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(RESEARCH ARTICLE)

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Roles of polymorph nuclear neutrophil elastase and thrombomodulin in patients with acute pancreatitis in the context of neutrophil extracellular traps

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Abstract

The goal of the study was to evaluate progression of acute pancreatitis (AP) based on measurement of plasma levels of polymorph nuclear neutrophil (PMN) elastase, thrombomodulin (TM), lipopolysaccharide (LPS), thrombin-antithrombin III complex (TAT), and platelet counts associated with neutrophil extracellular trap (NETs).

The subjects were 42 patients with AP, including 30 classified with mild AP (MAP) and 12 with severe AP (SAP), and 20 normal adult controls.

Plasma levels of TM, PMN elastase, LPS, and TAT were measured by ELISA. Platelets were measured by a routine method. TM molecular subspecies were isolated from plasma using beads and analyzed by reverse-phase HPLC. After these measurements, ulinastatin was administered to 16 patients.

TM, PMN elastase, LPS, and TAT levels in patients with AP were significantly higher than those in controls. Furthermore, these four markers were significantly higher in SAP cases than in MAP cases. Platelet counts significantly decreased in the order of SAP, MAP, and controls. Eight TM subspecies were present in plasma and most of these increased with aggravation of AP. The areas under the ROC curves for PMN elastase, TM, LPS, and TAT were 0.814, 0.827, 0.766, and 0.860, respectively. The markers were normalized by ulinastatin treatment in 16 patients with AP. At onset of focal AP, PMN elastase accumulates in platelets and leukocytes in NETs, and is released from neutrophils. This then releases TM from blood vessel walls, which activates coagulation and leads to systemic aggregation. Treatment with ulinastatin is effective for this disorder.

Keywords: Polymorph nuclear Neutrophil Elastase (PMN-Elastase); Thrombomodulin (TM); Neutrophil Extracellular Traps (Nets; Thrombin-Ant thrombin Complex (TAT); Lipopolysaccharide (LPS); Neutrophil

1. Introduction

Pancreatitis originates in acinar cells, in which premature activation of pancreatic proteases results in cell injury and can ultimately provoke self-digestion of the pancreas by its own proteases. This process is paralleled by a pronounced immune reaction that amplifies disease severity. Acute pancreatitis (AP) is an inflammatory diseases with varied severity, ranging from mild local inflammation to severe systemic involvement resulting in substantial mortality. The pathophysiological findings of local and systemic micro thrombosis in this disease are associated with vascular derangements, including endothelial dysfunctions, increased leukocyte migration to tissues, and activation of coagulation [1, 2].

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Neutrophils release neutrophil extracellular traps (NETs) and antibacterial granules through NETosis to kill and prevent spread of pathogens [3, 4]. Activation of NETs first requires recruitment, aggregation and activation of neutrophils, macrophages, platelets, lipopolysaccharide (LPS) and pancreatic enzymes. Subsequent scaffold formation may require constriction and occlusion of vessels, acinar cells, and secretory pancreatic ducts [4, 5]. Interactions of polymorph nuclear neutrophils (PMNs) with the endothelium are mediated by adhesion molecules (selectins and integrins), and PMN elastase in an important enzyme in this process [6].

Thrombomodulin (TM) is an endothelial cell (EC) membrane protein that acts as a cofactor for activation of plasma protein C and also inhibits the procoagulant properties of thrombin. Exposure of ECs to neutrophils (PMN elastase, phospholipase A_2, or hydrogen peroxide, lipid peroxide, endotoxin) in vitro promotes release of TM, and EC damage is associated with pathological conditions such as pancreatitis, diabetes mellitus, and disseminated intravascular coagulation (DIC) [7, 8].

NETs may be important in aggravation of AP and it has been suggested that an increase in soluble TM antigen in plasma is a marker of EC damage. Soluble TM consists of fragments (molecular subspecies) of various molecular weights, which are probably proteolytic ally degraded from cellular TM or derived from damaged ECs (7, 8). In addition, neutrophils stimulated by LPS secrete PMN elastase into blood, and the PMN elastase is involved in pancreatic tissue injury and aggravation of the systemic inflammatory response, thus causing critical processes in development of AP (9).

In this study, we measured PMN elastase released from neutrophils, TM released in blood due to vascular injuries, platelets that accumulate in blood and tissues with neutrophils, LPS produced from abnormalities in TLRs due to inflammation that stimulates neutrophils, and TAT as an indicator of thrombin production as markers of progression of AP.

2. Methods

2.1. Subjects

Table 1 Background of patients with AP

Item	Severe AP		Severe AP	Mild AP
	Non-survivors	Survivors		
Female	1	1	2	6
Male	6	4	10	24
Alcohol intake	4	3	7	10
Bile stone	1	0	1	6
Idiopathic	1	2	4	6
ERCP	0	0	0	2
S-amylase (u/L)	12787 ± 8542	10764± 10363	12174 ± 9349	7249 ± 4175
U-amylase (u/L)	18967 ± 6698	14786 ± 9853	15770±7451	6983 ± 4751
Lipase (u/L)	4478 ± 3471	4021 ± 3482	4102 ± 3375	886 ± 542
WBC (/μl)	1.8376±7546	12352±7864	17533 ± 8514	10823 ± 7382
Platelets (10^4/μl)	16.0±8.4*	17.8±6.2*	17.3±6.0*	18.6±5.4*
	(3.7-37.2)	(7.8-30)	3.7-37.2)	(8.7-30.3)
APACHE-II	13.7±4.7	11.9±6.1	12.9±5.3	5.2±3.1
	21-5	21-2	21-2	14-1

The subjects were 42 patients with AP, including 30 classified with mild AP (MAP) and 12 with severe AP (SAP), and 20 healthy age-matched adult controls. The patients had a mean age of 52.3±15.7 years. The baseline characteristics of the

patients are shown in Table 1. Diagnosis of AP was based on abdominal signs associated with high pancreatic enzymes, and morphological abnormalities consistent with AP on contrast-enhanced computed tomography and ultrasonography performed within 24 h of admission. Severity of AP was assessed using Ranson's criteria (10), APACHE-II criteria, JSS score, criteria for Intractable Disease of the Pancreas issued by the Japanese Ministry of Health, Labour and Welfare, and the Revised Atlanta Classification (11, 12). Multiple organ failure (MOF) was assessed using APACHE-II at the time of blood sampling. Of the 12 SAP cases, there were 7 deaths due to sepsis and MOF, and 5 survivors.

2.2. Determination of plasma PMN elastase, thrombomodulin and lipopolysaccharide

Measurements of PMN elastase with α1 complex, TM, LPS, and TAT levels were performed using commercial sandwich ELISA kits: PMN elastase® (Biovender Lab Med Inc., Czech Republic) for PMN elastase; TM test Teijin® (Teijin Diagnostics, Japan) for TM, Toxi-color® (Seikagaku Kougyou Co., Japan) for LPS, and TAT test® (Teijin Diagnostics) for TAT, with modifications. The intra- and inter-assay coefficients of variation were 7.9-11.2%.

2.3. Effect of ulinastatin on LPS-stimulated PMN elastase release from granulocytes

Granulocytes were separated from human blood using Ficol (Daiichi Kagaku. Tokyo, Japan), suspended in saline (20×10^4 /0.1ml), and combined with endotoxin (B4.011. 1.76 ng/0.1 ml, Difco, USA) and human plasma (content: α1-antitrypsin). This mixture was incubated for 3 h at 37ºC. Control samples included granulocytes, human plasma, and saline only. Ulinastatin (Miraclid®, Motida Pharmaceutical Co., Tokyo, Japan) was added to each sample at levels of 100, 1000, 5000, and 1.0×10^4 U / 0.1 ml, and PMN elastase generated in these samples was measured by ELISA.

2.4. Isolation and analysis of TM from plasma

TM was separated from plasma by affinity chromatography using magnetic beads coated with antihuman polyclonal TM antibody (Teijin, Tokyo, Japan). Plasma was diluted 10-fold with 1/15 M PBS (pH 6.4) and a 200-µl sample was added to the beads (200 µg; Dynabeads M-280, Sweden). The mixture was incubated for 30 min and then washed three times in PBS with 0.1% Tween 40. An elution buffer (1/15 M PBS with 1.0 M NaCl) was then added and the mixture was incubated for 20 min. Finally, the beads were removed in order to isolate TM. The TM isolated from plasma was analyzed by high-performance liquid chromatography (HPLC) using a TSK gel G3000WXL (Tosoh, Tokyo, Japan) column and a solution of 25 mM PBS (pH 6.8) with 0.3 M NaCl. The flow rate was 1.0 ml/min and the detection wavelength was 280 n (13).

Molecular weight standards were obtained from Pharmacia (Uppsala, Sweden). Assays of TM activity and antigen were performed as previously described (13).

2.5. Determination of white blood cell counts, platelet counts, and pancreatic enzymes

Serum and urine amylase, white blood cell counts, and platelet counts in patients and controls were measured by routine laboratory procedures. Venous blood samples were taken on admission. Totals of 62 and 42 blood samples were collected in SAP and MAP cases, respectively. All samples were collected in a tube containing 1/10 (v/v) 3.8% sodium citrate. Plasma was separated by centrifugation at 3000 rpm for 10 min at 4ºC and stored at -75ºC until assayed.

2.6. Statistical analysis

Values are expressed as mean ± standard deviation (SD). An unpaired Student t-test was used for comparison of mean values, with $P<0.05$ considered significant. The area under the curve (AUC) of the receiver operator characteristic (ROC) curve was determined for each marker.

3. Results

3.1. Plasma PMN elastase, TM, LPS and TAT levels and platelet counts

PMN elastase in surviving patients with SAP and MAP peaked during hospitalization at 0-3 days, and slowly decreased and normalized thereafter, but PMN elastase in non-survivors elevated again at 14 days after admission (Figure 1A).

PMN elastase was also significantly higher in SAP and MAP cases compared to controls, in non-survivors compared to survivors among patients with SAP, and in SAP cases compared to MAP cases (Figure 1B). Platelet counts in all patients with AP were significantly lower than those in controls. Platelet counts in surviving patients with SAP and MAP

decreased during hospitalization at 0-3 days, and then slowly increased and normalized thereafter. However, platelet counts in non-survivors decreased again at 14 days after admission (Figure 1 C, D).

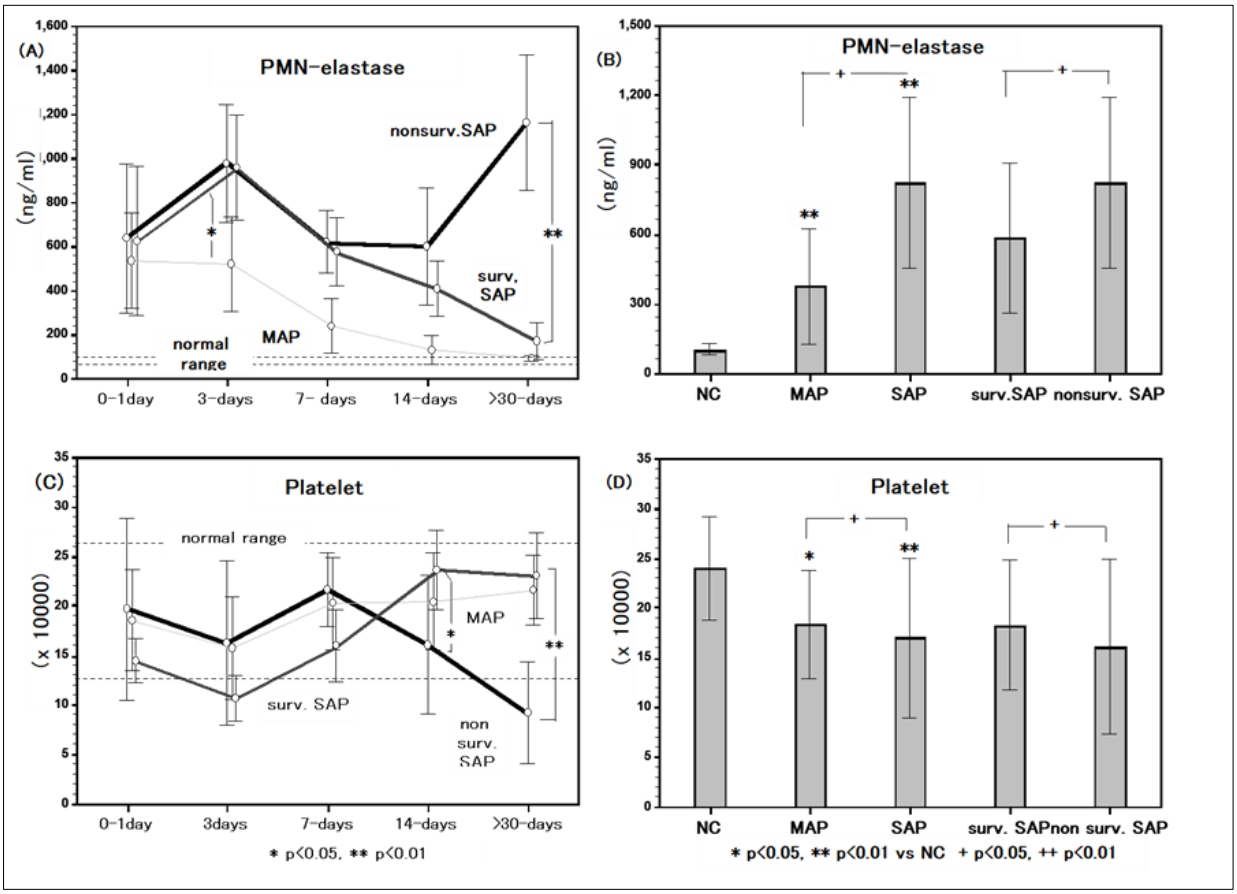

Figure 1 Changes of plasma levels of PMN elastase,, and platelet counts in patients with AP and healthy controls. Data are shown as mean ± SD

LPS and TAT levels in survivors with SAP and MAP elevated during hospitalization at 0-3 days, and slowly decreased and normalized thereafter, whereas both levels in non-survivors elevated again at 14 days after admission. LPS and TAT levels at >30 days after admission differed significantly between survivors and non-survivors. Plasma LPS was not measured in controls. TAT was significantly higher in SAP and MAP cases than in controls, and was higher in non-survivors compared to survivors among patients with SAP, although this difference was not significant (Figure 2 A-D).

3.2. Plasma TM and molecular subspecies

sTM levels in almost all patients peaked during admission at 0-3 days, and slowly decreased and normalized thereafter (Figure 3A), similarly to the changes in PMN elastase. Plasma TM was significantly higher in all AP patients compared to controls, in SAP cases compared to MAP cases, and in non-survivors compared to survivors among patients with SAP (Figure 3B).

3.3. Plasma TM molecular subspecies

Retention times of TM subspecies on HPLC and relationships with molecular weight are shown in Figure 3C. Most peak intensities for TM subspecies were in the order of SAP, MAP, and controls, but those for the 14 kDa subspecies had the order of controls, SAP, and MAP, and those for the 36 kDa subspecies had the order of MAP, controls, and SAP (Figure 3D).

3.4. Inhibitory effect of ulinastatin on release of PMN elastase from granulocytes

Release of PMN elastase from granulocytes was stimulated by treatment with LPS, and the effect of ulinastatin on this process was examined. The results showed dose-dependent inhibition of PNM elastase release by ulinastatin (Figure 4).

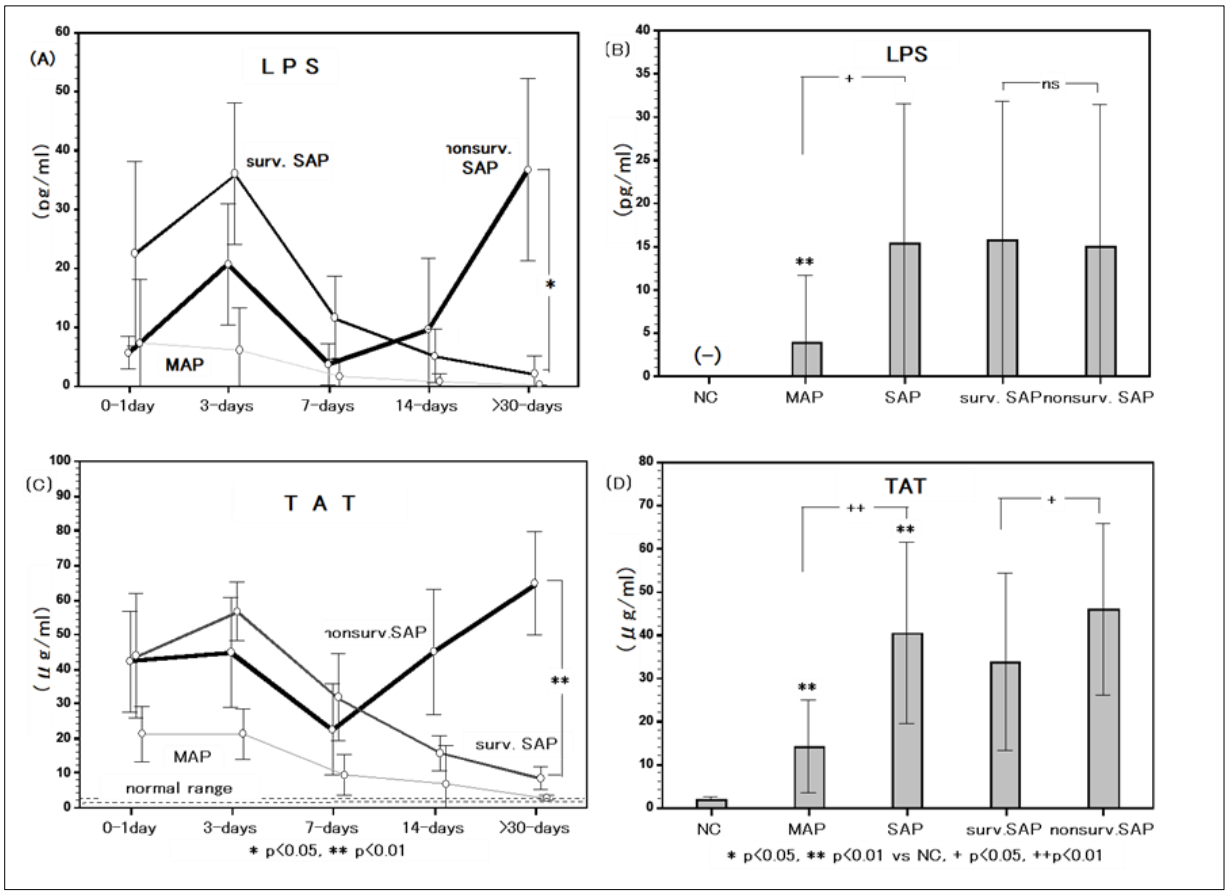

Figure 2 Changes of plasma levels of LPS and TAT in patients with AP and healthy controls. Data are shown as mean ± SD.

3.5. Correlations of PMN elastase with TM, LPS, platelet counts and TAT

The correlation coefficients for PMN elastase with TM, LPS, platelet counts and TAT were 0.814 (n=149, p<0.01), 0.602 (n=144, p<0.01), -0.253 (n=127, p<0.05) and 0.795 (n=146, p<0.01), respectively (Figure 5); and those for sTM with LPS, platelet counts, and TAT were 0.640 (n=147, p<0.01), -0.324 (n=127, p<0.05) and 0.795 (n=146, p<0.01), respectively(Figure 5).

3.6. ROC curve analysis of PMN elastase, TM, LPS, platelet counts, and TAT

The cutoff values distinguishing MAP from SAP were 530 ng/ml, 29 ng/ml, 4 pg/ml, 9.8 x 10^4, and 25 μg/ml for PMN elastase, TM, LPS, platelet counts, and TAT, respectively, and the respective AUCs of the ROC curve were 0.814, 0.827, 0.766, 0.367, and 0.860 (Figure 5).

3.7. Treatment of patients with AP with ulinastatin

Changes in TM and PMN elastase were examined after treatment of 16 AP cases (SAP 5, MAP 11) with ulinastatin (Miraclid® Mochida). The 5 patients with SAP received ulinastatin at 50×10^4 U/day for 30 days, and the 11 patients with MAP were treated with ulinastatin at 30×10^4 U/day for 14 days. Symptoms were relieved by ulinastatin and there were significant decreases in the levels of TM and PMN elastase (Figure 6).

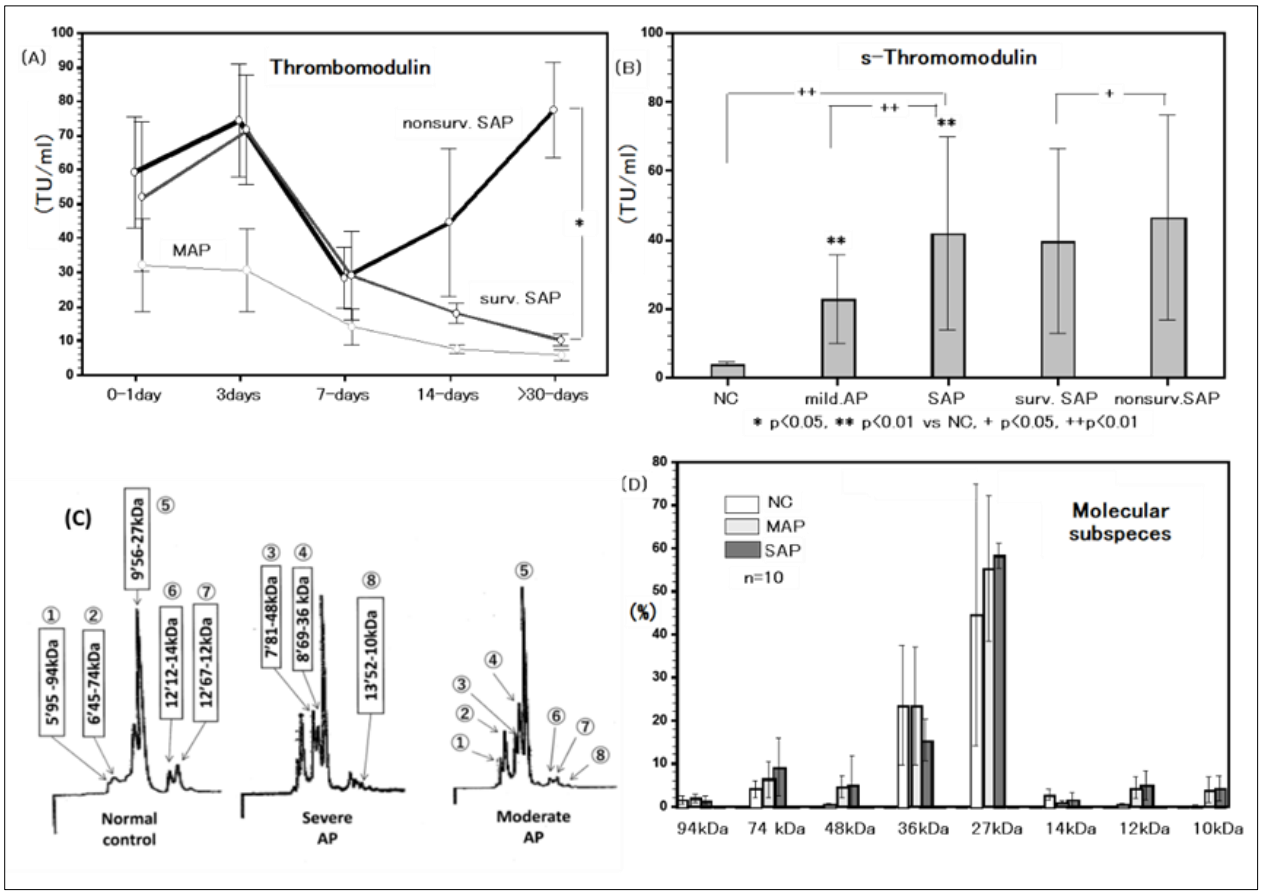

Figure 3 Changes of plasma levels of thrombomodulin (TM) and profiles of TM molecular subspecies in patients with AP and healthy controls. Data are shown as mean ± SD

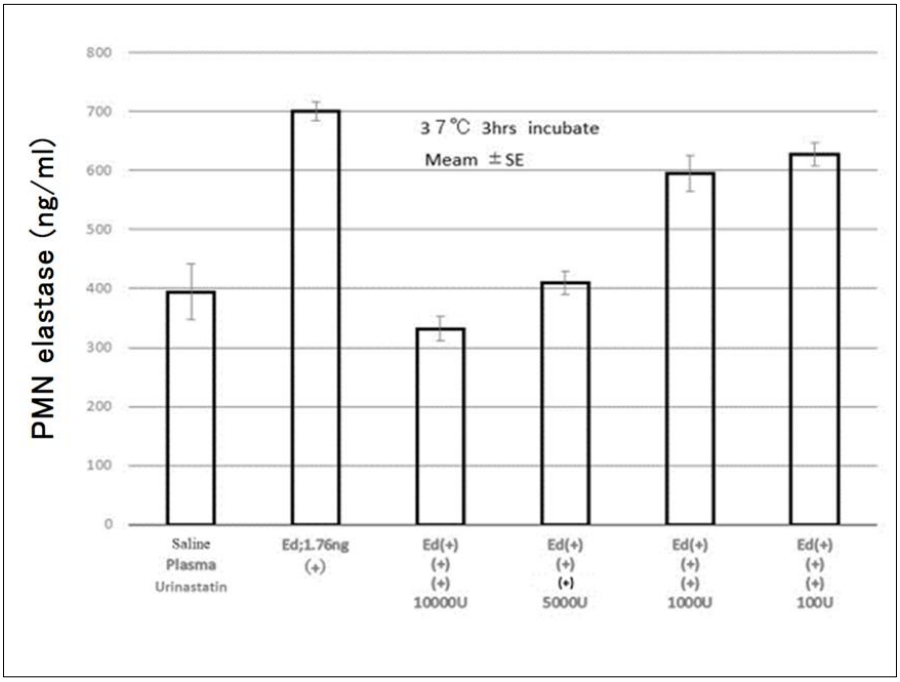

Figure 4 Effects of ulinastatin on release of PMN elastase from granular leukocytes in vitro. Data are shown as mean ± SE.

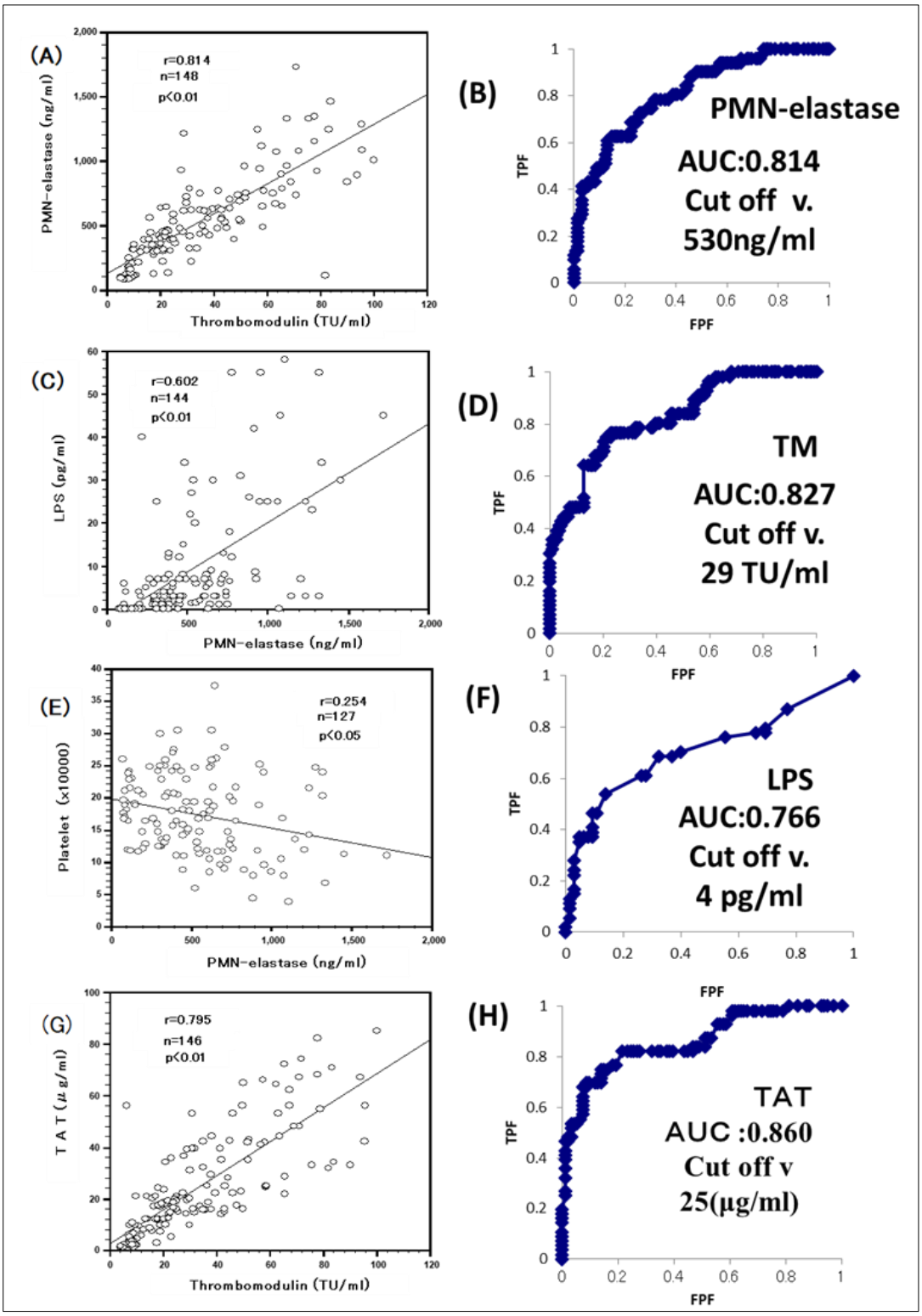

Figure 5 Correlations of PMN elastase with TM, LPS, platelet counts and TAT, and AUCs of ROC curves and cut-off values for these markers in patients with AP.

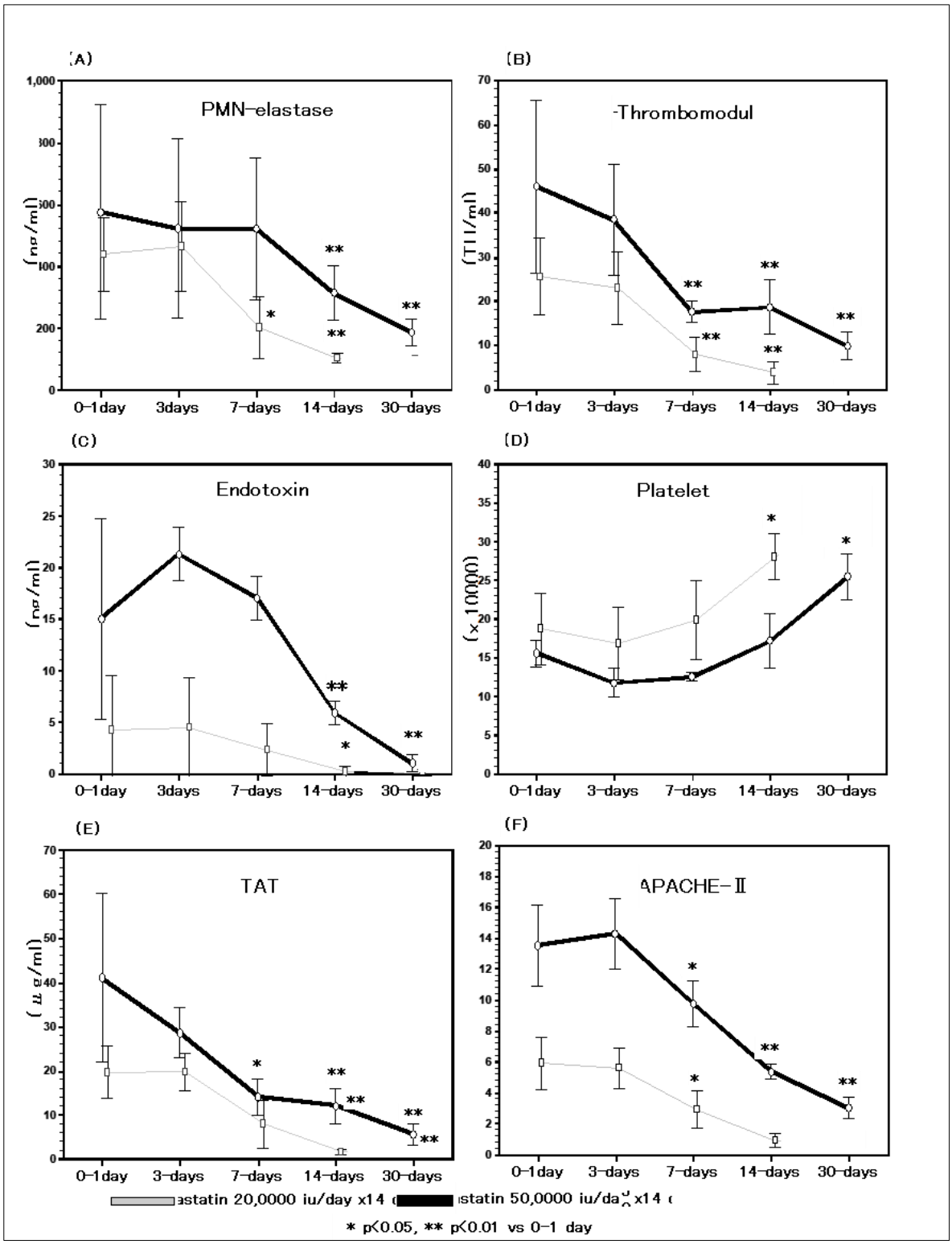

Figure 6 Changes in PMN elastase, TM, platelet counts, LPS, and TAT after ulinastatin administration in 16 patients with AP. Data are shown as mean ± SD

4. Discussion

Onset of focal AP requires initial release of inflammatory mediators from acinar cells and abnormal activation of trypsinogen, followed by occlusion of acinar cells and pancreatic secretor ducts by NETs with recruitment of inflammatory cells such as PMN neutrophils, platelets and macrophages. Local and severe systemic early pathological

events in AP are associated with vascular disorders, including endothelial activation and injury, increased vascular permeability, and activated coagulation [5, 14].

PMN elastase is a protease released by activated neutrophils as an early defense following pancreas injury. PMN elastase cleaves E-cadherin, which leads to reduced cell-cell contacts and formation of pancreatic edema and cell damage, and thus, this enzyme is associated with the predicted severity of AP and respiratory failure [6, 14].

In particular, PMN elastase is involved in pancreatic tissue injury and aggravation of the systemic inflammatory response, which are critical processes in AP. In experimental AP models and in severe AP, proteins activated by PMN elastase increase leukocyte adhesion and platelet transmigration of leukocytes through the vascular wall. We have found that PMN elastase can be generated from granular leukocytes by LPS and pancreatic juice stimulation in vitro (9, 15, 16). In the current study, PMN elastase levels were strongly positively correlated with TM, LPS and TAT, but negatively correlated with platelet counts. The area under the ROC curve for PMN elastase was 0.814, and the cutoff value between SAP and MAP was 530 ng/ml [15-18].

Human TM has five structural regions: a lectin-like domain with roles in inflammation, innate immunity, and cancer; a domain C-terminal to the lectin-like domain; a region comprising 6 epidermal growth factor (EGF)-like repeats with roles in coagulation and fibrinolysis; and transmembrane and cytoplasmic domains [19]. The relationship between the EGF repeats and TM subspecies are important for hemostasis. [19]**.** TM is an endothelial cell (EC) membrane protein that acts as a cofactor for activation of protein C, and also inhibits the procoagulant properties of thrombin. Activation of protein C by the thrombin-TM complex downregulates the coagulation cascade by degrading cofactors Va and VIIIa by limited proteolysis [6, 20].

These anticoagulant and cytoprotective activities may contribute to the beneficial in vivo effects of activated protein C. Plasma TM levels can be measured easily, but only a few studies have considered plasma levels of the TM subspecies. Several previous reports have evaluated TM in DIC, renal failure, and acute respiratory distress syndrome (ARDS), but particular subspecies have not been examined [6, 20, 21].

Elevated plasma TM is a marker of endothelial cell injury and is regarded as a parameter of activity in vasculitides [21, 22]. PMN elastase degrades protein C and protein S in vitro, which may weaken the defense mechanism of protein C in AP [23, 24].

Previous reports have indicated cut-off values of TM of 32-75 ng/ml for aggravation of AP. Our ROC analysis indicated an area under the ROC curve for TM of 0.827 and a cut-off of 29 TU/ml for distinguishing SAP from MAP. TM from plasma of healthy controls had seven subspecies with molecular weights between 94 and 12 kDa, but there were eight TM subspecies from 94 to 1.0 kDa in SAP cases. The 1.0 kDa subspecies was barely detected in controls, whereas increased levels of many subspecies were detected in AP patients. These results suggest that the severity of AP is closely related to vascular injury. In addition, all the TM subspecies are likely to have thrombin binding sites, and therefore, each subspecies can induce thrombin-catalyzed activation of protein C [6, 13, 15].

LPS binds to CD14 on monocytes and endothelial cells, and activates these cells, while PMN elastase is secreted into blood from activated granulocytes by stimulation with LPS [6, 25, 26]. LPS was only weakly correlated with platelet count in our patients, which may be because the actions of LPS and platelets are associated with various intermediate functions, including a decrease of platelet counts for thrombosis formation via activation of coagulation and recruitment [25, 26]. Pancreatic acinar cells respond to LPS during progression of mild, subclinical AP. LPS ultimately promotes thrombin formation via aggregation and activation of platelets and neutrophils. TAT showed relatively strong correlations with other markers, but had a negative correlation with PMN elastase. Synthesis of thrombin results in anticoagulant activity via activation of the protein C system [26, 27].

Recent studies have shown that platelet and leukocyte recruitment by macrophages is induced via actions of NETs. Macrophages can clear pathogens, tissue debris, and necrotic and apoptotic cells through phagocytosis, and thus, play an important role in AP, since tissue injuries induced by NETs are caused by cell debris and thrombus formation. Organ failure in the late stage is caused by long-term continuous neutrophil-derived inflammation and endothelial injury [27, 28]. These processes are paralleled by a pronounced immune reaction that amplifies disease severity, such as system inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS). Therefore, effective targeted inhibition of PMN elastase may be a promising treatment strategy to reduce the incidence of severe AP.

Administration of ulinastatin improved and normalized symptoms and five markers in patients with AP. There are many therapeutic agents (r-TM, r-PC, r-AT-III, gabexate mesilate, nafamostat mesilate) for AP, but ulinastatin has the advantage of being relatively inexpensive and having few side effects [29]. Many fatal complications of SAP are closely related to NET formation, and further studies of therapeutic strategies for AP are needed to address this issue.

5. Conclusion

At onset of focal AP, PMN elastase is released from neutrophils in NETs. Subsequently, TM is released from vessel epithelial cells, which activates coagulation and leads to systemic aggregation. Treatment with ulinastatin is effective for this disorder.

Compliance with ethical standards

Acknowledgments

We would like to thank Teijinn Co., Ltd. for providing various ELISA-kits.

Disclosure of conflict of interest

None of the authors have a conflict of interest.

Statement of ethical approval

Our study was approved by the ethics committee of Hijirigaoka Hospital and complies with the Treaty of Helsinki. All patients admitted to Hijirigaoka Hospital (Hokkaido, Japan) from January 2015 to October 2021 were included in the primary analysis.

Author Contributions

Concept, design, and supervision: S.U., K.G.; Resources, materials, data collection and processing: Y.F, T.M.; Analysis and interpretation: K.G. ; Literature search and manuscript writing: S.U.; Critical review: K.G.

Statement of informed consent

All patients gave informed consent and all agreed to donate blood samples and allow their clinical information to be used in the study. Control samples were collected from 20 healthy staff members in our hospital.

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