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(RESEARCH ARTICLE)

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Role of the protein C system in aggravation of acute pancreatitis

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Abstract

The goal of the study was to identify risk factors for coagulation and fibrinolysis in aggravation of acute pancreatitis (AP) based on 11 markers and APACHE-II score.

The subjects were 42 patients with AP (12 severe AP and 30 mild AP) and 20 healthy controls. Measurements of antithrombin III (AT-III), plasminogen (Plg), platelet counts, lipopolysaccaride (LPS), thrombomodulin (TM), protein C (PC), activated protein C (APC), APC/PC ratio, free-protein S (f-PS), thrombin-antithrombin complex (TAT), and tissue-type plasminogen activator·PAI-1 (t-pA·PAI-1) complex and clinical scores were performed using ELISA kits and clinical examinations of patients.

Fluctuations of most markers in the course of AP indicated abnormal activation from onset to 3 days. After 7 days, non-survivor severe AP (SAP) cases showed abnormal marker reactivation, while survivor SAP and mild AP (MAP) cases had gradual normalization of markers. At 7 days after onset and during all stages of progression of AP, there were significant differences between non-survivor SAP and survivor SAP cases for 5 and 8 markers, respectively, but APC and t-pA·PAI-1 complex were common to both. The TAT/APC and t-pA·PAI-1/APC ratios, which reflect APC generation, were higher in survivor SAP than in non-survivor SAP cases. A decrease in APC generation leading to excessive thrombin synthesis may be a risk factor for coagulation and fibrinolysis in aggravation of AP.

Keywords: Protein C; Activated Protein C; Thrombin-Antithrombin Complex; Tissue Type Plasminogen Activator·PAI-1 Complex; Thrombin; Acute Pancreatitis

1. Introduction

Acute pancreatitis (AP) is an inflammatory disease of varied severity, ranging from mild local inflammation to severe systemic inflammatory involvement that results in substantial mortality. The initial event is activation and retention of digestive enzymes in acinar cells, with subsequent cellular injury. The acinar cells release inflammatory mediators, which leads to recruitment of neutrophils, formation of free radicals, and activation of the complement system. The neutrophils and macrophages generate additional cytokines and other substances, and the amplified inflammatory response exacerbates pancreatic injury and systemic organ failure [1,2].

AP usually resolves within a week without local or systemic complications, but the disease may also progress to a generalized inflammation phase, which is also referred to as systemic inflammatory response syndrome (SIRS). Subsequently, a phase of mixed inflammatory response with transient organ failure and local complications occurs, followed by a final phase of suppressed inflammatory response, referred to as compensatory response syndrome (CARS), that manifests as severe AP associated with persistent organ failure [3,4]. In this phase, downregulation of the

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immune system leads to higher susceptibility of pancreatic and peripancreatic tissue to infection from the gut. The ensuing sepsis and multiorgan failure (MOF) are the major causes of late morbidity and mortality in severe AP.

The causative and sensitizing factors for AP include refluxed bile acids, hypercalcemia, ethanol, hypertriglycemia, and acidosis [5]. However, despite advances in investigational modalities and research techniques, the exact pathogenesis of AP is unclear. In particular, the mechanism of aggravation of AP is still unknown. Regarding pathomorphological findings in AP, parietal circular intravacular microthrombosis accompanied by endothelial desquamation is important. Therefore, in this study, markers of coagulation and fibrinolysis were measured to identify risk factors for these processes in development and progression of AP.

2. Methods

2.1. Subjects

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 [6], 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 [7, 8]. 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.

Table 1 Background of patients with severe and mild acute pancreatitis

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)	15.7 ± 8.4 (3.7-37.2)	17.3 ± 5.9 (7.8-30)	15.9 ± 5.0 (3.7-37.2)	18.6 ± 5.4（8.7-30.3)
APACHE-II	13.7 ± 4.7 (21-5)	11.9 ± 6.1 (21-2)	12.9 ± 5.3 (21-2)	5.2 ± 3.1 (14-1)

2.2. Determination of plasma antithrombin-III (AT-III), lipopolysaccharide (LPS), and thrombin-antithrombin complex (TAT)

AT-III, LPS, and TAT were measured using sandwich ELISA kits: Testchimu® (Sekisui Medical Co., Tokyo, Japan) for AT-III, Toxi-color® (Seikagaku Kougyou Co., Japan) for LPS, and TAT test® (Teijin Diagnostics, Osaka, Japan) for TAT, with modifications.

2.3. Determination of plasma protein C (PC), activated protein C (APC), and free-protein S (f-PS)

PC, f-PS, and TAT were measured using sandwich ELISA kits: protein C test Teijin®, protein S test Teijin®, and TAT test® (all Teijin Diagnostics), with modifications. APC was measured by the method of Liew et al. with modifications, using anti-human APC monoclonal antibody (protein C test Teijin®) as the first antibody and determination of the chromogenic activity of bound APC by addition of S-2366 chromogenic substrate (Diapharma, Malmo, Sweden) in coating buffer [9].

2.4. Determination of plasma plasminogen, tissue-plasminogen activator (t-pA·PA1) complex, and thrombomodulin (TM)

Measurements were performed using sandwich ELISA kits: Plasminogen Teijin® (Teijin Diagnostics, Osaka, Japan) for plasminogen; Tint Elize™, Imulyse (Biopool.AB, Umeá, Sweden) for t-PA·PA1 complex, and TM test Teijin® (Teijin Diagnostics) for TM, with modifications. The intra- and interassay coefficients of variation were 7.8-12.6%.

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.

3. Results

3.1. Fluctuation of each marker in the course of AP

AP cases were divided into SAP and MAP groups using APACHE-II and JJS classes. SAP cases were further divided into non-survivors and survivors. Serial changes of markers were examined using measurements made at 0-1, 2-3, 7, 14, and ≥30 days.

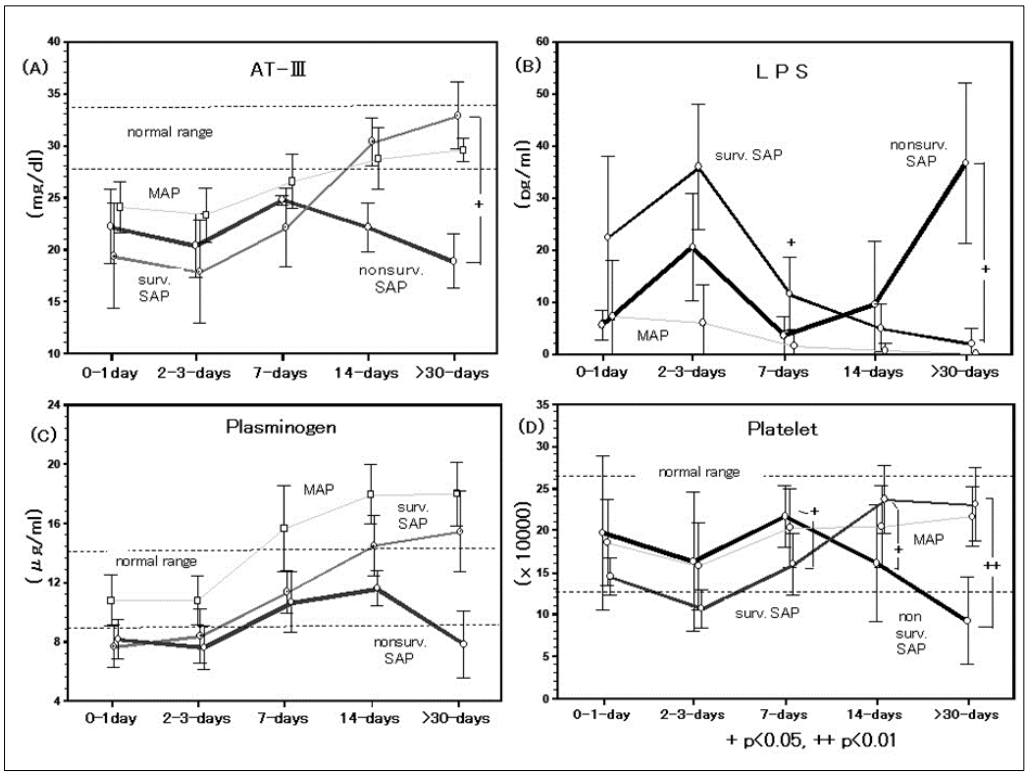
3.2. Fluctuations of AT-III, LPS, platelet counts, and plasminogen levels in AP

AT-III and LPS levels in survivor SAP and MAP cases peaked during hospitalization at 0-3 days, and slowly decreased and normalized thereafter. However, AT-III and LPS levels in non-survivors reversed after 7 days and elevated again at 14 days after admission, whereas survivor SAP and MAP cases normalized as the course progressed. Plasminogen levels in AP patients showed similar changes. Platelet counts in non-survivors had a course below the normal range, but those in survivor SAP and MAP cases normalized over time [Figure 1].

At ≥30 days after onset, there were significant differences in AT-III and LPS levels and platelet counts between survivor and non-survivor SAP cases [Figure 1].

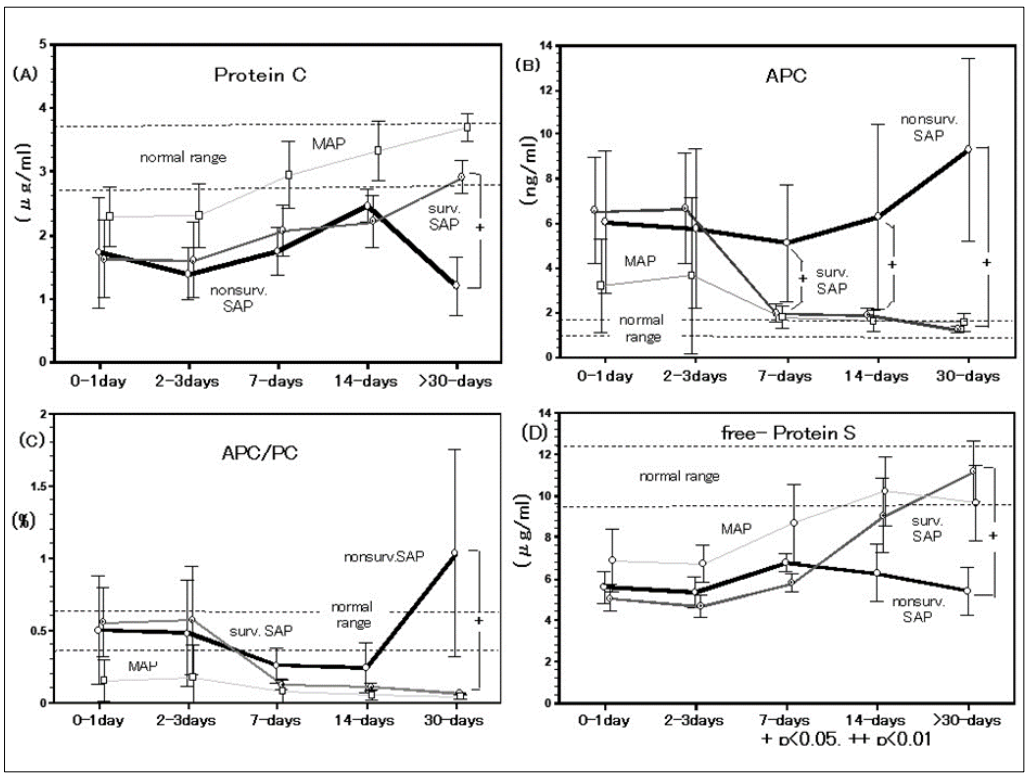
3.3. Fluctuations of PC, APC, APC/PC and free-PS levels in AP

PC and f-PS levels were below their normal ranges from onset of AP, and were low in non-survivor SAP cases from beginning to end. In contrast, these levels normalized over time in survivor SAP and MAP cases. APC increased from immediately after onset, and increased again in non-survivor SAP cases at 7 days after onset. At ≥30 days after onset, APC levels differed significantly in survivor SAP and non-survivor SAP cases [Figure 2].



Data are shown as mean ± SD; Normal range (mean±SD)

Figure 1 Fluctuations of AT-III, LPS, and plasminogen and platelet counts in patients with acute pancreatitis

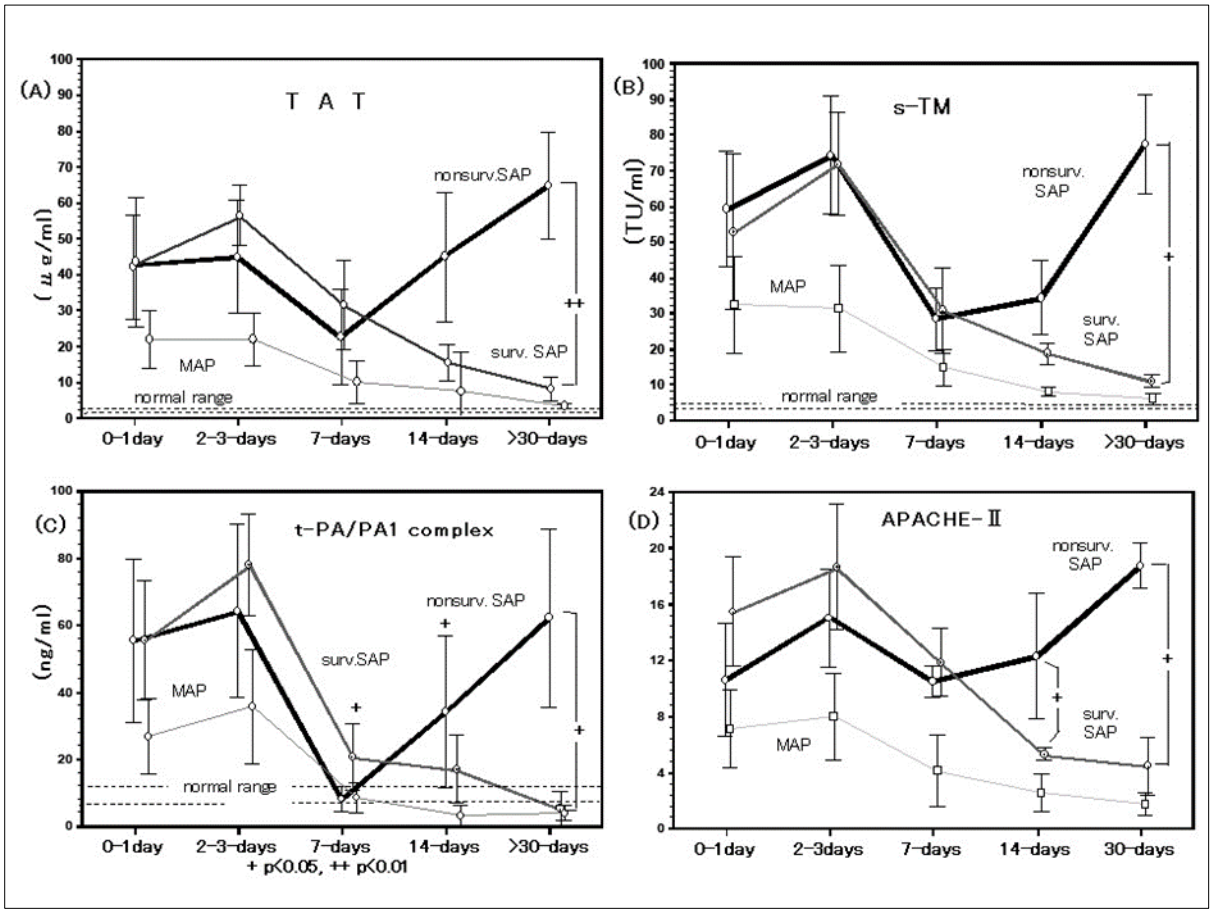


Data are shown as mean ± SD; Normal range (mean±SD)

Figure 2 Fluctuations of PC APC, APC/PC and f-PS levels in patients with acute pancreatitis

3.4. Fluctuations of TAT, soluble TM, t-pA·PAI complex, and APACHE-II scores in AP

AP, TAT, sTM, and t-pA·PAI-1 complex levels were higher than their normal ranges from onset of AP. These levels remained high in non-survivor SAP cases from start to end, but tended to normalize in survival SAP and MAP cases. Changes of APACHE-II scores followed similar patterns. At ≥30 days after onset, there were significant differences in AP, TAT, sTM, and t-pA·PAI-1 complex levels in survivor SAP and non-survivor SAP cases [Figure 3].



Data are shown as mean ± SD. Normal range (mean±SD)

Figure 3 Fluctuations of TAT, soluble TM, and t-pA·PAI complex levels, and APACHE-II scores in patients with acute pancreatitis

3.5. Correlations between APACHE-II scores and 11 markers in patients with AP

Correlation coefficients with APACHE-II scores were 0.807 for TAT, 0.763 for TM, -0.726 for AT-III, -0.687 for PC, 0.687 for t-pA·PAI-1 complex, -0.668 for plasminogen, 0.634 for LPS, -0.60 for f-PS, 0.537 for APC/PC, 0.512 for APC, and -0.451 for platelet count.

3.6. Plasma levels of markers in patients with AP

There were significant differences between SAP cases and controls for 9 markers (AT-III, TM, PC, APC, APC/PC, f-PS, platelet counts, LPS, TAT) and APACHE II scores; and between MAP cases and controls for 7 markers (AT-III, PC, APC, APC/PC, f-PS, LPS, TAT, t-pA·PAI-1 complex) and APACHE-II scores. In progression of AP, 8 markers differed significantly in survivors and non-survivors (plasminogen, TM, PC, APC, APC/PC, TAT, f-PS, LPS) [Figure 4]**.** All coefficients for these comparisons were significant ($p<0.05$).

Figure 4. Plasma levels of markers in patients with acute pancreatitis Data are shown as mean ± SD. (n) represents the number of measurements

	NC (n=20)	Severe AP	Mild AP	Non-survival SAP	Survival SAP
Antithrombin-Ⅲ (AT-Ⅲ : mg/dl)	30.1 ±2.6	21.4 ±4.6 ** (56)	23.8±3.4** (96)	21.2 ±3.4 (31)	21.6±5.7 (25)
Plasminogen (Plg :μg/ml)	11.7±2.5	10.0±3.2 (56)	12.6±3.5 (96)	9.1±2.4 (31)	11.3±3.8 (25)
Platelet (Plt: x10^4)	19.7±6.9	15.9±5.0* (56)	18.6±5.4 (92)	15.7±8.4 (31)	17.3±5.9 (25)
Lipopolysaccaride (LPS:pg/ml)	0(−)	16.2±16.3** (54)	3.8±7.5** (92)	16.6±16.6 (31)	15.8±16.3 (23)
Thrombomodulin (TM:TU/ml)	3.9±0.7	41.8±28.3** (56)	15.1±16.6 (94)	46.2±30.5 (31)	22.8±27.2 (25)
Protein C (PC: μg/ml)	3.2±0.5	1.9±0.7** (56)	2.5±0.6* (96)	1.7±0.7 (31)	2.1±0.7 (25)
Activated Protein C (APC:ng/ml)	1.5±0.3	5.5±4.0** (56)	2.7±2.5* (96)	6.6±4.2 (31)	3.9±3.0 (25)
APC/PC (%)	0.5±0.1	4.1±4.5** (56)	1.3±1.6* (96)	5.1±5.1 (31)	2.7±3.1 (25)
free Protein S (f-PS: μg/ml)	11.3±1.4	6.3±2.1* (56)	7.8±2.1* (96)	5.8±1.1 (31)	7.0±2.8 (25)
Thombin-antithrombin complex (TAT:μg/ml)	2.1±0.4	40.1±20.6** (56)	15.0±10.0** (96)	44.8±19.9 (31)	34.1±20.3 (25)
tPA・PA1complex (ng/ml)	7.3±3.6	47.0±29.8** (56)	20.3±19.0* (96)	47.2±30.9 (31)	23.6±22.3 (25)

*Mean ±SD (n) * p <0.05, * *p <0.01 vs NC, +p <0.05, ++p<0.01 ; non-survival vs survival, SAP vs MAP*

3.7. Comparison of markers in non-survivor and survivor SAP cases 7 days after AP onset

Markers that differed between non-survivor and survivor SAP cases at 7 days after the onset of disease are shown next:10.6±2.3 (5) and 11.3±1.6 (5) for plasminogen, 27.5±14.6 (4) and 29.5±14.6 (5) for TM, 1.8±0.4 (5) and 2.1±0.5 (5) for PC, 5.0±2.6 (6) and 2.2±0.5 (5) for APC, 24.1±13.7 (5) and 35.9±13.1(5) for TAT, 3.5±4.0 (4) and 11.6±7.8 (5) for LPS, 6.75±0.53 (4), and 5.6±0.48 (5) for f- PS, and 6.6±2.3 (3) and 21.6±12.0 (5) for t-pA·PAI-1 complex, respectively. TAT, LPS, f-PS, t-PA·PAI-1 complex, and APC differed significantly between these cases. (n) represents the number of measurements.

3.8. Markers of APC generation and clinical outcome in progression of AP

During progression of AP, PC was significantly lower and APC and the APC/PC ratio were significantly higher in non-survival SAP cases compared to survival SAP cases. APC and the APC/PC ratio also differed significantly over the whole course and in late phases of AP in survival SAP cases [Figure 5].

Figure 5 Fluctuations of TAT, soluble TM, and t-pA·PAI complex levels, and APACHE-II scores in patients with acute pancreatitis. (n) represents the number of measurements

	Period	Non-survivor SAP	Survivor SAP	Controls
P C (μ g /ml)	0-30 days	1.7±0.7(31) +	2.1±0.7(25)	3.2±0.5(20)
	7-30 days	1.8±0.7(20) +	2.4±0.5(15)	
APC (ng/ml)	0-30 days	6.6±4.2(31) +	3.9±3.0(25) ┐	1.5±0.3(20)
	7-30 days	7.0±4.2(20) ++	2.0±0.5(15) ┘ +	
APC/PC (%)	0-30 days	0.50±0.50(31) +	0.31±0.30(25) ┐	0.5±0.1(20)
	7-30 days	0.52±0.59(20) +	0.88±0.38(15) ┘ ++	
TAT/APC	0-30 days	8.72±5.19**(31)	9.31±5.31** (23)	1.16±0.40(20)
	7-30 days	8.38±4.48**(18)	9.58±5.80**(13)	
t-PA · PAI-1/APC	0-30 days	8.35±5.28*(28)	8.86±5.19*(24)	4.38±2.25(20)
	7-30 days	6.18±4.51 (16)	7.53±5.59(14)	
TM/APC	0-30 days	13.54±13.51**(31)	10.22±4.92 **(25)	2.65±0.88(20)
	7-30 days	8.90±4.91**(19)	10.25±4.96**(15)	
f-PS/APC	0-30 days	1.21±0.78**(31) +	3.11±2.57**(25) ┐	7.59±2.35 (20)
	7-30 days	1.20±0.81**(18) ++	8.47±4.31 (15) ┘ ++	

• *Mean ±SD, *p <0.05, * *p <0.01 vs Controls; +p <0.05, ++p <0.01 non-survor SAP vs survor SAP*

Data are shown as mean ± SD

TAT/APC and t-pA·PAI-1/APC ratios in non-survivor SAP cases were lower than in survivor SAP cases over the entire course. The TAT/APC ratio over the entire course was lower than that at 7-30 days, and the t-pA·PAI-1/APC ratio in non-survivors at 7-30 days after AP onset was lower than that in survivors; however, these differences were not significant. These results suggest that APC production in survivor SAP cases was higher than that in non-survivor SAP cases in the late phase of progression of AP.

The TM/APC ratio in non-survival SAP cases was higher than in survival SAP cases, but lower in non-survival SAP in the later phase of AP, with neither difference being significant. The TM/APC ratio in survival SAP cases was significantly lower than in the anaphase of AP.

f-PS was significantly higher in SAP and MAP cases than in controls, and significantly higher in SAP compared to MAP. The f-PS/APC ratio was significantly lower in non-survivor SAP cases than in controls, and significantly lower in non-survivor SAP cases compared to survivor SAP cases during progression of AP. The f-PS/APC ratio in survivor SAP cases in the later stage of AP was significantly higher than over the entire course [Figure 5].

4. Discussion

AP initiates local inflammation and injury in the pancreas, and the inflammation is then amplified due to SIRS and MOF. AP has a high incidence and mortality caused by an uncertain pathophysiological mechanism. Excessive systemic inflammation associated with AP is a consequence of uncontrolled or dysregulated activation of the immune system [10]. Both local and systemic AP are associated with vascular dysfunctions, including endothelial activation and injury, dysregulation of vasomotor tone, increased vascular permeability, increased leukocyte migration to tissue, and activation of coagulation. Endothelial cells have several important functions beyond being a barrier between blood and tissues, including control of vascular pressure and permeability, activation and adhesion of platelets and leukocytes, and coagulation and fibrinolysis, all of which are relevant in AP [11].

Activation of coagulation leads to stimulation of inflammatory mechanisms, with binding of FVII to tissue factor (TF) being the main trigger for coagulation activation. The complex formed by TF and activated FVII in the presence of FX stimulates protease-activated receptors (PARs). Thrombin serves as a serine protease to activate PARs expressed by platelets and numerous immune cells, including monocytes, lymphocytes, macrophages, dendritic cells, and mast cells, as well as by endothelial cells. When a fibrin thrombus is formed, t-pA forms a trimolecular complex with plasminogen that has loose binding with fibrin, which accelerates plasmin generation and subsequent fibrin dissolution. In contrast, anti-fibrinolytic and stabilized hemostatic thrombus is protected from fibrinolysis by cross-linked α_2-antiplasmin (α_2AP) and active thrombin activatable fibrinolysis inhibitor (TAFIa) through removal of a C-terminal lysine residue of fibrin. TAFI is generated through APC by TM and thrombin in vessel walls [12-14].

In the current study, fluctuation of markers of coagulation and fibrinolysis in progression of AP could be approximately divided into two periods. Most markers in the course of AP were abnormally activated from onset to 3 days. After 7 days, cases were divided into non-survivor SAP showing further marker elevation and survivor SAP and MAP, in which markers gradually normalized. Plg, TM, PC, APC, APC/PC, TAT, f-PS and t-pA·PAI-1 complex had significant differences between survivors and non-survivors, and LPS, t-pA·PAI-1 complex, f-PS and platelet counts differed significantly between non-survivor SAP and survivor SAP cases from 7 days after onset. The common markers in these two comparisons are APC, f-PS, and t-pA / PAI-1complex, which suggests that the PC system is an important risk factor in AP.

The PC pathway provides a natural anticoagulant feedback mechanism. PC is activated by thrombin bound to TM located on the endothelial cell surface. The endothelial cell protein C receptor (EPCR) localizes PC on the endothelial cell membrane and enhances activation of PC [9,15,16]. Whereas the anticoagulant activity of APC is inhibited when APC is complexed with EPCR, EPCR is a required cofactor for the antiapoptotic activity of APC. Activation of PAR-1 by APC is EPCR-dependent, and components of the PC pathway have both anticoagulant activity and an anti-inflammatory function [9.15,16]. Activation of PC requires thrombin, and TAT is a marker of thrombin inhibition. There are significant positive correlations between TAT and t-pA·PAI-1 complex, and APC. Because t-pA forms a complex with PAI-1, it is considered to be an index that reflects PAI-1 in generation of APC. Thus, we assumed that the TAT/APC and t-pA·PAI-1 complex/APC ratios are indexes of APC function. Our results showed that these ratios were higher in survivor SAP cases in different stages of AP, indicating greater APC function than in non-survivor SAP cases.

PS acts as a cofactor with APC and with tissue factor pathway inhibitor, and also has functions in the complement pathway through binding to C4b-bp. APC can lower fibrinolytic activity through inhibition of PAI-3 [17]. Thus, APC, and TM have been proposed as antithrombotic medications. We found that PS and PC were lower in patients with AP than in controls, and f-PS and the f-PS/APC ratio were lower in SAP cases than in controls. APC is a physiologically important anticoagulant and is profibrinolytic through inactivation of PAI-1. Activation of plasminogen to produce fibrinolytic activity requires a complex of t-pA and PAI-1, but t-pA decreases in vascular endothelial cells as the PAI-1 level increases in blood. Therefore, a high level of t-pA·PAI-1 complex inhibits fibrinolysis paradoxically and will promote thrombus formation [12,18]. The differences in changes in APC generation in non-survival and survival SAP cases over the course of AP suggest that the PC mechanism cannot handle overproduction of thrombin. These results also indicate that the PC system can work efficiently in patients with AP. At onset of AP, vascular injury or endotoxin and inflammatory cytokines initiate the coagulation cascade, ultimately resulting in thrombin generation and thrombus formation. The thrombin-TM complex rapidly converts PC to APC. Excess thrombin then complexes with TM, a receptor on the surface of vascular endothelial cells. APC generation is augmented by EPCR, which binds circulating PC and is present as the thrombin-TM complex. Since TM and EPCR are endothelial receptors that are part of the machinery required to generate APC in response to elevation of thrombin, it has been hypothesized that downregulation of EPCR and TM in pathologic conditions in which there is systemic endothelial dysfunction may be impaired by inflammatory severity [9,19,20]. This can also be inferred from the correlations of thrombin markers with APACHE-II scores during the course of AP. Most importantly, congenital and acquired PC or PS deficiencies are associated with severe recurrent thrombotic events [19].

In experimental SAP, treatment with APC decreases inflammation markers, increases pancreatic expression of EPCR and TM, and reduces severity of pancreatic morphological changes, including necrosis [21-23]. However, Kyhälä et al. found that APC did not alleviate coagulative disorders in patients in the APCAP study; rather, APC was associated with restricted recovery from coagulopathy**,** although no serious bleeding occurred in APC-treated patients [24]. Thus, there is a need for accumulation of results for APC treatment in more cases of AP. Platelets, which promote neutrophil extracellular traps (NETs), and LPS, which changes intestinal permeability, are also immunosuppressive factors (anti-inflammatory, anticoagulant, and cytoprotective functions) with APC in SAP[25-27].

Systemic inflammation seen in SAP is commonly associated with thrombotic disorders, and activation of coagulation may further aggravate inflammation. These findings suggest the need for studies of hemostasis and immunological mechanisms in AP.

5. Conclusion

Generation of excessive thrombin occurs with aggravation of AP. APC generation in SAP is a risk factor for coagulation and fibrinolysis mechanisms that cannot handle excessive thrombin, and this in turn produces hypercoagulability and frequent thrombosis.

Compliance with ethical standards

Acknowledgments

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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.

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.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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