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ORIGINAL ARTICLE

Genetic analysis of patients with deep vein thrombosis during pregnancy and postpartum

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Abstract Deep vein thrombosis (DVT) is a serious pregnancy-related complication. Recent studies indicate that the genetic background for DVT differs with ethnicity. In our study, we enrolled 18 consecutive Japanese patients who had developed DVT during pregnancy and postpartum. We performed a genetic analysis of three candidate genes for DVT, protein S, protein C and antithrombin, in these patients. We found that four patients had missense mutations in the protein S gene, including the K196E mutation in two patients, the L446P mutation in one patient, and the D79Y and T630I mutations in one patient, as well as one patient with the C147Y mutation in the protein C gene. All five patients with genetic mutations had DVT in their first two trimesters. Nine of the patients without genetic mutations developed DVT in the first two trimesters, and four in the postpartum period. Thus, genetic mutations in the protein S gene were predominant in pregnant Japanese DVT women, and DVT in pregnant women with genetic mutations occurred more frequently at the early stage of pregnancy than postpartum. Considering the rapid decrease in protein S activity during pregnancy, we may need to assess thrombophilia in women before pregnancy.

Keywords Deep vein thrombosis · Protein S · Thrombophilia · Pregnancy

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1 Introduction

Venous thromboembolism is the leading cause of maternal deaths in Western countries [1]. The incidence of pregnancy-related venous thromboembolism was 13 per 10,000 deliveries [2]. A 30-year population-based study reported that the unadjusted incidence of deep vein thrombosis (DVT) was 151.8 per 100,000 woman-years [3]. Most studies have found that the risk for thrombosis were 3–12 times higher in postpartum than during pregnancy [3, 4]. One study, however, reported twice as many events antenatally as postpartum [5]. Most of these studies involved patients in Western countries. A study in Japan showed that pulmonary thromboembolism occurred in 0.02% of total births, and the mortality rate was 2.5 per 100,000 deliveries [6]. Women with pregnancy-related thrombosis tend to have inherited thrombophilia, thus the prevention of DVT during pregnancy and postpartum is important for pregnant women. Therefore, the identification of inherited or acquired thrombophilia in pregnant women is urgently needed for the prevention of pregnancy-related thrombosis.

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In Caucasian populations, two thrombotic mutations, the factor V Leiden mutation and the prothrombin G20210A mutation, for venous thromboembolism are widely distributed, with 30–60% of women with pregnancy-related thrombosis having these mutations [7, 8]. Both mutations are well-established risk factors for venous thromboembolism during pregnancy and postpartum in Caucasian populations. Several prophylactic therapies for pregnant women, such as heparin administration in the perinatal period, are recommended based on the type of thrombophilia and history of thrombosis [9]. However, these two genetic mutations are not found in the Japanese population [10, 11]. Thus, Caucasians and Japanese have clear genetic differences for thrombosis [12].

Deficiencies in protein S, protein C, and antithrombin are well-known risk factors for DVT [13]. The frequency of protein C deficiency and antithrombin deficiency in the general Japanese population was estimated to be 0.13 and 0.15%, respectively, and was comparable to the Caucasian population [12, 14–16]. The frequency of protein S deficiency in Japanese, however, seemed to be higher than that in Caucasians [17, 18], although the assays for plasma protein S levels differed among the studies. Actually, the frequency of protein S deficiency in 2,690 individuals randomly selected from the general Japanese population was estimated to be 1.12%, higher than reported in Caucasian populations (0.03–0.13%) [17, 18]. In a study of Japanese patients with venous thromboembolism, the frequency of inherited protein S deficiency was higher than that in Caucasian patients [19, 20]. It was recently reported that 17% of Japanese patients with venous thromboembolism had genetic mutations in the protein S gene [20]; this was much higher than in selected Caucasian patients with thromboembolism (1.4–8.6%) [13]. Furthermore, we and others reported the significant association with a missense mutation, K196E, in the protein S gene and venous thromboembolism in Japanese populations [19, 21, 22]. The carriers of this mutation showed low protein S activity [19, 23]. The prevalence of this mutant allele in the general Japanese population was about 0.009, suggesting that a substantial proportion of the Japanese population carried the protein S E-allele and was at risk of developing DVT [12, 19, 21, 22, 24]. This mutation seems to be ethnically specific, because it has not so far been identified in Caucasians.

It is well recognized that plasma levels of protein S activity and antigen are significantly reduced during pregnancy [25] and in oral contraceptive users [26]. The activities of protein S, protein C, and antithrombin can be affected at the acute stage of thrombotic events or after antithrombotic therapies. Therefore, the plasma assay may have an intrinsic limitation for the diagnosis of thrombophilia, and alternative ways to diagnose thrombophilia are expected. Genetic analysis might fulfill this requirement if it is applicable.

In this study, we performed DNA analysis for the genes of protein S, protein C, and antithrombin in patients with DVT during pregnancy and postpartum. We measured their plasma activities of protein S, protein C, and antithrombin. Based on these analyses, we described the clinical characteristics of the DVT events in patients with genetic mutation.

2 Materials and methods

2.1 Study patients

In this study, 18 consecutive patients with DVT during pregnancy and postpartum were enrolled from two tertiary perinatal centers: the National Cerebral and Cardiovascular Center and the Osaka Medical Center and Research Institute for Maternal and Child Health. Both centers are located in the Osaka Prefecture, which has the third-largest population in Japan. Postpartum was defined as the first 3 months after delivery. DVT was diagnosed by ultrasonography, venography, or magnetic resonance imaging angiography. We enrolled only patients with symptomatic DVT. Each patient's age, body mass index, gestational weeks of DVT onset, complications of pregnancy, delivery mode, and other information were reviewed.

The protocol of this study was approved by the Ethics Review Committee of the National Cerebral and Cardiovascular Center and by that of the Osaka Medical Center and Research Institute for Maternal and Child Health. Only those who had given written informed consent for genetic analyses were included.

2.2 Activity measurements of protein S, protein C, antithrombin, and antiphospholipid syndrome screening

The plasma samples were obtained after at least 3 months' postpartum and at least 3 months without the use of warfarin. Samples were subjected to a thrombophilia screening, including prothrombin time, activated partial prothrombin time, and activities of protein S, protein C, and antithrombin. Protein S activity was measured as cofactor activity for activated protein C on the basis of the activated partial thromboplastin time assay using Staclot protein S (Diagnostica Stago, Asnieres, France) [18]. Protein C amidolytic activity was measured using S-2366 as a chromogenic substrate and Protac derived from *Agkistrodon contortrix* venom as the activator [16]. Antithrombin activity was measured as a heparin cofactor activity using chromogenic substrate S-2238 (Chromogenix AB, Stockholm, Sweden) [16, 27]. Samples were also subjected to an antiphospholipid syndrome screening of

lupus anticoagulant, anticardiolipin antibody, and anti-β2-glycoprotein-I antibody [28].

2.3 DNA sequencing of protein S, protein C, and antithrombin genes

We sequenced the entire coding region of protein S, protein C, and antithrombin genes in 18 patients with DVT. The method of direct sequencing using the 96-capillary 3730xl DNA Analyzer (Applied Biosystems Japan, Tokyo, Japan) has been described previously [20, 29]. We have adopted the numbering standards of the Nomenclature Working Group, wherein the A of the ATG of the initiator Met codon is denoted as nucleotide +1, and the initial Met residue is denoted as amino acid +1 [30].

3 Results

3.1 DVT history of enrolled patients

We enrolled 18 Japanese symptomatic DVT patients in this study, and only one patient had previous DVT event. All patients were negative for the antiphospholipid syndrome. Thirteen patients were primiparous and five were multiparous. One patient without genetic mutation had a history of miscarriage. One patient without genetic mutation had a history of first trimester artificial abortion that was also complicated with DVT at the time. As an additional risk factor, two out of 13 DVT patients without genetic mutation showed hyperemesis, but all five patients with genetic mutation did not show hyperemesis. Other risk factors such as bed rest, preeclampsia, multiple pregnancy, and preterm labor were not observed in all 18 patients. One patient without genetic mutation had the travelers' thrombosis in the first trimester. One patient without genetic mutation showed paradoxical embolism after DVT postpartum.

3.2 Identification of genetic mutation in DVT patients

We sequenced the coding regions of the protein S, protein C, and antithrombin genes in the 18 DVT patients and identified missense mutations in the protein S gene in four cases, and in the protein C gene in one case, but not in the antithrombin gene (Table 1). Two patients, cases 1 and 2, had the K196E mutation in the protein S gene; this is the most popular thrombophilic mutation in the Japanese population [19, 21, 24]. These two patients had protein S anticoagulant activity above 50% (Table 1). Case 3 had a missense mutation, L446P, in the protein S gene. Case 4 had two missense mutations, D79Y and T630I, in the protein S gene with very low anticoagulant activity of 4%, with family history of DVT in her father. The protein S anticoagulant activities during pregnancy in cases 2, 3, and 4 were decreased to 25, <20, and <1%, respectively. Case 5 had the C147Y mutation in the protein C gene with 45% amidolytic activity. Her protein C activity did not change during pregnancy (Table 1). None of the 18 patients with DVT had nonsynonymous mutations in the antithrombin gene. All patients were not obese with body mass index between 18 and 24. Case 1, 2, and 3 had term vaginal delivery; however, case 4 and 5 had cesarean section due to other obstetric indication.

3.3 Onset of DVT in patients with genetic mutation

Table 2 shows the onset of the DVT events in patients with or without genetic mutation. DVT was found in all five patients with genetic mutations in their first and second trimesters, but not in postpartum. In 13 patients without genetic mutations, DVT events occurred in postpartum for four patients and in the first and second trimesters for nine patients. Two out of four patients without genetic mutation underwent cesarean section. Thus, DVT in pregnant patients with genetic mutation tended to occur in the first and second trimesters and not postpartum.

4 Discussion

Although the relationship between DVT and genetic mutations in protein S, protein C, and antithrombin genes is well established, the clinical courses of DVT patients with genetic mutation among Japanese women during pregnancy and postpartum have not been well characterized. Recent genetic analysis of inherited thrombophilia revealed ethnic differences in DVT between Caucasians and Asians [19, 21], suggesting that the study of venous thromboembolism within individual ethnic populations is highly valuable [12]. It has been established that Caucasians have factor V Leiden mutation and prothrombin G20210A mutation as genetic risk factors for DVT, whereas Japanese do not carry them [10, 11]. However, Japanese have the K196E mutation in the protein S gene as a genetic risk for DVT [19, 21, 22]. The study of DVT in a Japanese population without factor V Leiden mutation or prothrombin G20210A mutation may reveal different clinical characteristics and give rise to hitherto unrecognized issues. In particular, sub-group analyses, such as DVT during pregnancy and postpartum, would be valuable. In the present study, we enrolled 18 pregnant Japanese women with DVT and found that five out of 18 patients (28% patients) had genetic mutations in the protein S or protein C gene. None carried mutations in the antithrombin gene.

The question of when DVT events occur in pregnant women with genetic mutations has been debated. Studies of

Table 1 Nonsynonymous mutations identified in protein S and protein C genes in patients ($n = 18$) with DVT during pregnancy and postpartum

Patient	cDNA[a]	Region	Amino acid change	Protein S[b] or protein C[c] activity (%)	Protein S[b] or protein C[c] activity, during pregnancy (%)	Age	Gravida	Parity	Body mass index	Family history	Other complications of pregnancy	Onset of DVT (weeks of gestation)	Delivery mode	Recurrence of DVT	Complication of PTE (weeks of gestation)
Protein S gene															
Case 1	c.586	Exon 6	K196E	57[b]	n.d.	30	1	1	18.6	None	None	27	TVD	None	27
Case 2	c.586	Exon 6	K196E	68[b]	25[b]	27	0	0	20.3	None	None	10	TVD	None	None
Case 3	c.1337	Exon 12	L446P	13[b,d]	<20[b]	30	0	0	18.8	None	None	27	TVD	None	None
Case 4	c.235	Exon 3	D79Y	4[b]	<1[b]	35	0	0	22.5	Father	None	6	C/S	None	None
	c.1889	Exon 15	T630I												
Protein C gene															
Case 5	c.440	Exon 6	C147Y	45[c]	57[c]	28	0	0	24.2	None	None	20	C/S	None	None

TVD term vaginal delivery, *C/S* cesarean section, *PTE* pulmonary thromboembolism

[a] Position from A of initial ATG in cDNA

[b] Protein S anticoagulant activity

[c] Protein C amidolytic activity

[d] Protein S activity was obtained under warfarin treatment

pregnant Caucasian women have reported a 3- to 12-times higher risk of thrombosis postpartum than during pregnancy [3, 4]. On the other hand, a large retrospective study found that events were twice as likely during pregnancy as postpartum [5]. In our new study, we found that Japanese patients with genetic mutations manifested DVT events in their first two trimesters (Table 2). In particular, pregnant Japanese patients with genetic mutation had no DVT events postpartum. Although this trend went against previous findings [3, 4], it was consistent with the results that there were twice as many DVT events during pregnancy as postpartum [5]. DVT onset at the early stage of pregnancy in patients with genetic mutation might be reasonable, since genetic mutation accelerates DVT onset, and patients with mutation might have DVT events in their early stage of pregnancy.

In the present study, we enrolled 18 pregnant Japanese women with DVT and found that four out of 18 patients (22% patients) had genetic mutations in the protein S gene. A previous study on thrombophilia activity screening in Japanese patients with DVT reported a high prevalence of protein S deficiency [31], and this was later confirmed by genetic analysis [19]. Taken together with these previous findings, our study reinforced the theory that protein S deficiency is an important risk factor for DVT in Japanese. This observation was in stark contrast to the case in Caucasians, in whom factor V Leiden and prothrombin G20210A mutations are involved in almost 50% of all DVT cases in pregnant women [8]. It is well known that the level of protein S activity was decreased immediately after pregnancy [25]. Therefore, predisposed thrombophilia should be considered in the care of patients with pregnancy-related complications, and antithrombotic prophylactic therapy might be applicable for those patients. Also, it might be good for women of child-bearing years to know their own thrombophilic nature.

A previous study reported on DNA sequence analyses of the protein S, protein C, and antithrombin genes in 173 Japanese DVT patients [20]. In this study, 55 patients (accounting for 32% of total patients) had nonsynonymous mutations in one of three genes. Among the three genes, mutations in the protein S gene were predominant, being found in 29 patients (17% of the total). Among various nonsynonymous mutations in the protein S gene, the K196E mutation was most prevalent. It was found in one out of 55–70 Japanese individuals, from analyses of general Japanese populations [19, 21, 22, 24]. In our study, we sequenced three genes in 18 patients with pregnancy-related thrombosis and identified missense mutations in five patients (accounting for 28% of the patients). Among five patients, four (22% of the total) had missense mutations in the protein S gene, which reconfirmed the predominance of inherited protein S deficiency in Japanese patients with

Table 2 Onset of DVT according to trimester of pregnancy and postpartum, and according to delivery mode

	Onset of DVT				Delivery mode			Complication of PTE
	First trimester	Second trimester	Third trimester	Postpartum period	Artificial abortion	Term vaginal delivery	Term cesarean section	
Patients with genetic mutation								
Protein S ($n = 4$)	2	2	0	0	0	3	1	1
Protein C ($n = 1$)	0	1	0	0	0	0	1	0
Total ($n = 5$)	2	3	0	0	0	3	2	1[a]
Patients without genetic mutation								
Total ($n = 13$)	4	5	0	4[c]	1[d]	8	4	1[b]

PTE pulmonary thromboembolism

[a] PTE events with genetic mutation occurred during the second trimester

[b] PTE events in the patients without genetic mutation occurred postpartum after cesarean section

[c] Two out of 4 patients without genetic mutation underwent cesarean section

[d] First trimester

DVT. Two of these patients had K196E mutation. Thus, K196E mutation in the protein S gene would be a genetic risk for not only DVT in general, but also for pregnancy-related DVT.

There are limitations to the present study. This was a small-scale retrospective study with 18 patients. We performed genetic analysis in those patients and identified five patients with genetic mutation. To understand the DVT risk in pregnant Japanese patients with inherited or acquired thrombophilia, we will have to recruit patients consecutively and perform thrombophilic screening, including genetic analysis, in the future evaluation.

In conclusion, we identified inherited thrombophilia in pregnant Japanese women with DVT and found protein S deficiency to be a predominant cause of thrombophilia. By DNA sequence analysis, we found two patients with a K196E mutation in the protein S gene that is prevalent in the Japanese population. Since pregnant women showed reduced protein S levels, a diagnosis of protein S deficiency based on its activity has an intrinsic limitation. Since the onset of DVT tends to occur at an early stage during pregnancy, the genetic analysis might be an alternative diagnostic tool.

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Regular Article

Predictive blood coagulation markers for early diagnosis of venous thromboembolism after total knee joint replacement

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ABSTRACT

Pulmonary embolism development may be prevented if asymptomatic venous thromboembolism (VTE) can be predicted and treated preoperatively or soon after total knee arthroplasty (TKA). The purpose of this study was to evaluate whether asymptomatic VTE can be predicted by blood coagulation markers preoperatively or early after TKA. This prospective single-centre study enrolled 68 patients (6 men, 62 women; mean age: 71 years) who underwent TKA between September 2004 and August 2009. Sixteen-row multidetector computed tomography was performed 4 days before and after surgery for diagnosis of asymptomatic VTE. Blood samples were taken to measure the plasma levels of soluble fibrin monomer complex (SFMC), D-dimer and cross-linked fibrin degradation products by leukocyte elastase (e-XDP) at 4 days preoperatively, and at 1 hour, 1 day and 4 days postoperatively. The preoperative SFMC, D-dimer and e-XDP levels did not differ significantly between the thrombus ($n=36$) and no-thrombus ($n=32$) groups. D-dimer and e-XDP levels showed the most significant increases at days 4 and 1, respectively, after surgery in the thrombus group. With cut-off points of 7.5 μg/ml for D-dimer and 8.2 U/ml for e-XDP, the sensitivities were 75% and 75%, and the specificities were 63% and 59%, respectively. By multiple logistic regression analysis, D-dimer at day 4 and e-XDP at day 1 postoperatively were independent markers for early diagnosis of VTE (odds ratio = 1.61 and 1.19, $P=0.01$ and 0.04, respectively). The postoperative occurrence of new asymptomatic VTE may be predicted by D-dimer at day 4 and e-XDP at day 1 after TKA.

Introduction

After arthroplasty, it is extremely important to prevent the development of postoperative venous thromboembolism (VTE), particularly symptomatic and fatal pulmonary embolism (PE), in orthopaedic surgery [1]. Since the 1990s, antithrombotic therapies using agents such as unfractionated and low molecular weight heparin have been administered to patients after surgery. However, despite the implementation of aggressive antithrombotic protocols, including those mandated by the American College of Chest Physicians (ACCP) [2], the incidence of fatal PE remains at 0.15% [3] and that of symptomatic PE remains at 0.41% [4] after total knee arthroplasty (TKA), with no changes since the 1990s. Pellergrini et al. [5] reported that 17% of patients with untreated deep vein thrombosis (DVT) experienced symptomatic PE after total hip arthroplasty (THA). While it is thought that prophylactic antithrombotic treatments are necessary to prevent postoperative fatal and symptomatic PE, previous reports found no differences in the incidences of fatal or symptomatic PE, regardless of whether or not prophylactic antithrombotic therapy was given [3,4,6], and that the infection rate was increased owing to haematoma caused by haemorrhage [7–9] and coagulation abnormalities [10] associated with prophylactic antithrombotic therapy early after surgery. It is also important for orthopaedic surgeons to avoid these complications, because such infections can last a lifetime or the patients can have a relapsing course if they achieve remission from the infection. The routine administration of prophylactic antithrombotic treatment is not recommended in the Japanese Guideline for Prevention of Venous Thromboembolism [11].

Abbreviations: ACCP, American College of Chest Physicians; e-XDP, Cross-linked fibrin degradation products by leukocyte elastase; DVT, deep vein thrombosis; MDCT, multidetector-row computed tomography; PE, pulmonary embolism; SFMC, Soluble fibrin monomer complex; THA, Total hip arthroplasty; TKA, total knee arthroplasty; VTE, Venous thromboembolism.

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Based on these observations, it is clinically important to detect asymptomatic VTE that may cause fatal or symptomatic PE before or shortly after surgery without prophylactic antithrombotic treatments to reduce postoperative infections in low-risk patients, and to start antithrombotic therapy only in those patients who need it [12].

The purpose of this study was to determine whether the postoperative occurrence of new asymptomatic VTE can be predicted by preoperative and postoperative measurements of blood coagulation markers in patients undergoing TKA, and to identify independent markers that will facilitate early diagnosis of asymptomatic VTE. We performed a prospective study using multidetector-row computed tomography (MDCT), which can detect PE and DVT simultaneously, to evaluate the predictive accuracy of various blood coagulation markers as indicators for postoperative asymptomatic VTE.

Materials and methods

Patients

The study protocol was approved by the Ethics Review Board of our university. This prospective single-centre study enrolled patients who underwent TKA at our institution between September 2004 and August 2009 and gave consent to participate in the study (Fig. 1). The necessary sample size was calculated for an alpha of 0.05 and a power of 0.90 using the statistical software 'G*Power 3' [13,14], and found to be 67. For exclusion criteria, patients with a past history of symptomatic VTE, cerebral haemorrhage, cerebral infarction, cardiac infarction and drug allergy to a contrast medium were excluded from the study. In addition, patients with liver disease, renal disease and congenital clotting factor deficiencies and those undergoing antithrombotic therapy or haemodialysis were excluded from the study. Patients with asymptomatic VTE by preoperative MDCT were also excluded from the study (Fig. 1). However, patients with hypertension, diabetes mellitus and rheumatoid arthritis were included in this study.

We finally enrolled 68 patients with low risk factors who underwent TKA for osteoarthritis (45 knees) or rheumatoid arthritis (23 knees). The patients comprised 6 men and 62 women, with a mean age of 71 years (range, 49–84 years). TKA was performed under general anaesthesia in all patients and a pneumatic tourniquet was used. During and after the surgery, the patients wore elastic stockings and used an intermittent pneumatic compression device until the initiation of walking training, in accordance with the Japanese Guideline for Prevention of Venous Thromboembolism [11]. No postoperative prophylactic antithrombotic therapy was administered. If the patients developed symptomatic VTE and if VTE was detected by MDCT, the study was discontinued and aggressive antithrombotic therapy was initiated.

MDCT

For diagnosis of VTE, 16-row MDCT was performed at 4 days preoperatively (day of admission) and 4 days postoperatively (Fig. 2), the point at which the incidences of PE and VTE are reported to be high [15] and the earliest point at which the patients could comfortably undergo MDCT during the postoperative period. The slice thicknesses were 2 mm in the thoracic region and 5 mm from the abdomen to the lower limbs. The window levels were 40–60 and 40–50 and the window widths were 400–500 and 200–400, respectively. A single radiologist (M.D.) evaluated the MDCT images in a blinded manner before and after the surgery. The incidence of postoperative new asymptomatic VTE was calculated.

Patients with asymptomatic VTE ($n=7$) by preoperative MDCT were excluded from the study. Preoperative MDCT revealed asymptomatic PE of the pulmonary segmental artery in one patient, proximal asymptomatic DVT in one patient and distal asymptomatic DVT in five patients (Fig. 1). These patients did not show D-dimer abnormalities. Preoperative MDCT revealed no asymptomatic VTE in the 68 patients included in the study. For the patients with proximal asymptomatic DVT and asymptomatic PE, antithrombotic therapy was initiated because the occurrence of fatal or symptomatic PE was considered likely [16].

The thrombus group was defined as patients with new asymptomatic VTE detected by MDCT after the surgery, and the no-thrombus group was defined as patients without asymptomatic VTE by MDCT after the surgery.

Blood coagulation markers

Blood samples were taken to measure the plasma levels of soluble fibrin monomer complex (SFMC), D-dimer and cross-linked fibrin

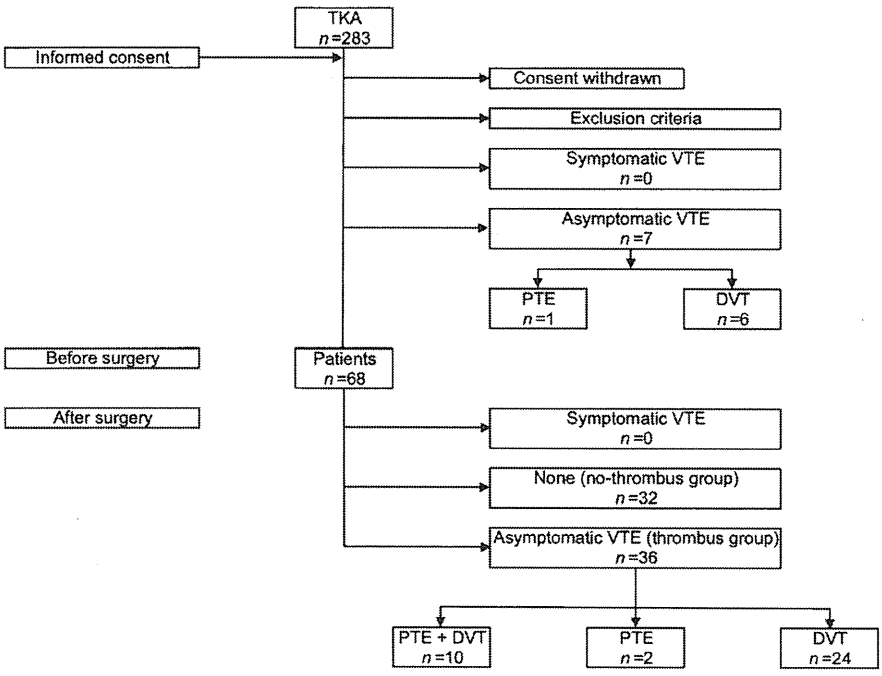

Fig. 1. Flowchart of the 283 patients undergoing TKA during the study period. *n*, number of patients.

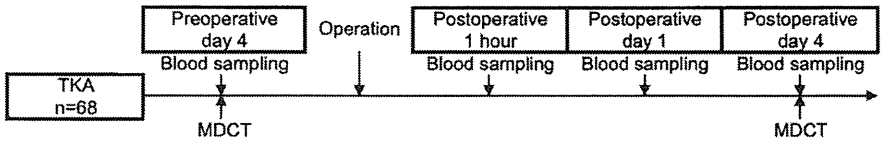

Fig. 2. Study protocol.

degradation products by leukocyte elastase (e-XDP) at 4 days preoperatively, and then at 1 hour, 1 day and 4 days postoperatively (Fig. 2). Citrated plasma samples were stored at −80 °C until analysis. The plasma SFMC levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) using the monoclonal antibody IF-43 [17]. Plasma D-dimer levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation) using the monoclonal antibody JIF-23 [18]. Plasma e-XDP levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation) using the monoclonal antibody IF-123 [19].

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 11.0 software (SPSS, Chicago, IL, USA). SFMC, D-dimer and e-XDP levels were analysed by the Shapiro–Wilk test if they did not fit a normal distribution. SFMC, D-dimer and e-XDP levels were compared at 4 days preoperatively and at 1 hour, 1 day and 4 days preoperatively using the Friedman test. If a significant difference was noted, the data were compared using the Wilcoxon signed rank test and corrected using Bonferroni's inequality. SFMC, D-dimer and e-XDP levels were compared between the thrombus and no-thrombus groups using the Mann–Whitney *U* test. Sex and diabetes mellitus distributions were compared between the thrombus and no-thrombus groups using Fisher's exact test, while hypertension distributions were compared between the thrombus and no-thrombus groups using the chi-square test. Age, volume of intraoperative haemorrhage, operation time, other presurgical factors and blood markers were compared using an unpaired *t*-test. Multiple logistic regression analyses were used to determine whether blood coagulation markers were independent predictors of the postoperative occurrence of new asymptomatic VTE or were affected by other factors. The level of statistical significance was set at $P<0.05$ for all tests.

Results

No patients developed symptomatic VTE after TKA in this study (Fig. 1). Postoperative MDCT revealed asymptomatic VTE in 36 patients (thrombus group) and no VTE in 32 patients (no-thrombus group) (Fig. 1). Aggressive antithrombotic therapy was initiated in the 36 patients in whom new asymptomatic VTE was detected on postoperative MDCT.

Preoperative blood coagulation markers

There were no significant differences in preoperative SFMC, D-dimer and e-XDP levels between the thrombus and no-thrombus groups ($P=0.13$, $P=0.18$ and $P=0.15$, respectively; Fig. 3).

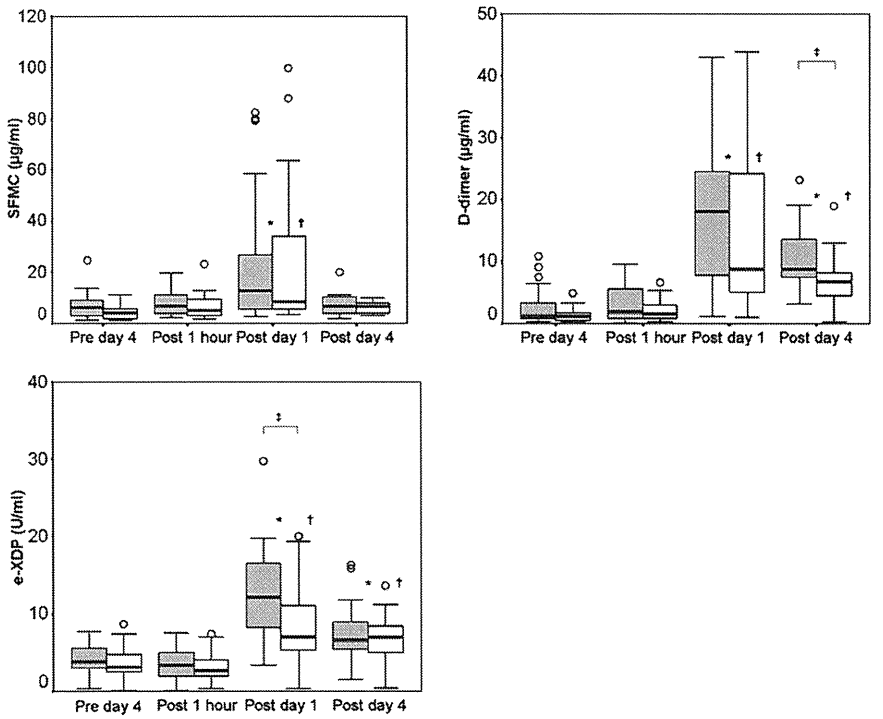

Fig. 3. Preoperative and postoperative SFMC, D-dimer and e-XDP levels. Gray boxes, thrombus group; white boxes, no-thrombus group. The circles are outliers. *$P<0.05$ versus the preoperative level in the thrombus group by the Wilcoxon signed-rank test with correction by Bonferroni's inequality. †$P<0.05$ versus the preoperative level in the no-thrombus group by the Wilcoxon signed-rank test with correction by Bonferroni's inequality. ‡$P<0.05$, thrombus group versus no-thrombus group by the Mann–Whitney *U* test.

Postoperative blood coagulation markers

SFMC

SFMC levels differed significantly at day 1 postoperatively in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, a significant increase in the SFMC level was observed at postoperative day 1 (median: 12.8 μg/ml; interquartile range: 5.3 to 27.2) compared with the preoperative value (median: 6.2 μg/ml; interquartile range: 3.1 to 9.3; $P=0.01$). Similarly, in the no-thrombus group, a significant increase in the SFMC level was observed at postoperative day 1 (median: 8.5 μg/ml; interquartile range: 5.6 to 34.9) compared with the preoperative value (median: 4.2 μg/ml; interquartile range: 2.2 to 6.4; $P=0.01$). However, postoperative changes in the SFMC level at postoperative day 1 did not differ significantly between the thrombus and no-thrombus groups ($P=0.85$; Fig. 3). SFMC levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour (median: 6.9 μg/ml; interquartile range: 4.3 to 11.4; $P=0.10$; and median: 5.3 μg/ml; interquartile range: 3.1 to 9.8; $P=0.07$, respectively) and 4 days (median: 6.8 μg/ml; interquartile range: 4.1 to 10.6; $P=0.27$; and median: 6.6 μg/ml; interquartile range: 4.2 to 8.2; $P=0.06$, respectively) postoperatively (Fig. 3).

D-dimer

D-dimer levels differed significantly at postoperative days 1 and 4 in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, significant increases in D-dimer levels were observed at postoperative day 1 (median: 18.1 μg/ml; interquartile range: 7.3 to 25.8) and day 4 (median: 8.8 μg/ml; interquartile range: 7.4 to 13.7) compared with the preoperative value (median: 1.3 μg/ml; interquartile range: 0.8 to 3.9; $P=0.01$ for both time points). In the no-thrombus group, significant increases in D-dimer levels were observed at postoperative day 1 (median: 8.8 μg/ml; interquartile range: 4.8 to 25.2) and day 4 (median: 6.8 μg/ml; interquartile range: 4.3 to 8.6) compared with the preoperative value (median: 1.2 μg/ml; interquartile range: 0.6 to 1.8; $P=0.01$ for both time points). With regard to the postoperative changes, the D-dimer level at postoperative day 4 was significantly higher in the thrombus group than that in the no-thrombus group ($P=0.01$; Fig. 3). With a cut-off D-dimer level of 7.5 μg/ml, the sensitivity was 75%, the specificity was 63% and the likelihood ratio (sensitivity/1–specificity) was 2.03 for predicting postoperative asymptomatic VTE (Fig. 4). D-dimer levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour postoperatively (median: 1.9 μg/ml; interquartile range: 0.9 to 5.6; $P=0.39$; and median: 1.5 μg/ml; interquartile range: 0.9 to 3.4; $P=0.09$, respectively) (Fig. 3).

e-XDP

The e-XDP levels differed significantly at postoperative days 1 and 4 in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, significant increases in e-XDP levels were observed at postoperative day 1 (median: 12.2 μg/ml; interquartile range: 7.9 to 18.2) and day 4 (median: 6.7 μg/ml; interquartile range: 5.3 to 9.0) compared with the preoperative value (median: 3.8 μg/ml; interquartile range: 3.0 to 5.6; $P=0.01$ for both time points). In the no-thrombus group, significant increases in e-XDP levels were observed at postoperative day 1 (median: 7.0 μg/ml; interquartile range: 5.3 to 11.1) and day 4 (median: 7.0 μg/ml; interquartile range: 4.6 to 8.4) compared with the preoperative value (median: 3.1 μg/ml; interquartile range: 2.6 to 4.7; $P=0.01$ for both time points). With regard to the postoperative changes, the e-XDP level at postoperative day 1 was significantly higher in the thrombus group than that in the no-thrombus group ($P=0.01$; Fig. 3). With a cut-off e-XDP level of 8.2 U/ml, the sensitivity was 75%, the specificity was 59% and the likelihood ratio was 1.84 for predicting postoperative asymptomatic VTE (Fig. 4). The e-XDP levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour postoperatively (median: 3.3 U/ml; interquartile range: 2.0 to 5.1; $P=0.33$; and median: 2.7 U/ml; interquartile range: 2.0 to 4.4; $P=0.08$, respectively) (Fig. 3).

There was a significant difference in the minimum blood pressure, but no significant differences in age, sex, volume of intraoperative haemorrhage, operation time or other presurgical factors between the thrombus and no-thrombus groups (Table 1). Multiple logistic regression analyses revealed that the D-dimer level at postoperative day 4 differed significantly among the other factors and was an independent marker of postoperative new asymptomatic VTE by MDCT (odds ratio = 1.61, $P=0.01$; Table 2). Likewise, the e-XDP level at postoperative day 1 differed significantly among the other factors and was an independent marker of postoperative new asymptomatic VTE (odds ratio = 1.19, $P=0.04$; Table 2).

Discussion

Recently, MDCT has been used as a technique to identify VTE. In addition, MDCT is able to diagnose PE to the level of the subsegmental pulmonary arteries and can provide rapid and objective detection and

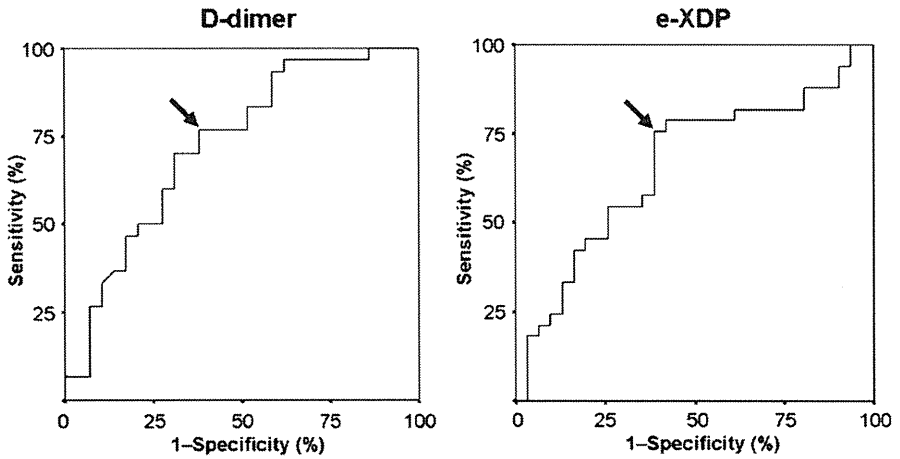

Fig. 4. Receiver-operator characteristic curves. Left: At a cut-off point of 7.5 μg/ml for the D-dimer level at postoperative day 4, the sensitivity is 75% (95% confidence interval: 73 to 77), the specificity is 63% (95% confidence interval: 60 to 66) and the likelihood ratio is 2.03. Right: At a cut-off point of 8.2 U/ml for the e-XDP level at postoperative day 1, the sensitivity is 75% (95% confidence interval: 73 to 77), the specificity is 59% (95% confidence interval, 56 to 62) and the likelihood ratio is 1.84.

Table 1
Patient characteristics.

		Thrombus group (n=36)	No-thrombus group (n=32)	P		95% Confidence interval
					(Odds ratio)	
Sex, male:female		1:35	5:27	0.09*	0.15§	0.02 to 1.40
Hypertension:normotension		18:18	14:18	0.61†	1.29¶	0.50 to 3.35
Diabetes mellitus:normoglycaemia		2:34	4:28	0.41*	0.41#	0.07 to 2.42
					*(Difference**)*	
Age	years	71 (68 to 73)††	72 (68 to 75)††	0.82‡	-0.5	-5 to 4
Intraoperative haemorrhage	ml	167 (0 to 344)††	175 (4 to 346)††	0.95‡	-7	-252 to 236
Operation time	min	161 (147 to 174)††	150 (140 to 160)††	0.23‡	10	-7 to 27
Before surgery						
Height	cm	148 (146 to 151)††	150 (148 to 153)††	0.25‡	-2	-6 to 1
Weight	kg	57 (54 to 60)††	57 (52 to 61)††	0.91‡	0	-5 to 5
Body mass index	kg/m^2	26 (25 to 27)††	25 (23 to 27)††	0.51‡	1	-2 to 3
Blood pressure (maximum)	mmHg	128 (124 to 133)††	127 (121 to 132)††	0.61‡	2	-5 to 9
Blood pressure (minimum)	mmHg	74 (71 to 77)††	67 (63 to 70)††	0.01‡	8	4 to 12
Pulse rate	/min	73 (70 to 76)††	71 (68 to 73)††	0.18‡	3	-1 to 7
Temperature	°C	36.5 (36.4 to 36.6)††	36.5 (36.4 to 36.7)††	0.58‡	-0.1	-0.2 to 0.1

*Fisher's exact test; †chi-square test; ‡unpaired t-test.
§Male/female; ¶hypertension/normotension; #diabetes mellitus/normoglycaemia; **thrombus-no thrombus; ††mean (95% confidence interval).

measurement of thrombi in both PE and DVT [20–24]. In patients in whom symptomatic PE is suspected, the usefulness of this modality is supported by its high sensitivity and specificity, which have been reported to be 100% and 89% for PE using pulmonary angiography as the reference standard [20], 100% and 96.6% for proximal DVT using doppler sonography as the reference standard [21] and 93% and 97% for distal and proximal DVT using doppler sonography and venography as the reference standard [22], respectively. Additionally, sensitivity and specificity have been reported to be 100% and 97% for distal DVT, 100% and 97% for proximal DVT, respectively, using conventional venography as the reference standard in patients in whom symptomatic DVT was suspected [23]. However, it is unknown how the sensitivity and specificity for only distal DVT of MDCT can be estimated, because there are few studies that have estimated sensitivity and specificity; overall, the sensitivity (ranges from 71 — 100%) and specificity (ranges from 93 — 100%) of MDCT are high [24]. A highly sensitive and specific imaging examination is necessary for early detection of VTE. However, there are problems with this approach that limit its practicality, such as exposure to radiation [25,26], invasive administration of contrast medium, potential for drug allergy, cost of equipment and the frequent imaging required to detect VTE, the occurrence of which is unpredictable. Therefore, initial evaluation of the presence of VTE using blood markers is preferable, with only high-risk patients examined by imaging techniques, to reduce the potential risks associated with radiation or contrast exposure and to improve the cost-effectiveness.

There were no blood coagulation markers for predicting early postoperative asymptomatic DVT after TKA until 1997 [27,28]. In 2000, Rever at al. [29] performed venography after TKA and reported that the SFMC level was significantly elevated in patients with asymptomatic DVT at postoperative days 3 and 6. However, they concluded that there was no clinically significant cut-off point. Similarly, the present study could not establish that the SFMC level was associated with asymptomatic VTE after TKA or an independent marker for predicting the postoperative occurrence of new asymptomatic VTE.

In 1998, Bounameaux et al. [30] performed venography and D-dimer measurements at day 3 after TKA. They found that the D-dimer level was significantly elevated in patients with asymptomatic DVT and that the sensitivity and specificity were 58.8% and 73.5%, respectively, at a cut-off level of 3000 μg/ml. In the present study, the D-dimer level at postoperative day 4 was significantly higher in the thrombus group than that in the no-thrombus group. The sensitivity, specificity and likelihood ratio of the D-dimer level at postoperative day 4 using a cut-off point of 7.5 μg/ml were 75%, 63% and 2.03, respectively, for predicting postoperative asymptomatic VTE. Furthermore, by multiple logistic regression analysis, the D-dimer level at postoperative day 4 was an independent marker for predicting postoperative asymptomatic VTE, whereas, except for the minimal blood pressure, there were no significant differences in age, sex, volume of intraoperative haemorrhage, operation time and presurgical factors or blood markers between the thrombus and no-thrombus groups.

Table 2
Multiple logistic regression analyses of blood coagulation markers and other factors.

		Postoperative day 1			Postoperative day 4		
		Odds ratio	95% Confidence interval	P	Odds ratio	95% Confidence interval	P
SMFC	μg/ml	1.00	0.98 to 1.02	0.84	1.00	0.97 to 1.04	0.88
D-dimer	μg/ml	0.97	0.93 to 1.02	0.29	1.61	1.12 to 2.20	0.01
e-XDP	U/ml	1.19	1.01 to 1.40	0.04	0.75	0.54 to 1.05	0.10
WBC	/μl	1.00	1.00 to 1.00	0.67	1.00	1.00 to 1.00	0.93
RBC	×10^4/μl	1.00	0.97 to 1.03	0.96	1.03	0.98 to 1.08	0.26
Hct	%	0.37	0.09 to 1.55	0.18	0.47	0.12 to 1.84	0.28
Hb	g/dl	13.49	0.30 to 604.51	0.18	3.34	0.06 to 239.21	0.52
Plt	×10^4/μl	1.05	0.94 to 1.17	0.40	1.16	0.97 to 1.38	0.10
Blood pressure (maximum)	mmHg	0.55	0.30 to 1.00	0.51	0.55	0.25 to 1.22	0.14
Blood pressure (minimum)	mmHg	2.31	0.84 to 6.40	0.11	2.73	0.93 to 8.00	0.07
Pulse rate	/min	0.91	0.42 to 2.00	0.82	0.75	0.29 to 1.94	0.55
Temperature	°C	1.2	0.46 to 3.08	0.72	12.47	0.88 to 177.10	0.06

WBC=white blood cell count; RBC=red blood count; Hct=haematocrit; Hb=haemoglobin; Plt, platelet count.

It has been reported that the level of e-XDP, which are the fibrin degradation products by leukocyte elastase released from activated leukocytes [31], is useful for the diagnosis and prognosis of disseminated intravascular coagulation [32–34] and the diagnosis of symptomatic DVT [35]. However, no previous studies have measured the e-XDP level before and after orthopaedic surgery to evaluate its usefulness for diagnosis of postoperative VTE. In this study, the e-XDP level at postoperative day 1 was significantly higher in the thrombus group than that in the no-thrombus group. The diagnostic sensitivity, specificity and likelihood ratio of the e-XDP level at postoperative day 1 using a cut-off point of 8.2 U/ml were 75%, 59% and 1.84, respectively, for predicting postoperative asymptomatic VTE. Therefore, the e-XDP level at postoperative day 1 is estimated to be a blood marker for early prediction of postoperative asymptomatic VTE. Furthermore, by multiple logistic regression analysis, the e-XDP level at postoperative day 1 differed significantly among the other factors and there were no significant differences in age, sex, volume of intraoperative haemorrhage, operation time or other presurgical factors, except for the minimal blood pressure, or blood markers between the thrombus and no-thrombus groups. Therefore, this study has established that the e-XDP level at postoperative day 1 and the D-dimer level at postoperative day 4 are associated with asymptomatic VTE after TKA and are independent markers for predicting the postoperative occurrence of new asymptomatic VTE. Recent studies have demonstrated that leukocyte elastase-mediated fibrinolysis is activated to varying degrees depending on the amount of systemic inflammation, such as a major surgical procedure, and sepsis as an alternative pathway to the plasminogen activator-plasmin system [32–36]. We consider that leukocyte elastase-mediated fibrinolysis may have been mainly activated in the early phase as an alternative pathway, and then plasmin may have been mainly activated in the late phase as the plasminogen activator-plasmin system in patients who developed asymptomatic VTE after TKA. If leukocyte elastase causes asymptomatic VTE and subsequently symptomatic and fatal PE, inactivation of leukocyte elastase may prevent the development of symptomatic and fatal PE.

The postoperative occurrence of new asymptomatic VTE could not be predicted from the preoperative SFMC, D-dimer or e-XDP levels. Dunn et al. [37], who performed venography after TKA or THA, and Bounameaux et al. [30], who employed venography after TKA, compared the preoperative D-dimer levels between patients who did and did not develop DVT postoperatively and reported no significant differences. Our results are consistent with these previous reports, and we could not establish that preoperative blood coagulation markers are associated with VTE after TKA.

In this study, the early diagnosis of new asymptomatic VTE that can be predicted by the D-dimer level at postoperative day 4 and the e-XDP level at postoperative day 1 after TKA may prevent PE development after TKA. Therefore, in daily clinical practice, we consider that measurements of e-XDP at postoperative day 1 and D-dimer at postoperative day 4 are necessary for early detection of asymptomatic VTE in patients who have low risk factors because they are independent markers. However, we need to further verify these measurements in larger studies to determine the adequate cut-off points, sensitivities and specificities of e-XDP at postoperative day 1 and D-dimer at postoperative day 4 for preventing symptomatic and fatal PE in the perisurgical period. One limitation of our study is that we do not know whether early detection of asymptomatic VTE prevents symptomatic and fatal PE. Therefore, we have continued to follow up the patients in daily clinics after completing this study, and no patients have suffered from symptomatic and fatal PE to date. Another limitation of our study is that MDCT was performed 4 days preoperatively and postoperatively, and the results therefore reflect the state of asymptomatic VTE at these time points. We believe that almost all asymptomatic VTEs may disappear spontaneously within 2 or 3 days postoperatively and that an asymptomatic VTE that does not disappear spontaneously within 2 or 3 days postoperatively can cause symptomatic and fatal PE in low-risk patients after TKA. This is because the incidence of PE was reported to be high at days 3 or 4 postoperatively after TKA [15]. Furthermore, that is the earliest point at which the patients had less pain and could comfortably undergo MDCT during the postoperative period. However, since MDCT was not performed from the operative day to postoperative day 3, it can be assumed that not all asymptomatic VTE were detected in the perisurgical period. Therefore, the incidence of postsurgical asymptomatic VTE may be underestimated, and the roles of e-XDP and D-dimer levels in predicting asymptomatic VTE in the perisurgical period must be further verified in larger studies.

Conflict of interest

The authors state that they have no conflict of interest.

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Regular Article

Local regulation of neutrophil elastase activity by endogenous α1-antitrypsin in lipopolysaccharide-primed hematological cells

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ABSTRACT

Neutrophil elastase released from activated neutrophils contributes in combating bacterial infection. While chronic inflammation results in anemia and decreased bone marrow activities, little is known about the effect of neutrophil elastase on hematological cell growth in severe inflammatory states. Here, we demonstrated that α1-antitrypsin, a physiological inhibitor of neutrophil elastase, functions as a regulator for cell growth by neutralizing neutrophil elastase activity in lipopolysaccharide-primed hematological cells. HL-60 cells were resistant to neutrophil elastase, as they also expressed α1-antitrypsin. The growth of HL-60 cells transduced with a LentiLox-short hairpin α1-antitrypsin vector was significantly suppressed by neutrophil elastase or lipopolysaccharide. When $CD34^+$ progenitor cells were differentiated towards a granulocytic lineage, they concomitantly expressed neutrophil elastase and α1-antitrypsin and prevented neutrophil elastase-induced growth inhibition. These results suggest that granulocytes might protect themselves from neutrophil elastase-induced cellular damage by efficiently neutralizing its activity through the simultaneous secretion of endogenous α1-antitrypsin.

Introduction

Neutrophil elastase is implicated in antimicrobial defense by degrading engulfed microorganisms [1–4]. Neutrophil elastase is a potent protease as it cleaves almost all connective tissue components as well as soluble proteins [5–7]. At the site of inflammation, neutrophil elastase released from azurophilic granules of the activated leukocyte is thought to mediate tissue destruction through its proteolytic cleavage of cell surface glycoproteins, extracellular matrix and junctional complexes [5,8]. The activity of neutrophil elastase is counteracted by endogenous inhibitors [9–11]. The serine protease inhibitor, α1-antitrypsin possesses potent anti-neutrophil elastase activities [12]. Abnormalities of α1-antitrypsin have been associated with liver damage arising from pathologic polymerization of the variant α1-antitrypsin, and with the development of pulmonary emphysema or panniculitis due to inflammatory stimuli leading to the unregulated activity of neutrophil elastase [13–16].

Neutrophil elastase is also known to provide feedback to granulopoiesis through direct proteolytic action on granulocyte-colony stimulating factor (G-CSF) [17]. Patients with mutations in the gene encoding neutrophil elastase (ELANE) display severe congenital neutropenia due to abnormal traffic of neutrophil elastase and induction of the unfolded protein response [18–20]. In addition, neutrophil elastase induces apoptosis of hematopoietic progenitor cells, which is prevented by a secretory proteinase inhibitor [17,21]. Chronic infection or inflammation results in anemia and decreased bone marrow activities [22,23], and hematopoietic efficacy declines with hematopoietic cell apoptosis and altered cytokine production [24,25]. However, little is known regarding the effect of neutrophil elastase on hematological cell growth or regulation by α1-antitrypsin in severe inflammatory states such as sepsis.

In this study, we investigated the possibility that the growth of hematological cells may be affected by the enzymatic activity of neutrophil elastase and that is regulated by endogenous alpha1-antitrypsin under the stimulation of lipopolysaccharide.

Materials and methods

Cell lines and cell cultures

The human leukemia cell lines HL-60 and K562 were obtained from the American Type Culture Collection (ATCC, Manassas VA, USA) and MEG-01 was purchased from the European Collection of Cell Cultures (ECACC, Down, UK). They were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated FBS (Gibco). Cells were adapted to serum free AIM-V Medium (Gibco) as needed. Human embryonic kidney 293 T cells

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were also purchased from the ATCC and grown in DMEM/F-12 medium (Gibco) supplemented with 10% heat-inactivated FBS. Human cord blood cells were isolated from healthy volunteers with informed consents according to the Declaration of Helsinki.

In vitro differentiation of human cord blood derived CD34$^+$ cells

Human cord blood-derived CD34$^+$ progenitor cells from four independent donors were isolated with a CD34 MicroBead Kit (Miltenyi Biotech, Auburn, CA, USA), and cultured with StemPro-34 SFM (Invitrogen, San Diego, CA, USA). These cells were differentiated into three lineages of hematological cells using appropriate cytokines: granulocytic (10 ng/mL GM-CSF, 10 ng/mL IL-3, and 50 ng/mL SCF), erythrocytic (3 U/mL EPO, 10 ng/mL IL-3 and 50 ng/mL SCF) and megakaryocytic (50 ng/mL TPO, 10 ng/mL IL-3, and 10 ng/mL IL-6) [26].

Measurement of neutrophil elastase activity

The enzymatic activity of neutrophil elastase was determined by an amidolytic reaction to a specific substrate, methoxysuc-AAPV-p-nitroanilide (Sigma-Aldrich, St. Louis, MO, USA) and measuring its absorbance at 405 nm with a Bechmark Plus Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA), as previously described [27].

Detection of human neutrophil elastase mRNA and related factor mRNA

RNA samples were subjected to reverse transcription-polymerase chain reaction (RT-PCR) using the following primer pairs: neutrophil elastase forward (5'-GTAAACTTGCTCAACGACATC-3') and reverse (5'-CTCACGAGAGTGCAGACGTT-3'); α1-antitrypsin forward (5'-CAGATCAACGATTACGTGGAGA-3') and reverse (5'-GCTTCATCATAGGGACCTTCAC-3'); and GAPDH forward (5'-AAGGTGAAGGTCGGAGTC-3') and reverse (5'-GAAGATGGTGATGGGATTTC-3'). The RT-PCR was performed using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA, USA) as previously described. In brief, reverse transcription was performed at 42 °C for 30 minutes followed by PCR. The initial denaturation step was at 95 °C for 5 minutes followed by 30 cycles of denaturation at 95 °C for 15 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 7 seconds.

Construction of lentiviral α1-antitrypsin shRNAi vectors

To knock down α1-antitrypsin, shRNAi vectors were constructed. As a control, a scramble sequence was inserted into the vector used. The piGENE hU6 vector (iGENE Therapeutics, Tokyo, Japan) was digested with the restriction enzymes EcoRI (Toyobo, Osaka, Japan) and HindIII (Fermentas Life Sciences, Ontario, Canada). The fragment incorporating the hU6 promoter and a BfuAI site was extracted with a MinElute Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and inserted into the pBC SK + vector (Stratagene, La Jolla, CA, USA), linearized with *Eco*RI and *Hind*III. After digestion with *Xba*I and *Xho*I (Toyobo), they were cut and inserted into the pLentiLox 3.7 vector (pLL3.7 vector; ATCC) previously treated with *Xba*I and *Xho*I. Short interfering RNAs (siRNAs) targeted towards human α1-antitrypsin were based on sequences within the NCBI database. Sense and antisense oligonucleotides of siRNA were designed by Hokkaido System Science (Sapporo, Japan). The sequences oligonucleotides used in this study were: short hairpin sense (5'-CACCGTTTGGGTATGTTTAGCATACGTGTGCTGTCCGTATGTTAAACATGCCTAAACTTTTT-3') and antisense (5'-GCATAAAAAGTTTAGGCATGTTTAACATACGGACAGCACACGTATGCTAAACATACCCAAAC-3'); scramble sense (5'-CACCGATCATAGATAGCACAGGTACGTGTGCTGTCCGTACTTGTGCTATCTGTGGTCTTTTT-3') and antisense (5'-GCATAAAAAGACCACAGATAGCACAAGTACGGACAGCACACGTACCTGTGCTATCTATGATC-3'). These oligonucleotides were ligated into pLL3.7 at the *Bfu*AI site. The pLL3.7 shRNAi plasmid was co-transfected with three plasmids containing PV, REV and VSV-G into 293 T cells. Viral RNA was purified using a QIAamp Viral RNA Mini kit (QIAGEN), and the number of viral particles was determined with a TaqMan PCR Core reagent kit (Applied Biosystems) and an ABI 7700 System (Applied Biosystems). The primers used for quantitative RT-PCR analysis were: forward (5'-GCTTTCATTTTCTCCTCCTT-3') and reverse (5'-GGCCACAACTCCTCATAA-3') along with a FAM-labeled probe (5'-ATCCTGGTTGCTGTCT-3').

Stimulation of hematological cells with neutrophil elastase or lipopolysaccharide (LPS)

Cultured hematological cells were treated with various concentrations of neutrophil elastase (Elastin Products, Owensville, MO, USA), α1-antitrypsin (Sigma-Aldrich) and sivelestat sodium hydrate (ONO Pharma, Osaka, Japan). For LPS stimulation, 1×10^6 cells were incubated with 10 ng/mL LPS (Sigma-Aldrich) at 37 °C for 45 minutes. After washing, cells were re-suspended in the medium with 100 nM N-formyl-Met-Leu-Phe (fMLP; Sigma-Aldrich) for another 45 minutes at 37 °C, as described previously [28].

Western blot analysis

Cells were lysed with a lysis buffer (10 mM Tris-HCl pH 7.6, 1% NP40, 0.15 M NaCl, 1 mM EDTA, 10 μg/mL aprotinin), and each sample consisted of 1×10^6 cells. After centrifugation, 10 μg of sample was applied to SDS-PAGE then electrically transferred to PVDF membranes. After blocking with 20 mM Tris-HCl, (pH 7.6) and 150 mM NaCl containing 2% bovine serum albumin (BSA) at 25 °C for 1 hour, the membrane was incubated with mouse anti-human α1-antitrypsin antibody (1:3000; Ikagaku, Kyoto, Japan) followed by goat anti-mouse IgG horseradish peroxidase-conjugated antibody (1:10000; Invitrogen) in 20 mM Tris-HCl, (pH 7.6), 150 mM NaCl containing 0.1% Tween20 for detection by an ECL Western Blot Detection system (GE Healthcare, Buckinghamshire, UK).

Enzyme-linked immunosorbent assays (ELISAs)

The levels of neutrophil elastase antigen were measured using a Human Elastase ELISA kit from Hycult biotechnology (Uden, Netherlands). The α1-antitrypsin antigen levels were measured using an AssayMax Human α1-Antitripsin Elisa Kit from Assaypro (St. Charles, MO, USA). Concentrations of transforming growth factor-β1 (TGF-β1) were determined by a Quantikine Human TGF-β1 Immunoassay kit from R&D Systems (Minneapolis, MN, USA). The manufacturer's recommended protocols were followed for each kit.

Immunohistochemistry

Cytospin slides containing 1×10^5 cells were prepared with a Cytospin3 (Shandon, Cheshire, UK). After fixation with ethanol, they were incubated with phosphate-buffered saline (PBS) containing 1% BSA and 1% non-immune goat serum for 10 minutes at 25 °C. After blocking, slides were stained for 2 hours at 4 °C using a rabbit anti-human neutrophil elastase antibody (1:200; Calbiochem, Darmstadt, Germany) or mouse anti-human α1-antitrypsin antibody (1:200; Ikagaku) diluted with PBS containing 1% BSA as primary reagents. After washing with PBS, they were incubated with Alexa Fluor-488 labeled goat anti-rabbit IgG antibody (1:500; Invitrogen) or Alexa Fluor-555 conjugated rabbit anti-mouse IgG antibody (1:500; Invitrogen) for 1 hour at 25 °C. Nuclei were stained with DAPI (Roche, Basel, Switzerland).

Terminal dUTP nick-end labeling (TUNEL) Assay

Cytospin slides with 1×10^5 cells were fixed with ethanol, and the TUNEL assay was performed using the DeadEnd Colorimetric

Apoptosis Detection System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Quantification of TUNEL positive cells was determined by mean percentage of apoptotic cells from the total number of cells counted in five fields per slide.

Statistical analysis

The SPSS statistical software package (SPSS, Chicago, IL, USA) was used for all statistical analyses of data. Variables not normally distributed were analyzed with the two-sided Mann-Whitney *U* test. A difference with $p<0.03$ was considered statistically significant.

Results

Effect of LPS on secretion of neutrophil elastase and proliferation in hematological cells

We stimulated three different hematological cell types, HL-60, K562 and MEG-01 cells with LPS. The antigen levels of neutrophil elastase were increased only in HL-60 cells (Fig. 1 A). However, the enzymatic activity of neutrophil elastase was markedly decreased until four hours after the stimulation (Fig. 1 B). LPS did not affect proliferation of any cell type (Fig. 1 C). Then, we cultured 1×10^5 cells/mL of HL-60, K562 and MEG-01 for 24 hours in the absence or presence of the supernatants derived from HL-60 cells that had been stimulated with 10 ng/mL LPS for one hour. Interestingly, the proliferation of K562 and MEG-01 cells was markedly inhibited in the presence of culture medium derived from LPS-stimulated HL-60 cells, although their supernatants did not suppress proliferation of naïve HL-60 cells (Fig. 1 D). These inhibitory effects on K562 and MEG-01 cells were canceled by a human neutrophil elastase specific inhibitor, sivelestat sodium (Fig. 1 D).

Effect of neutrophil elastase on proliferation of hematological cells

To determine whether neutrophil elastase could inhibit proliferation of hematological cells, HL-60, K562 and MEG-01 cells were cultured in the presence of neutrophil elastase. Proliferation of K562 and MEG-01 was significantly inhibited by treating with 1 nM neutrophil elastase for 72 hours (Fig. 2 A), and their proliferation was suppressed in a dose-dependent manner (Fig. 2 B). In contrast, neutrophil elastase had no effect on the proliferation of HL-60 cells. Both K562 and MEG-01 underwent apoptosis in a dose-dependent manner with neutrophil elastase (Fig. 2 C and D). There was no significant increase in the number of apoptotic cells when HL-60 cells were cultured in the presence of 5 nM neutrophil elastase.

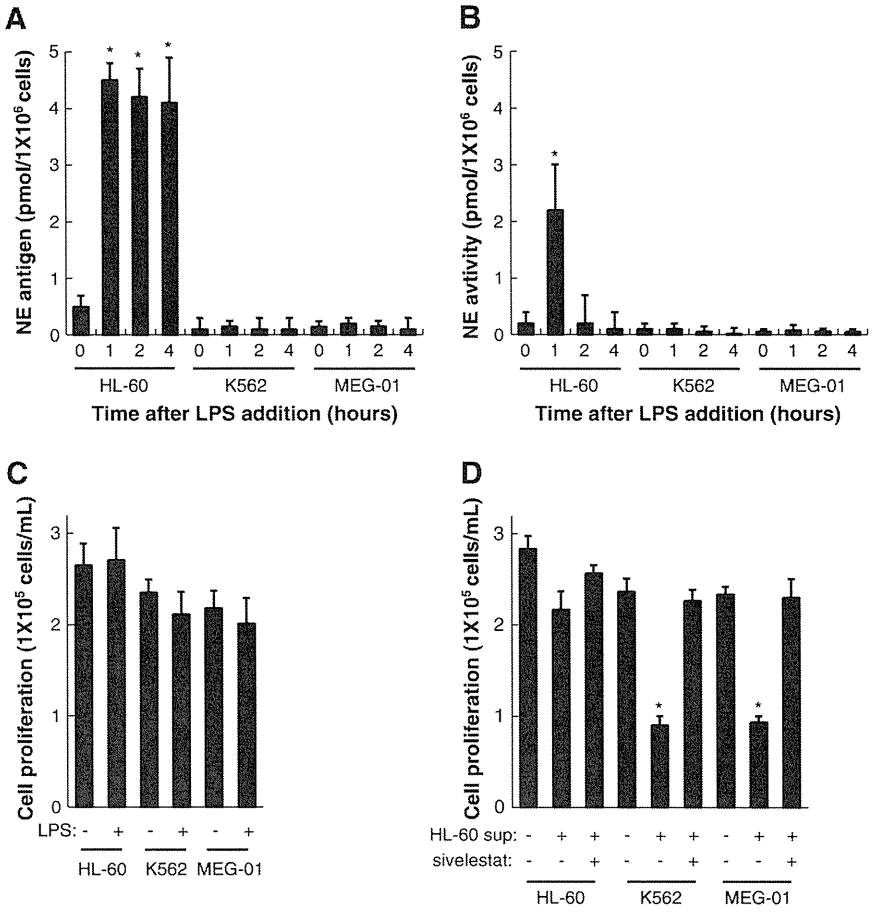

Fig. 1. Effects of LPS on secretion of neutrophil elastase and proliferation in hematological cells. A - B. 1×10^5 cells of three different hematological cell types HL-60, K562 and MEG-01 were stimulated with 10 ng/mL LPS for 0, 1, 2 and 4 hours. Neutrophil elastase antigen levels (A) and activity (B) were measured as described in the Materials and methods. C. 1×10^5 cells/mL HL-60, K562 and MEG-01 cells were cultured in the absence or presence of 10 ng/mL LPS. Cell numbers for each cell were counted at 24 hours by trypan-blue viable staining. D. 1×10^5 cells/mL of naïve HL-60, K562 and MEG-01 were cultured in the absence or presence of the supernatants derived from HL-60 cells that had been stimulated with 10 ng/mL LPS for 1 hour. HL-60, K562 and MEG-01 cells were also cultured in the presence of LPS-stimulated HL-60 supernatants treated with 100 nM sivelestat sodium. Cell numbers for each cell were counted at 24 hours. Values are mean ± SD; *$p<0.03$ compared with controls. (A), (B) and (C) $n=5$, (D) $n=3$. NE, neutrophil elastase.

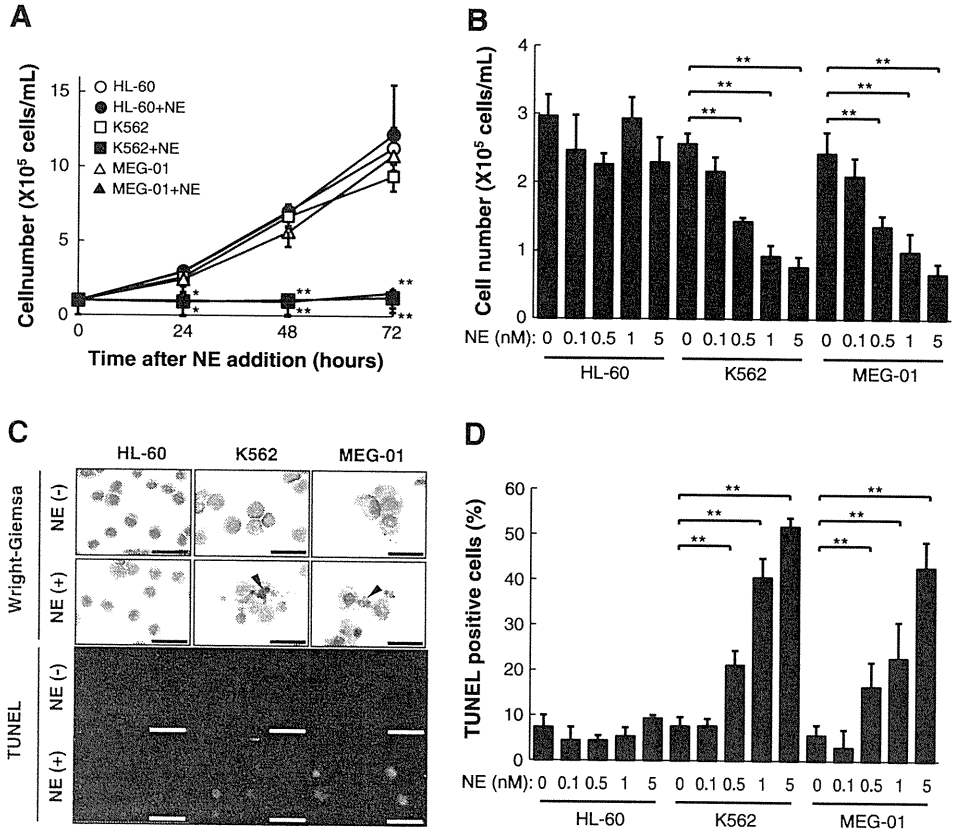

Fig. 2. Effects of neutrophil elastase on proliferation and induction of apoptosis in hematological cells. A. HL-60, K562 and MEG-01 cells were cultured in the absence or presence of 1 nM neutrophil elastase for 72 hours. Cell numbers for each cell were counted every 24 hours. B. HL-60, K562 and MEG-01 cells were cultured in the presence of 0–5 nM neutrophil elastase for 24 hours, and viable cell numbers were determined. C. Morphological changes in HL-60, K562 and MEG-01 cells were analyzed using Wright-Giemsa staining 24 hours post-stimulation with 1 nM neutrophil elastase (upper panels). For TUNEL labeling, apoptotic cells demonstrated a greater intensity of fluorescence (lower panels). Representative data from three independent experiments are shown. Arrow heads demonstrate apoptotic cells. Scale bars indicate 50 μm. D. The proportion of TUNEL-positive cells was calculated for each cell line treated with neutrophil elastase. Values are mean ± SD; $^{*}p<0.03$ and $^{**}p<0.01$ compared with controls. (A), and (B) $n=5$, (D) $n=3$. NE, neutrophil elastase.

HL-60 neutralizes neutrophil elastase activity by concomitant secretion of α1-antitrypsin

To clarify how HL-60 cells were not subject to apoptotic induction by neutrophil elastase, we measured residual antigen and enzymatic activity after its addition. We detected both antigen and enzymatic activity of neutrophil elastase in K562 and MEG-01 culture supernatants at levels similar to which they were added (Fig. 3 A and B). In HL-60 cultures, antigen levels of neutrophil elastase increased in a dose-dependent manner (Fig. 3 A), however enzymatic activity was not increased even after the addition of 0.3 or 1 nM neutrophil elastase (Fig. 3 B). Apoptotic effects on K562 and MEG-01 cultures were no longer evident when 1 nM neutrophil elastase was present together with its specific inhibitor, sivelestat sodium, at a concentration of 100 nM (Table 1), suggesting that cell type-specific apoptotic effects of neutrophil elastase might be based on its enzymatic activity. Thus, we focused on a natural inhibitor of neutrophil elastase, α1-antitrypsin. As shown in Fig. 3 C, HL-60 cells expressed α1-antitrypsin mRNAs, although the amount of mRNA did not increase after stimulation with neutrophil elastase. The α1-antitrypsin proteins were observed in the cytoplasmic granules of HL-60 cells but their appearance diminished following stimulation with neutrophil elastase (Fig. 3 D). Levels of α1-antitrypsin antigen in the culture medium of HL-60 cells significantly increased following stimulus with neutrophil elastase (Fig. 3 E). In contrast, α1-antitrypsin levels in HL-60 cell lysates were very low (Fig. 3 F).

Silencing of endogenous α1-antitrypsin expression with a shRNA lentiviral vector induces inhibition of HL-60 proliferation following the stimulation with neutrophil elastase

To confirm that the absence of α1-antitrypsin suppresses HL-60 growth when neutrophil elastase is present in the culture, we used siRNAs to knock down endogenous α1-antitrypsin expression. We examined the efficiency of the lentiviral shRNA for α1-antitrypsin with eGFP expression in HL-60 cells. As shown in Fig. 3 A, HL-60 cells transduced with the LentiLox-short hairpin or scramble α1-antitrypsin demonstrated expression of eGFP. Expression of α1-antitrypsin protein was significantly suppressed by transduction with LentiLox-short hairpin α1-antitrypsin (Fig. 4 B and C), demonstrating that silencing of endogenous α1-antitrypsin by a shRNA lentiviral vector system is effective in HL-60 cells. The proliferation of HL-60 cells transduced with the LentiLox-scramble α1-antitrypsin was not affected by neutrophil elastase (Fig. 4 D). However, α1-antitrypsin deficient HL-60 cells transduced with LentiLox-short hairpin sequences (Fig. 4 E) exhibited significant growth retardation after stimulation with neutrophil elastase (Fig. 4 D).

Effect of LPS on α1-antitrypsin deficient HL-60 cells transduced with a shRNA lentiviral vector

When HL-60 cells were stimulated with LPS, it was found that all cells secreted neutrophil elastase antigen regardless of whether they

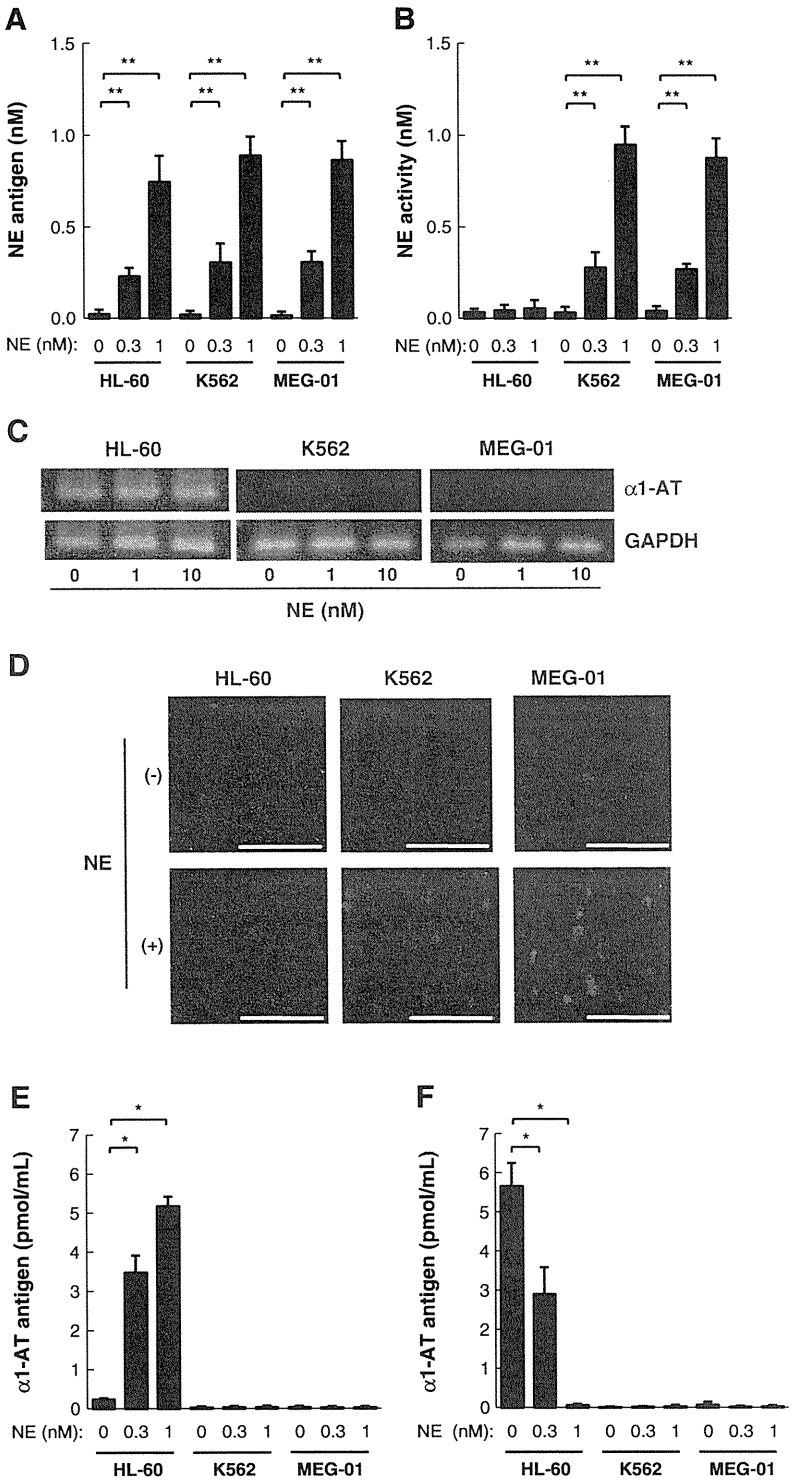

Fig. 3. HL-60 cells neutralize neutrophil elastase activity by secretion of α1-antitrypsin. A - B. Level of neutrophil elastase antigens (A) and enzyme activity (B) in a culture medium containing 1×10^5 cells/mL of HL-60, K562 and MEG-01 cells was measured 24 hours after the addition of neutrophil elastase (0, 0.3 and 1 nM), as described in the Materials and methods. C. HL-60, K562 and MEG-01 cells were cultured in the presence of 0–10 nM neutrophil elastase for 24 hours, and then α1-antitrypsin and GAPDH mRNA of each cell were analyzed by RT-PCR. D. HL-60, K562 and MEG-01 cells were cultured in the absence (upper panels) or presence (lower panels) of 1 nM neutrophil elastase for 24 hours, and then stained with anti-human α1-antitrypsin IgGs (red) and DAPI (blue) Scale bars indicate 50 μm. E-F. Levels of α1-antitrypsin antigens in culture medium (E) and cell lysate (F) of 1×10^5 cells/mL HL-60, K562 and MEG-01 cells were measured 24 hours after the addition of neutrophil elastase (0, 0.3 and 1 nM), as described in the Materials and methods. Values are the mean ± SD ($n = 3$, in each group). $^{**}p < 0.01$ compared with controls. NE, neutrophil elastase; α1-AT, α1-antitrypsin.

were transduced with LentiLox-scrambled or LentiLox-short hairpin α1-antitrypsin (Fig. 5 A). However, the neutrophil elastase activity in α1-antitrypsin deficient HL-60 cells was higher when compared to HL-60 controls or cells transduced with LentiLox-scrambled α1-antitrypsin (Fig. 5 B). Knock-down of α1-antitrypsin with the shRNA lentiviral vector efficiently suppressed α1-antitrypsin secretion

Table 1
Effect of sivelestat sodium on neutrophil elastase-induced apoptosis in hematological cells.

NE (nM)	sivelestat sodium (nM)	TUNEL positive cells (/1X103 cells)		
		HL-60	K562	MEG-01
0	0	4.7 ± 1.8	5.6 ± 1.9	3.0 ± 0.8
1	0	4.7 ± 0.6	47.2 ± 4.2**	39.8 ± 4.8**
1	10	4.2 ± 1.5	48.8 ± 5.4**	38.4 ± 2.5**
1	50	5.4 ± 2.3	34.7 ± 6.7**	28.0 ± 5.0**
1	100	4.4 ± 0.3	9.0 ± 2.2	7.7 ± 3.0

HL-60, K562 and MEG-01 cells were stimulated with 1 nM human neutrophil elastase in the presence of 0–100 nM of sivelestat sodium hydrate for 24 hours. TUNEL positive cells were calculated in each of 1×10^3 cells. Values are mean ± SD; n = 4; **$p < 0.01$ compared with controls. NE, neutrophil elastase.

following LPS-stimulus (Fig. 5 C). As expected, the growth of HL-60 cells transduced with LentiLox-short hairpin α1-antitrypsin was significantly suppressed when treated with LPS compared to cells transduced with LentiLox-scramble α1-antitrypsin (Fig. 5 D). Additionally, when HL-60 cells were stimulated with LPS in the presence of sivelestat sodium, levels of α1-antitrypsin in the culture medium did not increase (data not shown).

Effect of neutrophil elastase on in vitro differentiated hematological cells derived from CD34$^+$ progenitor cells

Human cord blood-derived CD34$^+$ progenitor cells were treated with differentiation protocols to produce cells of granulocytic, erythrocytic and megakaryocytic lineages as previously described [29]. We found that

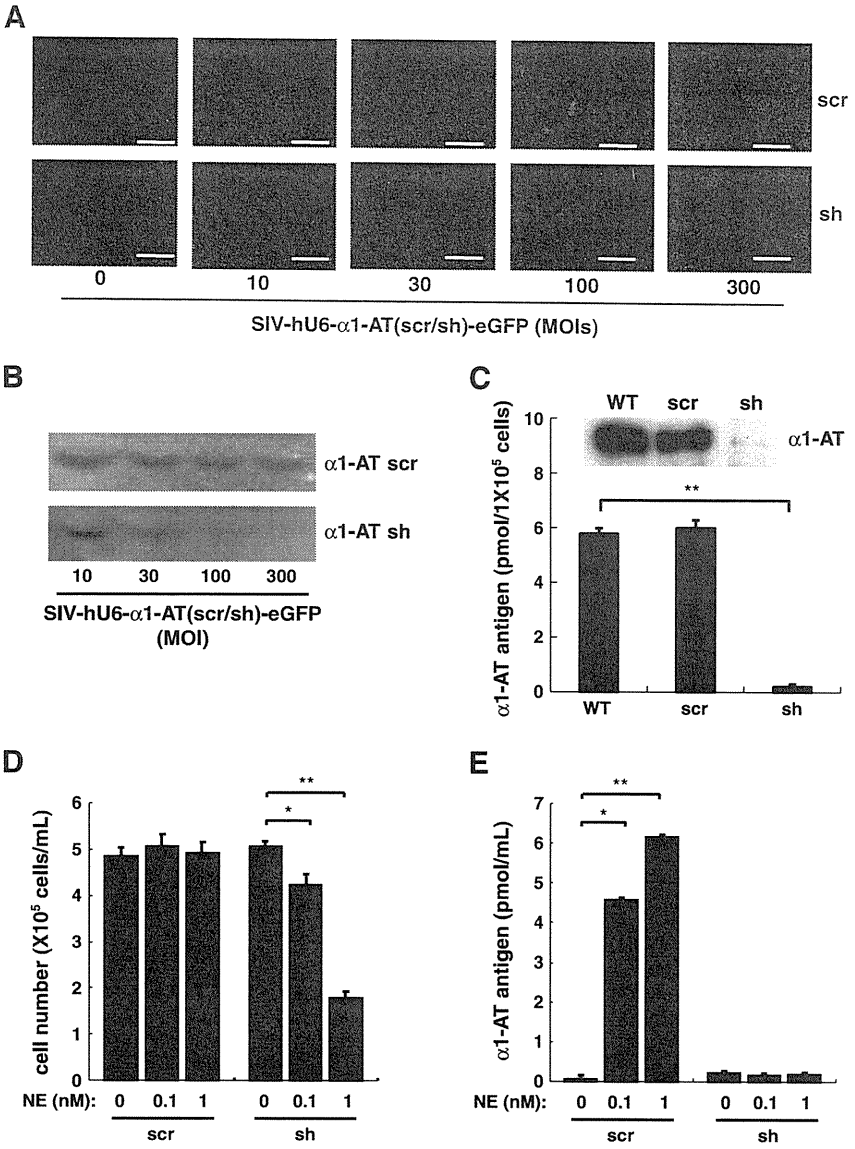

Fig. 4. Silencing of endogenous α1-antitrypsin expression with a shRNA lentiviral vector in HL-60 cells. A. HL-60 cells were transduced with LentiLox-scramble α1-antitrypsin (upper panels) or LentiLox-short hairpin α1-antitrypsin (lower panels) at multiplicities of infection (MOIs) of 0–300. Representative data from three independent experiments are shown. Scale bars show 50 μm. B. Cell lysates of HL-60 cells transduced with LentiLox vectors were analyzed with SDS-PAGE followed by Western blotting with anti-human α1-antitrypsin IgGs. Representative data from three independent experiments are shown. C. Amounts of α1-antitrypsin antigen were measured by ELISA in the cell lysates of 1×10^5 HL-60 cells transduced without (WT, $n = 5$) or with LentiLoxs (scr, $n = 5$; sh, $n = 5$). D - E. HL-60 cells transduced with LentiLox-short hairpin α1-antitrypsin or LentiLox-scramble α1-antitrypsin were cultured in the absence ($n = 5$) or presence of 0.1 nM ($n = 5$) and 1 nM ($n = 5$) neutrophil elastase for 72 hours and viable cell numbers (D) and antigen levels of α1-antitrypsin (E) were measured. Values are mean ± SD; *$p < 0.03$ and **$p < 0.01$ compared with controls. NE, neutrophil elastase; α1-AT, α1-antitrypsin; WT, non-transduced; scr, LentiLox-scramble α1-antitrypsin transduced; sh, LentiLox-short hairpin α1-antitrypsin transduced HL-60 cells.

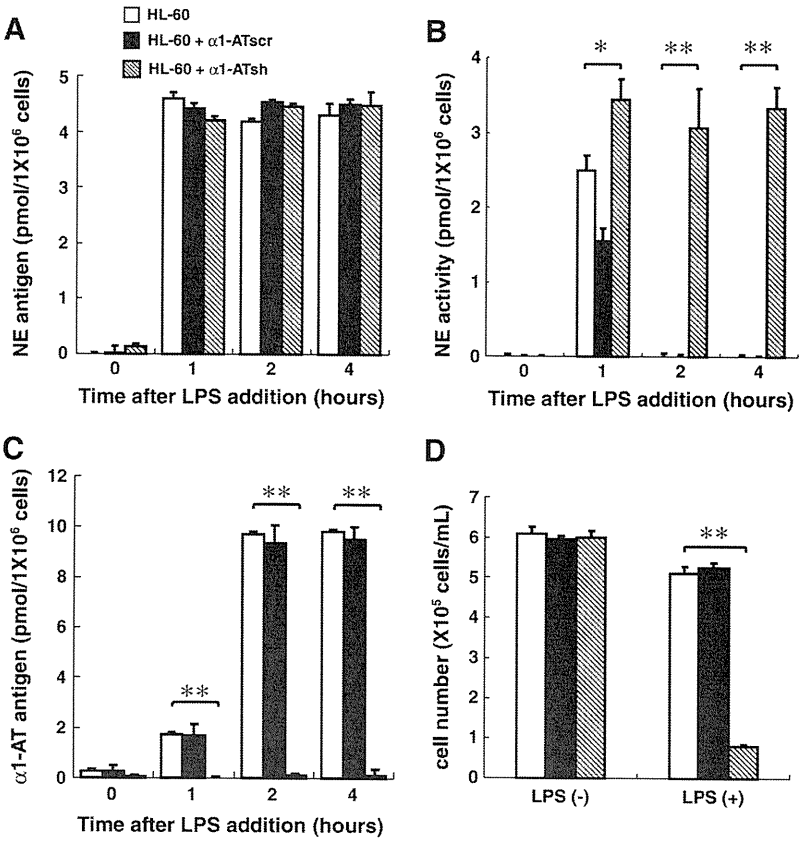

Fig. 5. Effects of LPS on α1-antitrypsin deficient HL-60 cells transduced with shRNA lentiviral vectors. A - C. 1×10^5 HL-60 cells ($n = 4$, bars), LentiLox-scramble transduced HL-60 cells ($n = 4$, shaded bars), LentiLox-short hairpin α1-antitrypsin transduced HL-60 cells ($n = 4$, hatched bars) were stimulated with 10 ng/mL LPS for 0, 1, 2 and 4 hours. Neutrophil elastase antigen levels (A), activity (B) and α1-antitrypsin antigen levels (C) were measured as described in the Materials and methods. D. 1×10^5 HL-60 cells ($n = 4$, bars), LentiLox-scramble transduced HL-60 cells ($n = 4$, shaded bars), LentiLox-short hairpin α1-antitrypsin transduced HL-60 cells ($n = 4$, hatched bars) were stimulated with 10 ng/mL LPS. After 48 hours, cell numbers of viable HL-60 cells were counted. Values are mean ± SD; $^*p < 0.03$ and $^{**}p < 0.01$ compared with controls. NE, neutrophil elastase; α1-AT, α1-antitrypsin; LPS, lipopolysaccharide.

progenitor cells could differentiate into three lineages, with the lineage of these cells determined by morphological assessment using a phase-contrast microscope, and detection of lineage-specific surface markers observed until 16 days after the induction of differentiation (Fig. 6 A). Interestingly, the only differentiated cells that concomitantly expressed neutrophil elastase with α1-antitrypsin mRNA were a granulocytic lineage, suggesting that this enzyme-inhibitor system may be up-regulated during granuloid cell differentiation. Proliferation of erythrocytic and megakaryocytic lineage cells was significantly suppressed by 1 nM neutrophil elastase as compared to granulocytic lineage cells (Fig. 6 D). Residual neutrophil elastase activity was found in erythrocytic as well as megakaryocytic cells with very little activity apparent in granulocytic cells (Fig. 6 E). However, only granulocytic lineage cells possessed α1-antitrypsin antigen in their culture supernatants after stimulation with neutrophil elastase (Fig. 6 F). We found that granulocytic lineage cells secreted significant amounts of active leukocyte elastase shortly after stimulation with LPS when compared to erythrocytic and megakaryocytic lineages (Fig. 6 B and C). When each lineage of cells was cultured with the supernatant derived from LPS-stimulated granulocytic cells, both erythrocytic and megakaryocytic lineage cells were significantly suppressed in their proliferation (Fig. 6 G).

Discussion

Neutrophil elastase is known to contribute towards combating bacterial infection [1–3]. Paradoxically, neutrophil elastase damages host tissues such as the intestine, kidney and lung during inflammation [10,30,31]. In our study, active neutrophil elastase was secreted not from K562 or MEG-01 but from HL-60 cells after stimulation of LPS (Fig. 1). In addition, the proliferation of K562 and MEG-01 was markedly inhibited after addition of culture medium derived from LPS-treated HL-60 cells, and the effects were canceled by sivelestat sodium. Several researchers have shown that neutrophil elastase induces endothelial and epithelial apoptosis [10,32]. We showed that neutrophil elastase significantly inhibited proliferation of K562 as well as MEG-01 cells by inducing apoptosis (Fig. 2). Interestingly, neutrophil elastase did not affect cell growth of HL-60 cells. Thus, the damage of hematological cells may involve a disturbance in the balance between neutrophil elastase activity and its inhibitor in response to LPS.

It has been reported that neutrophils respond to surface stimulation by secreting neutrophil elastase and α1-antitrypsin, and that there might be an inherent mechanism for damping the local effects of neutrophil elastase [33,34]. Apoptotic effects of neutrophil elastase on K562 and MEG-01 cultures were absent when the enzymatic activity of neutrophil elastase was neutralized by sivelestat sodium (Table 1) [31,35]. Only HL-60 cells exhibited resistance to apoptotic induction as they stored α1-antitrypsin, which was secreted rapidly after stimulation by neutrophil elastase (Fig. 3). These results suggest that cell type-specific effects of neutrophil elastase on hematological cell growth might be dependent on the neutralizing activity of neutrophil elastase by endogenous α1-antitrypsin.