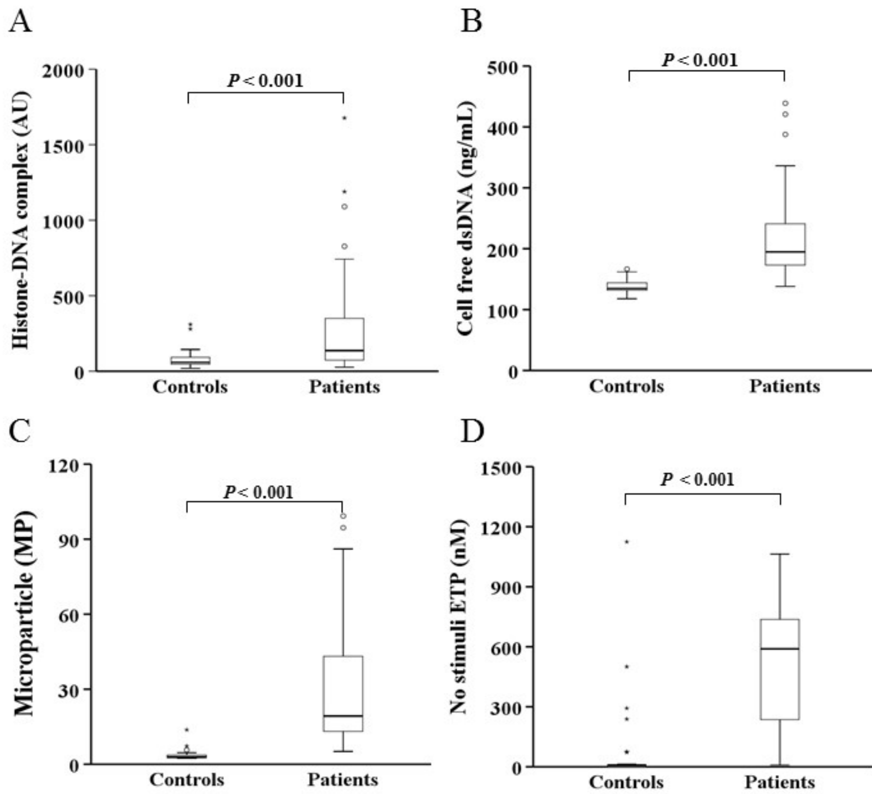

Figure 6. The circulating levels of NET and hypercoagulability markers is increased in patients with pancreatobiliary malignancy. The levels of (A) histone-DNA complex, (B) cell free dsDNA, (C) microparticle and (D) no stimuli ETP were measured in patients with pancreatobiliary malignancy (n = 62) and healthy controls (n = 30).

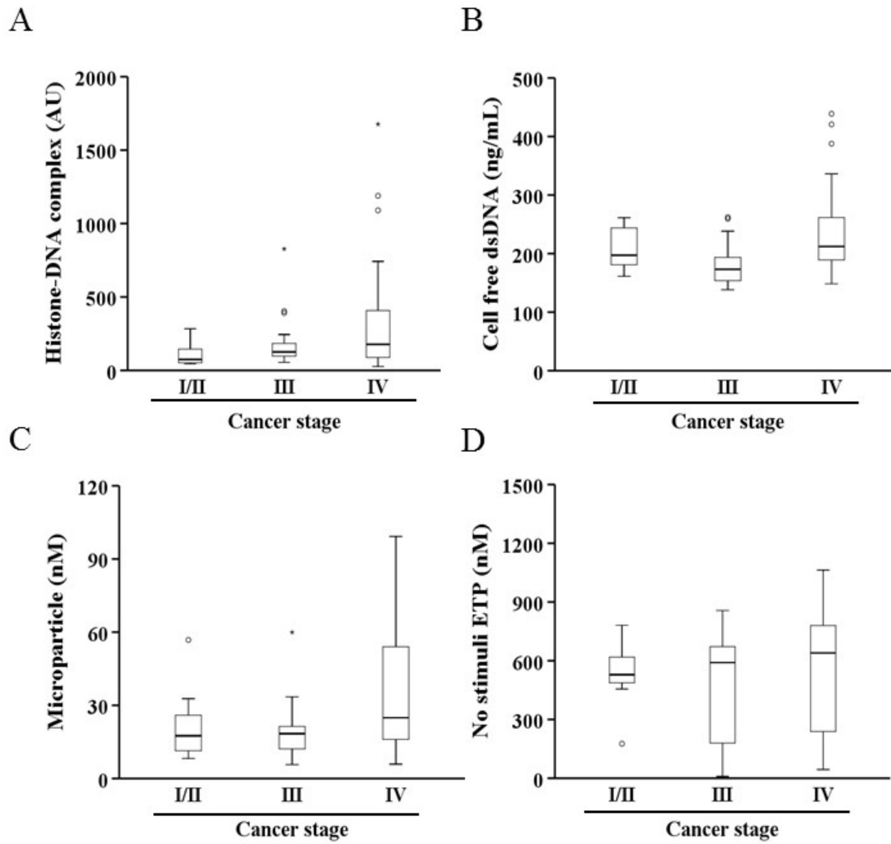

Figure 7. The circulating levels of NET and hypercoagulability markers tend to increase in relation to cancer stage of pancreatobiliary malignancy. The levels of (A) histone-DNA complex, (B) cell free dsDNA, (C) microparticle and (D) no stimuli ETP were shown in stage I/II (n = 8), stage III (n = 20) and stage IV (n= 34) of pancreatobiliary malignancy.

DISCUSSTION

Our study demonstrated that pancreatic cancer cell induced NET formation that was inhibited by PGE1 or antithrombin. The NET increased not only hypercoagulability through promotion of plasma thrombin generation, but also cancer progression through promotion of cancer cell migration and invasion and endothelial angiogenesis. In practice, both circulating NET and hypercoagulability markers were significantly increased in patients with pancreatobiliary malignancy and the NET markers well correlated with hypercoagulability markers.

In our results, pancreatic cancer cell could induce NET formation of whole blood in a dose-dependent manner. Our result reinforces the evidence of recent reports which showed cancer-induced NET formation *in vivo* and *in vitro* [7, 24]. To determine whether the NET formation was induced by other blood cells or cell-cell contact effect, cancer cell was cultured with isolated neutrophils. NET was actively formed even in isolated neutrophils mixed with pancreatic cancer cell, suggesting that there was no necessity of other blood cells for NET formation. Interestingly the cell-free CM harvested from cancer cells also induced NET formation and heat-treated CM abolished it, suggesting the role of soluble protein components via contact-independent way. Cancer cell releases extracellular microvesicles called as exosomes containing proteins, lipids and nucleic acids such as mRNA and miRNA [25]. Several reports have investigated the abilities of tumor-derived exosomes in neutrophil together with cancer metastasis. Tumor-derived exosomes (proteins and RNAs) induce not only neutrophil autophagy through

NF-κB pathway by interacting high mobility group box 1 (HMGB1) and TLR4 [26], but also neutrophil recruitment and infiltration by activating TLR3 [27]. These abilities of tumor-derived exosomes, autophagy and infiltration in neutrophil, enhance NET formation in pancreatic cancer [24]. Recently, it has been reported that tumor-derived exosomes induce NET formation [28]. According to our result showed that AsPC-1 cell free CM-induced NET formation was abolished by removal of proteins and nucleic acids by heat treatment, it is plausible that proteins and nucleic acids in cancer cell-derived exosomes are likely to be potential one of the NET inducers.

Since neutrophil can produce ROS that induces NET formation [29], role of ROS in the NET formation was investigated. The ROS activities were not significantly increased and the ROS inhibitor also did not inhibit the NET formation, suggesting that ROS is not involved in the pancreatic cancer-induced NET formation. This is consistent with previous report in which NET induced by pancreatic cancer did not depend on ROS generation [7].

Interestingly, PGE1 could inhibit both cancer cell and PMA-induced NET formation. PGE1 has various pharmacological activities such as anti-platelet, anti-inflammatory and vasodilating effects [3, 30]. PGE1 can induce intracellular cyclic adenosine monophosphate (cAMP) that inhibits NET formation through reduction of intracellular calcium ions [31]. Therefore, the cancer cell-induced NET formation is likely to occur through cyclic AMP production.

In our results, antithrombin significantly reduced the cancer cell-induced NET formation. Antithrombin exerts not only anticoagulant activity, but also anti-inflammatory activity [16]. As an anticoagulant, antithrombin

binds to thrombin, quenching thrombin activity that is the central protease in the coagulation system [32]. Antithrombin also binds to syndecan-4 on neutrophils, inducing to reduction of CXCL-2 expression and finally attenuates neutrophil migration to inflammatory site [33]. In our *in vitro* system, it is hard to differentiate which effects of antithrombin exert on the inhibition of cancer cell-induced NET formation. There has been an interesting study showing attenuation of endotoxemia through inhibition of NET formation by antithrombin in septic mice [16]. Considering that this beneficial effect of antithrombin is helpful to sepsis treatment, the inhibitory effect of antithrombin on the cancer cell-induced NET formation may deserve further study for clinical application to cancer in future.

TF is a surface receptor of AsPC-1 cell which can initiate coagulation system, producing thrombin [21]. Even when the TF on the surface of AsPC-1 cells was blocked by anti-TF neutralizing antibody, the cancer cell-induced NET formation was not inhibited, suggesting that the surface TF of cancer cell may not play a role in NET formation of our *in vitro* condition.

NET is mainly composed of cell free DNA and histones. Since the DNA has negative charged, it can activate the intrinsic coagulation pathway [34]. Hence, we speculate that the negative-charged DNA of NET may activate intrinsic coagulation pathway, finally producing thrombin. In reality, the thrombin generation in normal plasma was enhanced by NET in our result. Since thrombin is an essential protease in the coagulation system, NET-induced thrombin generation enhancement suggests a significant contribution of NET to hypercoagulability and thrombotic tendency in cancer.

It has been reported that NET induced cancer metastasis through

sequestering circulating tumor cells [15]. Our results showed that the NET increased cancer cell migration and invasion and this process was NET-specific because inhibitors of NET blocked the migration. More interestingly anti-TLR2 and anti-TLR4 neutralizing antibodies significantly blocked the NET-induced cancer cell migration, suggesting TLR2 and TLR4 play roles in the NET-induced cancer cell migration. Although previous reports showed that TLR2 and TLR4 were involved in cell migration and invasion [22, 23], there was until now no data about the ligands of cancer cell for TLR2 and TLR4. We showed NET by itself acts like a chemoattractant. According to the recent reports [35-37], HMGB1 was released from NET and was a ligand for TLR4. Hence, it is assumed that HMGB1-mediated TLR4 signaling is involved in the NET-induced cancer cell migration.

Angiogenesis is a complex morphogenetic process, in which the resting endothelium was switched to migratory endothelial phenotype, migrating through basement membrane where the endothelial cells proliferate and form endothelial sprouts [38]. Angiogenesis is an essential process for cancer progression [39]. Until now in our acknowledgment, there was no report about the effect of NET on angiogenesis. We showed that major component of NET, histone, promoted angiogenesis. This finding reinforces role of NET in cancer progression.

Among NET inhibitors, heparin and PSA are anionic substances which bind to positive charged-histone and neutralize the histone activity [6, 40], and DNase I degrades the DNA [41]. The NET inhibitors significantly blocked the NET-induced cancer cell migration and angiogenesis, suggesting the migration and angiogenesis are NET-specific processes. As therapeutic targets of cancer progression, the NET inhibitors can be expected for future

exploration.

There has been reports that circulating levels of NET markers were increased in various cancers [42, 43]. Although pancreatic cancer-induced NET could contribute to thrombosis in one *in vitro* study [7], there was no data showing circulating NET levels in patients with pancreatic cancer until now. Our data showed that circulating levels of histone-DNA complex and cell free dsDNA proposed as NET markers were significantly increased in patients with pancreatobiliary malignancy. Furthermore, circulating levels of hypercoagulability markers (microparticles and ETP) were also significantly increased in patients with pancreatobiliary cancers and the NET markers well correlated with hypercoagulability markers, suggesting that the NET-induced hypercoagulability actively occurs in pancreatobiliary malignancy.

This study has several limitations. First, we could not clarify mechanism of tumor-derived exosomes as NET inducer. However, it is assumed that mechanism of NET formation is involved in NF-κB/HMGB1/TLR4 and TLR3 signaling that can induce infiltration and autophagy in neutrophil. Understanding mechanism of tumor-derived exosomes in NET formation may be worth studying for cancer therapy. Second, we could not demonstrate the exact chemotactic factor(s) to cancer cell migration. However, HMGB1can be one of the candidate chemotactic factors, because it is released from NET and acts like chemoattractant of cancer cells. Future study is necessary to prove which chemotactic factors in NET participate in cancer cell migration. Lastly, we could not demonstrate the direct association of both NET and hypercoagulability markers with clinical thrombotic events, because the specimens were collected retrospectively and some of patients had old thrombotic histories that did not show any

association of the markers with thrombosis (data not shown).

In summary, this study demonstrated that pancreatic cancer cell induced NET formation in contact-independent and ROS-independent manners. Moreover, the cancer cell-induced NET formation was inhibited by PGE1 and antithrombin. The NET could promote not only hypercoagulability through enhancement of plasma thrombin generation, but also cancer progression through enhancements of cancer cell migration and angiogenesis (Figure 8). Practically in patients with pancreatobiliary malignancy, the elevated NET markers well correlated with the hypercoagulability makers. Taken together, these findings implicate cancer cell-induced NET formation plays an important role in enhancements of both hypercoagulability and cancer progression, suggesting inhibitory agents of NET formation such as PGE1 and antithrombin can be potential therapeutics to reduce both hypercoagulability and cancer progression.

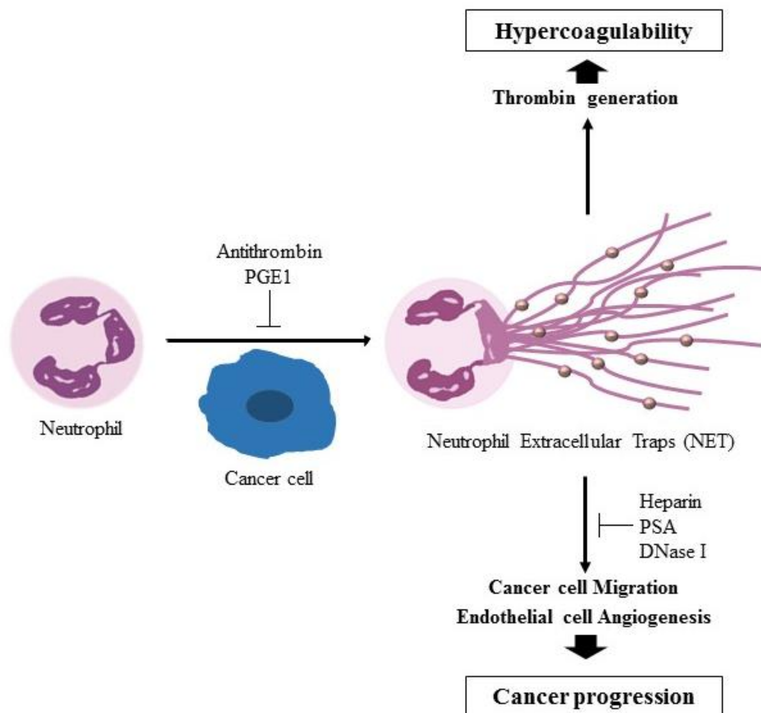

Figure 8. Potential mechanism that cancer cell-induced NET formation promotes both hypercoagulability and cancer progression. Cancer cell induces neutrophils to release NET, which is inhibited by prostaglandin E1 (PGE1, cAMP inducer) and antithrombin. The NET can promote hypercoagulability through enhancement of plasma thrombin generation. Moreover, the NET can promote cancer progression through enhancement of cancer cell migration and endothelial cell angiogenesis, which are inhibited by heparin, polysialic acid (PSA), DNase I.

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논 문 초 록

정 혜 수

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서론: 호중구는 여러 자극을 받으면 세포내의 DNA-histone complex등 그물망과 같은 구조들을 생성하는데, 이를 호중구세포외기질 (neutrophil extracellular traps, NET)이라고 부른다. 호중구와 NET 형성은 암 조직에 풍부하게 나타난다. 본 연구는 암세포가 어떻게 NET 형성을 유도하고 유도된 NET이 혈장 속 트롬빈 (thrombin) 생성과 암 진행을 촉진시키는지 조사했다.

방법: 췌장암세포주 (AsPC-1)에 의해 유도된 NET 형성 정도는 히스톤-DNA 복합체 농도로 측정하였다. 내인성트롬빈형성능 (endogenous thrombin potential, ETP) 검사는 thrombin generation assay를 통해 측정하였다. 시험관 내 실험을 통해 NET에 의한 암세포의 이동과 침윤 정도를 검사하였고 신생혈관형성 검사를 하였다. 62명의 췌담도암 환자와 30명의 건강정상인에서 혈중 NET 과 응고항진성 지표들을 평가하였다.

결과: AsPC-1은 NET 형성을 농도에 의존적으로 유의하게 증가시켰다. AsPC-1 배양으로부터 얻은 적응용배지도 NET 형성을 증가시켰다. 흥미롭게도 적응용배지를 열처리를 하여 단백질성분을 파괴했을

때 NET 형성이 억제되었지만, 지방성분을 제거한 적응용배지는 NET 형성을 계속 증가시킴으로써 단백질성분이 중요한 역할임을 보여주었다. 활성산소 (Reactive oxygen species, ROS) 억제제는 암세포 유도 NET 형성을 억제하지 못하였으나, 프로스타글란딘 E1 (PGE1, cyclic adenosine monophosphate 유도제)과 안티트롬빈 (antithrombin)은 췌장암세포에 의한 NET형성을 완전히 억제시켰다. NET은 정상 혈장 내 ETP를 촉진시켰다. 또한, NET은 암세포의 이동과 침윤 그리고 혈관형성을 촉진시켰고 이는 히스톤 결합제 (헤파린, polysialic acid), DNA 분해 효소와 toll-like receptor 중화항체에 의해 억제되었다. 췌담도암 환자에서, 증가한 혈중 NET 지표들이 응고항진성 지표들과 상관관계가 있었다.

결론: 본 연구는 암세포 유도 NET 형성이 응고항진성과 암 진행을 촉진시키는데 중요한 역할을 하고, 이는 PGE1과 안티트롬빈과 같은 NET 형성 억제제가 응고항진성과 암 진행을 줄이는데 가능성 있는 치료제 후보로 제안될 수 있다.

Keywords: 호중구세포외기질, 암세포, 응고항진성, 암진행성, 프로스타글란딘 E1 (PGE1), 안티트롬빈 (antithrombin)

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