Fluorogenic Assay of Activated Factor VII
Plasma Factor VIIa Levels in Relation to Arterial Cardiovascular Diseases in Japanese

Kazuomi Kario, Toshiyuki Miyata, Toshiyuki Sakata, Takefumi Matsuo, Hisao Kato

Abstract  Factor VII (FVII) plays an important role in initiation of the tissue factor–induced coagulation pathway. An increase in FVII coagulant activity (FVIIc) has been proposed as an independent risk factor for coronary artery disease. However, it remains uncertain whether high FVIIc levels are due to an increase in the activation of FVII or an increase in the concentration of FVII mass. We developed a new fluorogenic assay for plasma activated FVII (FVIIia) that used soluble tissue factor. The sensitivity of this assay ranged from 0.2 to 1000 ng FVIIa per milliliter of plasma. Plasma FVIIa levels were measured in 110 healthy subjects and 93 patients with hypertension, diabetes mellitus, and/or cardiovascular disease. The mean plasma FVIIa level in healthy Japanese individuals was 2.5 ng/mL, which was lower than that in Western subjects. Gel filtration analysis showed that most of the circulating FVIIa was in a free form, and binding of FVIIa to tissue factor in plasma was not detected. Aging increased both the FVIIa level and FVII mass, whereas menopause increased mainly the FVII mass. Elderly patients with arterial cardiovascular diseases showed increases in plasma FVIIa levels and FVIIa to FVII antigen (FVII:Ag) ratios. Among the elderly, arterial cardiovascular disease was more common in a high-FVIIa than in a low-FVIIa group. Plasma FVIIa levels were not correlated with serum levels of total cholesterol or triglycerides. The FVIIa level and the FVIIa-to-FVII:Ag ratio were positively correlated with fibrinogen level and negatively correlated with body mass index and serum albumin level in the elderly. In conclusion, aging, cardiovascular disease, and malnutrition increased plasma FVIIa levels. FVIIa levels were not correlated with lipid levels or hepatic synthesis, suggesting that FVIIa may be an independent risk factor for cardiovascular disease. (Arterioscler Thromb. 1994;14:265-274.)

Key Words  • factor VIIa assay  • factor VII coagulant activity  • factor VII antigen  • coronary artery disease  • cerebral infarction  • aging  • lipids
that such trace amounts of circulating FVIIa may initially activate FVII complexed with cell-surface tissue factor. Thus, measurement of FVIIa activity in plasma may be more important than determination of FVIIc or FVII mass and may help to resolve the controversies mentioned above. The extracellular domain of tissue factor loses the ability to promote FVII autoactivation or enhance the FXα-catalyzed activation of FVII but retains the ability to promote FVIIa-catalyzed activation of FXα. Using these characteristics, a coagulation assay that directly measures plasma FVIIa level without interference from the zymogen form of FVII has been developed, which uses a mixture of phospholipid and truncated soluble tissue factor.30,31

Most previous studies of FVII have been performed with adults less than 65 years old. We recently found that plasma FVIIc and FVII:Ag levels correlated positively with atherosclerosis and coronary artery disease in elderly Japanese individuals despite the low incidence of coronary artery disease in this country.2,13 In the present study, we developed a new fluorogenic assay for FVIIa, determined plasma FVIIa levels in young and elderly subjects, and compared FVIIa with FVIIc and FVII:Ag levels. Moreover, we investigated the relation between plasma FVIIa levels and arterial cardiovascular disease as well as normal aging, menopause, and serum lipids.

Methods

Assay of FVIIa

Human plasma FVIIa and human recombinant FVIIa were kindly provided by the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan, and Novo Nordisk Japan, Tokyo, Japan, respectively. The FVIIa concentration was determined by using a 1% extinction coefficient at 280 nm of 13.9 and a molecular weight of 50 000.32 Congenital human FVII-deficient plasma (<1% FVII activity) was purchased from George King Bio-Medical, Overland Park, Kan. Rabbit brain cephalin and protease-free bovine serum albumin (BSA) were purchased from Sigma Chemical Co, St Louis, Mo. Rabbit brain cephalin was prepared in saline according to the manufacturer’s instruction. A fluorogenic peptide substrate for thrombin, N-tetra-butoxycarbonyl-Val-Pro-Arg-4-methylcoumaryl-7-amide, was purchased from Peptide Institute Inc, Osaka, Japan. Recombinant soluble human tissue factor (sTF, residues 1 to 217) was expressed in yeast and purified according to a published method.30 The sTF concentration was determined by using a 1% extinction coefficient at 280 nm of 14.52 and a molecular weight of 37 000.33

The clot-based assay for FVIIa was originally developed by Morrissey et al.31 We modified it as follows. The first reagent was prepared by mixing 1 mL of congenital FVII-deficient plasma, 1.5 mL rabbit brain cephalin, and 5 mL Tris-buffered saline (0.02 mol/L Tris-HCl, pH 8.0, containing 0.15 mol/L NaCl). The sTF-negative start reagent was prepared by mixing 400 μL of 10 mmol/L N-tetra-butoxycarbonyl-Val-Pro-Arg-4-methylcoumaryl-7-amide, 250 μL of 1 mol/L CaCl2, and 10 mL Tris-buffered saline. Then the sTF-positive start reagent was prepared by adding 25 μL of 2.2 mmol/L sTF to 10.6 mL of the sTF-negative start reagent. A 5-μL aliquot of sample plasma was mixed with 225 μL of the first reagent (the ratio of FVII-deficient plasma to sample plasma was 6:1) and incubated for 30 seconds at 37°C. Then the reaction was started by adding 60 μL of the sTF-positive start reagent and 20 μL water (the final concentration of sTF was 10 mmol/L). The fluorescence generated was measured automatically by using a Cobas Fara II centrifugal analyzer (Roche Products Ltd, Welwyn Garden City, UK) with excitation at 380 nm and emission at 450 nm as described.33 Relative fluorescence intensity was determined by assigning 100 μmol/L 7-amino-4-methylcoumarin the value of 1.0. Fluorescence readings obtained at 30-second intervals were fed into a computer (NEC PC-9801Vm) and transformed into reaction times, which were defined as the time elapsed to reach a relative fluorescence of 0.05 (corresponding to that of 5 μmol/L 7-amino-4-methylcoumarin). Standard curves for calibrating the sTF fluorogenic assay for FVIIa were prepared by using various concentrations of human plasma FVIIa diluted with Tris-BSA buffer (0.02 mol/L Tris-HCl, pH 9.0, containing 0.15 mol/L NaCl and 0.1% BSA). To determine the basal level of hydrolysis of the fluorogenic substrate by plasma, the assay was performed without sTF by using the sTF-negative start reagent.

The effects of bilirubin, hemoglobin, triglycerides, and phospholipid on FVIIa activity were examined by using Interference Check kit A (Green Cross Inc, Osaka, Japan).

Gel filtration analysis was performed on a HiLoad 16/60 Superdex 200 column (Pharmacia, Uppsala, Sweden) equipped with a fast protein liquid chromatography system (Pharmacia). The column was equilibrated and eluted with Tris-buffered saline containing 0.05% NaN3 at room temperature and a flow rate of 1 mL/min. Five hundred microliters of plasma was applied to the column, 1-mL fractions were collected, and a 50-μL aliquot of each fraction was used in the FVIIa assay. Molecular-weight calibration of the column was performed with gel filtration standards (Bio-Rad, Richmond, Calif).

Other Assay Procedures

FVIIc was measured with a chromogenic assay autoanalyzer (Behring Chromoimeter, Behringwerke AG), a human placentalcalfed thromboplastin reagent (Chromogenix, Bevingwerke AG),36 and FVII-deficient plasma. FVII:Ag was determined with an enzyme-linked immunosorbent assay kit (Diagnostica Stago). Fibrinogen levels were determined with a one-stage clotting assay kit (Data-Fit, Dade). Plasma levels of FVIII and FX were determined by chromogenic assays with Testzyme VIII and Testzyme FX (Chromogenix AB), respectively. The plasma antithrombin III level was determined by a chromogenic assay with Berichrom antithrombin III (Behringwerke AG). Protein C amiodiolic activity was measured by using S-2366 (KabiVitrum AB) as the substrate and southern copperhead venom (Protac, Pentapharm, Basel, Switzerland) as the activator for protein C. In all of these studies, commercially available pooled plasma (CTS standard plasma, Behringwerke AG) was taken as 100%. Serum levels of total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and glucose were determined at the central laboratory of SRL Co Ltd, Tokyo, Japan, by the following enzymatic procedures. Serum total cholesterol and triglyceride levels were determined with commercial enzyme assay kits (Wako, Osaka, Japan). Serum HDL-C was determined by an enzymatic procedure after precipitation with phosphotungstic acid (Wako). Serum glucose was determined with a commercial enzyme assay kit (Kanto Chemicals, Tokyo, Japan). Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald equation. Serum levels of albumin, pseudocholinesterase, and uric acid were also measured with enzyme assay kits (Wako). The body mass index was calculated as weight (kg)/height squared (m2). The FVIIc-to-FVII:Ag ratio was calculated as an indicator of the proportion of FVIIa. The FVIIa-to-FVII:Ag ratio was also calculated as an indicator of the extent of activation of FVII to FVIIa by taking the mean plasma FVIIa level of young Japanese control subjects (2.1 ng FVIIa/mL) as 100%.

Subjects

Forty-two healthy young control subjects (18 men and 24 women; mean age, 29 years; range, 15 to 40 years) and 68 healthy elderly subjects (35 men and 33 women; mean age, 77 years; range, 64 to 95 years) were selected from among...
participants in annual health examinations. Some were living in the community and some were residents of a local home for the aged. They showed normal results in standard laboratory tests (leukoocyte count <8000/μL, C-reactive protein <0.8 mg/dL, serum creatinine <1.2 mg/dL, blood urea nitrogen <20 mg/dL, and aspartate aminotransferase or alanine aminotransferase <35 UI/L), did not have any current illnesses, and were not receiving any medical treatment. Twenty-nine hypertensive elderly subjects (15 men and 14 women; mean age, 80 years; range, 63 to 93 years) and 13 diabetic subjects (3 men and 10 women; mean age, 78 years; range, 62 to 92 years) were also studied. In addition, 22 elderly outpatients with coronary artery disease (18 with a prior myocardial infarction and 5 with stable angina), and 6 with both diseases were studied as arterial cardiovascular disease subjects. All patients with a prior myocardial infarction had abnormal Q waves on the electrocardiogram and had been stable for at least 6 months. Coronary angiography was performed in 8 subjects with a prior myocardial infarction and in 3 subjects with stable angina. Echocardiography was performed in the remaining patients and detected regional akinesis or hypokinesis. Cerebral infarction was diagnosed by neurological signs and brain computed tomography, and all of these patients had been stable for at least 3 months since the ictus. Some of these patients were receiving oral antiplatelet agents and antihypertensive therapy, but subjects receiving lipid-lowering agents were excluded from this study. Patients with a systolic blood pressure ≥160 mm Hg and/or a diastolic blood pressure ≥95 mm Hg on three occasions or those receiving antihypertensive therapy were diagnosed as having hypertension. Those with a fasting blood glucose level ≥160 mg/dL or receiving antidiabetic therapy were diagnosed as having diabetes mellitus. All of the subjects were ambulatory and had normal appetites.

After a minimum fasting period of 12 hours, blood samples for hemostatic determinations were collected into disposable, siliconized, evacuated glass tubes containing 3.8% trisodium citrate (9 vol blood to 1 vol 0.13 mol/L trisodium citrate solution), and blood samples in the second tube were used for the FVIIa assay. Samples were centrifuged at 3000 g for 15 minutes at room temperature within 1 hour of collection. Plasma was subsequently separated and stored in plastic tubes at -80°C until laboratory determinations were performed. At the time of assay, plasma samples were transferred to a water bath at 37°C for 10 minutes, and FVIIa levels were measured within 30 minutes after thawing. To avoid an artificial increase in FVIIa activity, care was taken not to leave thawed samples for long, even in the plastic tubes.

Statistical Analysis

Data are shown as mean±SD. One-way ANOVA and Welch’s t test were used for comparisons of mean values in the various groups. Mean values in samples from the same patient were compared by the paired t test, with P<0.05 indicating statistical significance. In addition, Pearson’s correlation coefficients were calculated for the different variables, and P<0.01 indicated statistical significance.

Results

Measurement of FVIIa

The original assay for FVIIa was the clot-based method.26 We modified this method for the Cobas Fara II centrifugal autoanalyzer by measuring the hydrolysis of a fluorogenic thrombin substrate instead of clot formation. Fig 1 shows typical reaction curves obtained with various concentrations of the FVIIa substrate (shown by the dashed line) corresponds to the hydrolysis of a certain amount of substrate by thrombin that is formed as a result of complex formation between plasma FVIIa and stTF. Fig 2 shows the standard curves for the routine assay of FVIIa with 10 nmol/L stTF. The reaction time required for a 0.05 increase in fluorescence was plotted versus the concentration of FVIIa on a log-log scale. A higher concentration of stTF (100 nmol/L) shortened the reaction time below that obtained in the routine analysis shown here, but the range of linearity was the same (0.2 to 1000 ng FVIIa/mL; data not shown). Prolonging the incubation time from 30 seconds to 5 minutes did not affect reaction time (data not shown). In the routine assays, 1.0, 2.0, 10, and 20 ng FVIIa/mL were always measured. The FVIIa levels in all plasma samples in our study were within this range. To determine the basal hydrolysis of the fluorogenic substrate, an assay without stTF was performed for every plasma sample, with the stTF-negative solution as the start reagent. The stTF-independent activity of the samples was below the sensitivity limit of the assay, indicating that there was no interference with FVIIa activity.

We performed recovery studies in which we added different amounts of FVIIa to a plasma sample and measured FVIIa levels. Normal plasma showed 2.3 ng FVIIa/mL. Addition of 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 ng FVIIa yielded values of 3.0, 3.4, 5.7, 8.3, 10.9, and 14.1 ng FVIIa/mL, respectively, which showed good recovery of the added FVIIa. Next we added a fixed amount of FVIIa (2.5 ng) to five different normal plasma samples and measured FVIIa levels twice. Five different normal plasma samples measured 1.94±0.62 ng FVIIa/mL plasma; addition of 2.5 ng FVIIa yielded 4.28±0.60 ng FVIIa, indicating that 2.34±0.30 ng FVIIa was recov-
erated. Next we diluted three normal plasma samples and measured FVIIa activity. The results are shown in Fig 2 (insert), in which the dilution curves for the three samples are parallel. As described above, addition of FVIIa to plasma samples and dilution of plasma samples did not affect the reported values.

We examined the effects of bilirubin, hemoglobin, triglycerides, and phospholipid on FVIIa activity. Bilirubin, up to 10.7 mg/dL plasma, did not affect the results, nor did up to 113 mg hemoglobin/dL plasma, but FVIIa activity was decreased when more than 113 mg hemoglobin/dL plasma was added. FVIIa activity was not influenced by up to 170 mg triglycerides or 182 mg phospholipid/dL plasma.

The interassay and intra-assay coefficients of variation were 4.2% and 1.3%, respectively. We examined the effect of varying sample conditions on the FVIIa fluorogenic assay in several ways. Triple freezing and thawing of seven samples from high-FVIIa elderly subjects and seven samples from low-FVIIa elderly subjects did not cause a significant change in FVIIa levels (data not shown). Samples stored in plastic tubes at 4°C or room temperature for more than 5 hours showed a slight increase in FVIIa activity, and samples stored in unsiliconized glass tubes at 4°C for 15 hours showed a marked increase (data not shown), indicating that cold activation occurred during storage, as described by Morrissey et al.1 There were no significant differences in FVIIa levels in the two plasma samples collected sequentially into two separate syringes (data not shown). There were also no significant differences between FVIIa levels in plasma obtained in the fasting and postprandial states (data not shown), as described by Morrissey et al.3 To study individual between-day variations, blood from the same individuals was collected six times over a period of 2 months and FVIIa levels were measured. Each subject showed a characteristic level of FVIIa, and there was little change during the 2-month period (Fig 3). Subjects 2, 3, and 4 constantly showed low FVIIa levels, whereas subjects 5, 6, and 7 showed high FVIIa activity. This was consistent with previous findings that showed that plasma samples obtained from three donors on three sequential days showed constant levels.31

**Gel Filtration Analysis**

It is well known that FVIIa binds very strongly to tissue factor.1,36 The presence of FVIIa in plasma gives rise to the question of whether FVIIa binds to tissue factor associated with lipids. To address this question, plasma (0.5 mL each) from three individuals was applied to a gel filtration column, and the FVIIa activity of the eluant was measured (Fig 4). Sample 1 was from a 30-year-old healthy subject (open triangles), sample 2 from an 88-year-old hypertensive man with a prior myocardial infarction (open circles), and sample 3 from an 87-year-old hypertensive woman with chronic cerebral infarction (closed circles). The plasma FVIIa levels were 2.8, 4.6, and 5.9 ng/mL, respectively. The peak FVIIa activity eluted in fractions 70 to 75. From the calibration curve obtained with the use of molecular-weight markers, the apparent molecular weight of FVIIa was calculated to be 70 000. Both VLDL-C and LDL-C eluted in fractions 41 to 47, and HDL-C eluted in fractions 55 to 65.
Fraction number (1 ml/tube)

**FIG 4.** Gel filtration analysis of plasma. A HiLoad 16/60 Superdex 200 column was equilibrated and eluted with Tris-buffered saline containing 0.05% NaN₃ at room temperature and a flow rate of 1 mL/min. Five hundred microliters of plasma was applied to the column and 1-mL fractions were collected. Sample 1 was from a 30-year-old healthy subject (A), sample 2 from an 88-year-old hypertensive man with a prior myocardial infarction (o), and sample 3 from an 87-year-old hypertensive woman with chronic cerebral infarction (•). Plasma activated factor VII (FVIIa) levels were 2.8, 4.6, and 5.9 ng/mL, respectively.

A small amount of FVIIa activity eluted in the later HDL fractions. Thus, most of the FVIIa activity in plasma eluted in a free form, indicating that it was not associated with membrane-bound tissue factor under conditions of gel filtration. In addition, FVIIa coeluted with FVIIc (data not shown).

### Age- and Sex-Related Changes of Plasma FVIIa Levels in Healthy Individuals

Table 1 shows plasma levels of FVIIa, FVIIc, and FVII:Ag in healthy young and elderly subjects. Morrissey et al have reported a significant correlation between FVIIa levels and increasing age in both males and females. We confirmed this correlation and furthermore found an age-related increase in the FVIIa-to-FVII:Ag ratio in healthy individuals of both sexes, although there was a wide range of values. In both sexes, FVIIc and FVII:Ag levels also increased with age. In young individuals, sex-related differences in FVIIa, FVIIc, and FVII:Ag were not significant. However, plasma FVIIc and FVII:Ag levels in elderly women were significantly higher than those in elderly men, whereas FVIIa did not differ significantly. The FVIIa-to-FVII:Ag ratio was greater in elderly men than in elderly women.

### Relation Between Plasma FVIIa Levels and Arterial Cardiovascular Disease

Table 2 shows plasma levels of FVIIa, FVIIc, and FVII:Ag in the elderly subjects with arterial cardiovascular disease. Elderly individuals with arterial cardiovascular disease showed significantly higher FVIIa levels than did healthy subjects, and the FVIIa-to-FVII:Ag ratio also tended to be higher in this group. When the patients with arterial cardiovascular diseases were divided into three groups, ie, patients with coronary artery disease, chronic cerebral infarction, or both, plasma FVIIa levels and the FVIIa-to-FVII:Ag ratio were highest in those with both coronary artery disease and chronic cerebral infarction, whereas there was no significant difference in the plasma FVIIa level or FVIIa-to-FVII:Ag ratio between those with coronary artery disease alone and chronic cerebral infarction alone (data not shown). Plasma FVIIa levels also tended to be increased in hypertensive or diabetic subjects compared with healthy subjects, but the difference was not significant. Similar trends were also observed for plasma FVIIc and FVII:Ag levels, but the differences were not significant.

We investigated the prevalence of arterial cardiovascular disease among the high-or low-plasma-FVIIa groups. The high-FVIIa group (n=22) was defined as having each parameter greater than the mean + 1 SD, and the low-FVII group (n=26) was defined by each parameter being below the mean - 1 SD. In the high-FVIIa group, 63% of subjects had arterial cardiovascular disease compared with only 27% in the low-FVIIa group. There was also an increased incidence of cardiovascular disease in the groups with high FVIIc, FVII:Ag, and FVIIa-to-FVII:Ag ratio levels, but the highest incidence of

### Table 1. Plasma Levels of FVIIa, FVIIc, and FVII:Ag in Healthy Elderly and Young Subjects

<table>
<thead>
<tr>
<th>Age, y</th>
<th>FVIIa, ng/mL</th>
<th>FVIIc, %</th>
<th>FVII:Ag, %</th>
<th>FVIIc/FVII:Ag</th>
<th>FVIIa/FVII:Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (n=18)</td>
<td>28 ± 7</td>
<td>2.2 ± 0.4</td>
<td>89 ± 18</td>
<td>95 ± 18</td>
<td>0.93 ± 0.15</td>
</tr>
<tr>
<td>Women (n=24)</td>
<td>29 ± 7</td>
<td>2.1 ± 0.4</td>
<td>97 ± 26</td>
<td>99 ± 17</td>
<td>0.93 ± 0.09</td>
</tr>
<tr>
<td>Men (n=35)</td>
<td>77 ± 7</td>
<td>2.6 ± 0.6†</td>
<td>109 ± 23†</td>
<td>100 ± 22</td>
<td>1.12 ± 0.21‡</td>
</tr>
<tr>
<td>Women (n=33)</td>
<td>75 ± 8</td>
<td>2.9 ± 0.8‡</td>
<td>135 ± 22‡</td>
<td>128 ± 28‡</td>
<td>1.08 ± 0.16‡</td>
</tr>
</tbody>
</table>

FVIIa indicates activated factor VII; FVIIc, factor VII clotting activity; and FVII:Ag, factor VII antigen.

Data are mean ± SD. Ratios of FVIIc to FVII:Ag and of FVIIa to FVII:Ag are also shown. The mean FVIIa value of young healthy subjects, 2.1 ng/mL, was considered as 100%.

*P < .05, †P < .01, ‡P < .001 compared with young control subjects of the same sex.

§P < .05, †P < .01, ‡P < .001 compared with young or elderly men.
TABLE 2. Plasma Levels of FVIIa, FVIIc, and FVII:Ag in Elderly Subjects With Arterial Cardiovascular Diseases

<table>
<thead>
<tr>
<th></th>
<th>Healthy Subjects</th>
<th>Hypertensive Subjects</th>
<th>Diabetic Subjects</th>
<th>Arterial Cardiovascular Disease Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of men/women</td>
<td>35/33</td>
<td>15/14</td>
<td>3/10</td>
<td>24/27</td>
</tr>
<tr>
<td>Age, y</td>
<td>77±7</td>
<td>80±6</td>
<td>77±8</td>
<td>81±8</td>
</tr>
<tr>
<td>FVIIa, ng/mL</td>
<td>2.7±0.7</td>
<td>2.9±0.9</td>
<td>3.2±0.7*</td>
<td>3.3±0.9†</td>
</tr>
<tr>
<td>FVIIc, %</td>
<td>122±26</td>
<td>126±24</td>
<td>133±30</td>
<td>130±28</td>
</tr>
<tr>
<td>FVII:Ag, %</td>
<td>113±28</td>
<td>118±24</td>
<td>122±28</td>
<td>120±30</td>
</tr>
<tr>
<td>FVIIc/FVII:Ag</td>
<td>1.10±0.20</td>
<td>1.08±0.18</td>
<td>1.13±0.30</td>
<td>1.11±0.21</td>
</tr>
<tr>
<td>FVIIa/FVII:Ag</td>
<td>1.26±0.37</td>
<td>1.26±0.33</td>
<td>1.34±0.32</td>
<td>1.43±0.41†§</td>
</tr>
</tbody>
</table>

FVIIa indicates activated factor VII; FVIIc, factor VII clotting activity; and FVII:Ag, factor VII antigen. Data are mean±SD. Ratios of FVIIc to FVII:Ag and of FVIIa to FVII:Ag are also shown. The mean FVIIa value of young healthy subjects, 2.1 ng/mL, is considered as 100%.

*P<.05, †P<.02, ‡P<.001 compared with healthy subjects.
§P<.05 compared with hypertensive subjects.

arterial cardiovascular disease was clearly in the high-FVIIa group.

Relation Between Plasma FVIIa, FVIIc, and FVII:Ag Levels

Correlations between plasma FVIIa, FVIIc, and FVII:Ag levels in the 161 elderly subjects are shown in Fig 5. Plasma FVIIa levels had a weak, positive correlation with both FVIIc (Fig 5A, r=.541) and FVII:Ag (Fig 5B, r=.476). The former correlation has been reported previously. The correlation between FVIIc and FVII:Ag (Fig 5C, r=.646) was the strongest among these three parameters. However, the FVIIc-to-FVII:Ag ratio had no correlation with the FVIIa level (Fig 5D, r=.019).

![Fig 5](http://atvb.ahajournals.org/)

Scatterplots showing correlations between activated factor VII (FVIIa) and FVII clotting activity (FVIIc) (A), FVIIa and FVII antigen (FVII:Ag) (B), FVIIc and FVII:Ag (C), and FVIIa and the FVIIc-to-FVII:Ag ratio (D) in 161 elderly subjects: o, 68 healthy subjects; a, 42 hypertensive or diabetic subjects; x, 45 subjects with coronary artery disease or chronic cerebral infarction; and +, 6 subjects with both coronary artery disease and chronic cerebral infarction.
Our method is fully automated, so the assay has high reproducibility (interassay and intra-assay clot-based assays previously described require 45 or 50 mL of plasma and 5 mL of sample plasma; the automated fluorogenic assay for directly measuring plasma FVIIa of FVII-deficient plasma and 5 or 10 mL of sample plasma requires only 30 mL FVIIa levels in 28 plasma samples simultaneously. A single assay requires only 30 μL of FVII-deficient plasma and 5 μL of sample plasma; the automated clot-based assays previously described require 45 or 50 μL of FVII-deficient plasma and 5 or 10 μL of sample plasma. Our method is fully automated, so the assay has high reproducibility (interassay and intra-assay coefficients of variation were 4.2% and 1.3%, respectively). This fast, less expensive, and accurate FVIIa assay method may be advantageous when many plasma FVIIa assays, as in an epidemiological study, are required. The sensitivity of our method ranged from 0.2 to 1000 ng FVIIa/mL (Fig 2). Since the sensitivity of the clotting method previously reported by two independent groups ranged from 0.01 to 10 000 ng FVIIa/mL, our method was somewhat insensitive. However, all samples from healthy subjects and patients with various diseases had values exceeding 1 ng FVIIa/mL, indicating that our method was applicable for the current study. We measured the sTF-independent activity with an sTF-negative start reagent to determine the intrinsic activated protease activity in the plasma samples and found that none of the samples showed significant activity. We also tested the effect of varying conditions of blood collection and storage. Repeated freezing and thawing (up to three times) did not affect FVIIa activity (data not shown). However, samples stored in plastic tubes at 4°C or 37°C showed a gradual increase in FVIIa activity, and samples stored in unsiliconized glass tubes at 4°C showed a marked rise in activity that might have been due to cold activation. Thus, it is important to avoid long-term storage of samples after thawing. The results of the sample handling described in this article confirmed previous findings. The present study confirmed that trace levels of FVIIa were present in normal healthy young and elderly subjects as reported previously (Table 1). The normal range in healthy Japanese was 1.2 to 5.1 ng/mL, and the mean plasma FVIIa level was 2.5 ng/mL, which was lower than the values of 3.6 and 4.3 ng/mL determined by coagulation assay in Western subjects. We also performed the fluorogenic FVIIa assay with the use of

Table 3. Correlations of FVIIa, FVIIc, and FVII:Ag With Various Parameters in 161 Elderly Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FVIIa</th>
<th>FVIIc</th>
<th>FVII:Ag</th>
<th>FVIIa/FVII:Ag</th>
<th>FVIIc/FVII:Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index</td>
<td>-0.220†</td>
<td>-0.032</td>
<td>0.051</td>
<td>-0.271†</td>
<td>-0.108</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.002</td>
<td>0.113</td>
<td>0.070</td>
<td>-0.101</td>
<td>0.036</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.036</td>
<td>0.053</td>
<td>-0.029</td>
<td>-0.001</td>
<td>0.074</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.130</td>
<td>0.342§</td>
<td>0.272‡</td>
<td>-0.141</td>
<td>0.027</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.167</td>
<td>0.252†</td>
<td>0.324§</td>
<td>-0.145</td>
<td>-0.132</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.263‡</td>
<td>0.394§</td>
<td>0.212†</td>
<td>0.104</td>
<td>0.149</td>
</tr>
<tr>
<td>LDL-C</td>
<td>0.041</td>
<td>0.226†</td>
<td>0.182</td>
<td>-0.156</td>
<td>0.017</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>-0.132</td>
<td>-0.075</td>
<td>0.066</td>
<td>-0.233‡</td>
<td>-0.149</td>
</tr>
<tr>
<td>Pseudocholinesterase</td>
<td>0.021</td>
<td>0.313§</td>
<td>0.257‡</td>
<td>-0.235†</td>
<td>0.012</td>
</tr>
<tr>
<td>Albumin</td>
<td>-0.236†</td>
<td>0.111</td>
<td>0.009</td>
<td>-0.258‡</td>
<td>-0.091</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.086</td>
<td>0.101</td>
<td>0.110</td>
<td>-0.012</td>
<td>-0.032</td>
</tr>
<tr>
<td>Uric acid</td>
<td>-0.036</td>
<td>-0.027</td>
<td>-0.059</td>
<td>0.051</td>
<td>0.058</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.253‡</td>
<td>0.219†</td>
<td>0.127</td>
<td>0.117</td>
<td>0.096</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>0.166</td>
<td>0.176</td>
<td>0.190</td>
<td>0.004</td>
<td>-0.031</td>
</tr>
<tr>
<td>Factor X</td>
<td>0.154</td>
<td>0.487§</td>
<td>0.445§</td>
<td>-0.255†</td>
<td>0.013</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>0.144</td>
<td>0.497§</td>
<td>0.501§</td>
<td>-0.318§</td>
<td>-0.066</td>
</tr>
<tr>
<td>Protein C</td>
<td>0.314§</td>
<td>0.632§</td>
<td>0.596§</td>
<td>-0.256†</td>
<td>-0.053</td>
</tr>
</tbody>
</table>

FVIIa indicates activated factor VII; FVIIc, factor VII clotting activity; FVII:Ag, factor VII antigen; HDL-C, high-density lipoprotein cholesterol; and LDL-C, low-density lipoprotein cholesterol. Pearson’s correlation coefficients are shown.

†P<.01, ‡P<.001, §P<.0001.

Relation Between FVIIa and Lipids or Hemostatic Factors

Table 3 shows correlations between plasma FVIIa, FVIIc, and FVII:Ag levels with the levels of lipids and other hemostatic factors in the 161 elderly subjects. Plasma FVIIc and FVII:Ag levels were positively correlated with serum levels of total cholesterol, triglycerides, and HDL-C, which confirms our previous results. Interestingly, however, the correlation of FVIIa with total cholesterol and triglyceride levels was not statistically significant. FVIIa levels were negatively correlated with body mass index and serum albumin level but positively correlated with plasma fibrinogen, HDL-C, and protein C levels. Plasma FVIIc and FVII:Ag levels were positively correlated with the levels of lipids, pseudocholinesterase, FX, antithrombin III, and protein C, all of which are synthesized by the liver. These data indicate that the plasma FVIIa level is independent of the FVIIc or FVII:Ag level and does not correlate with lipid levels. The positive correlations of plasma FVIIc and FVII:Ag levels with other plasma proteins might be attributable to their synthesis in the liver.

Discussion

Using a centrifugal autoanalyzer, we developed a new fluorogenic assay for directly measuring plasma FVIIa levels. The method takes only 20 minutes to measure FVIIa levels in 28 plasma samples simultaneously. A single assay requires only 30 μL of FVIII-deficient plasma and 5 μL of sample plasma; the automated clot-based assays previously described require 45 or 50 μL of FVIII-deficient plasma and 5 or 10 μL of sample plasma. Our method is fully automated, so the assay has high reproducibility (interassay and intra-assay co-efficients of variation were 4.2% and 1.3%, respectively). This fast, less expensive, and accurate FVIIa assay method may be advantageous when many plasma FVIIa assays, as in an epidemiological study, are required. The sensitivity of our method ranged from 0.2 to 1000 ng FVIIa/mL (Fig 2). Since the sensitivity of the clotting method previously reported by two independent groups ranged from 0.01 to 10 000 ng FVIIa/mL, our method was somewhat insensitive. However, all samples from healthy subjects and patients with various diseases had values exceeding 1 ng FVIIa/mL, indicating that our method was applicable for the current study. We measured the sTF-independent activity with an sTF-negative start reagent to determine the intrinsic activated protease activity in the plasma samples and found that none of the samples showed significant activity. We also tested the effect of varying conditions of blood collection and storage. Repeated freezing and thawing (up to three times) did not affect FVIIa activity (data not shown). However, samples stored in plastic tubes at 4°C or 37°C showed a gradual increase in FVIIa activity, and samples stored in unsiliconized glass tubes at 4°C showed a marked rise in activity that might have been due to cold activation. Thus, it is important to avoid long-term storage of samples after thawing. The results of the sample handling described in this article confirmed previous findings. The present study confirmed that trace levels of FVIIa were present in normal healthy young and elderly subjects as reported previously (Table 1). The normal range in healthy Japanese was 1.2 to 5.1 ng/mL, and the mean plasma FVIIa level was 2.5 ng/mL, which was lower than the values of 3.6 and 4.3 ng/mL determined by coagulation assay in Western subjects. We also performed the fluorogenic FVIIa assay with the use of
recombinant FVIIa obtained from Novo Nordisk (Denmark) as the standard and obtained essentially the same standard curve as that obtained with the plasma-derived agent. Thus, we conclude that plasma FVIIa levels are lower in Japanese than in Western individuals. Japanese subjects have been reported to have lower FVIIc levels than Westerners. For the measurement of FVIIc and FVII:Ag, we used commercially available pooled plasma as a 100% control. This was prepared from 100 healthy young nonsmoking German men aged 20 to 40 years. The mean plasma FVIIc levels in healthy young Japanese subjects in this study were lower (90%) than that of the control pooled plasma obtained from Germany, supporting previous observations. This low FVIIc level might account for the low FVIIa level in our Japanese population.

We performed the gel filtration study to evaluate the state of FVIIa in plasma. If FVII is activated in a tissue factor–dependent manner, FVIIa must be associated with membrane-bound tissue factor because the dissociation constant between FVIIa and tissue factor is very small. Small amounts of FVIIa, not complexed with membrane-associated lipids could elute in a high-molecular-weight fraction under gel filtration conditions. However, the gel filtration study shown in Fig 4 did not support this assumption. A small amount of FVIIa activity eluted in the later HDL fractions, but most of the FVIIa activity in plasma eluted in a free form. Thus, most of the FVIIa in plasma was present in a free form and was not complexed with membrane-bound tissue factor. The small amount of FVIIa that eluted in the later HDL fractions is of interest. It has been proposed that FVII in plasma binds to chylomicrons, VLDL, and LDL and that this association is important for the activation of FVII by FXIIa because lipids could serve as a negative surface and promote FXII activation. Furthermore, it has been proposed that the phospholipase C–sensitive fraction of FVII forms a complex with phospholipids in plasma and that the FVII present in such complexes is markedly activated. However, we did not detect any direct association of FVIIa with lipids in the gel filtration experiment. We suspect that the FVIIa–lipid dissociation constant is relatively high, so the complex would start to dissociate as soon as filtration started.

Confirming previous reports, we found an age-related increase in plasma FVIIa levels as well as an age-related increase in the FVIIa-to-FVII:Ag ratio in normal subjects of both sexes (Table 1). Age-related increases were also found in plasma FVIIc and FVII:Ag levels of both sexes, as described. These results suggest that normal aging increases both the FVIIa level and FVII mass and that the increase in the former is greater. Although FVIIa levels in elderly subjects were not significantly different in men and women, FVII:Ag and FVIIc levels were higher in women, whereas the FVIIa-to-FVII:Ag ratio was higher in men. These data indicate that menopause mainly increases FVII mass and does not accelerate the activation of FVII to FVIIa. These sex-related differences were absent in healthy young subjects. Menopause in the elderly is associated with decreased FVIIa levels, but this has not been reported to result from an increase in FVII mass. We confirmed the increases in FVIIc and FVII:Ag after menopause and also found that menopause did not accelerate the activation of FVII to FVIIa because the FVIIa-to-FVII:Ag ratio was lower in elderly women than elderly men, as the increase in FVIIa after menopause is mainly due to increased FVII mass.

Although an extensive prospective study is required to confirm the association between disease risk and FVIIa levels, we undertook an initial study to determine the link between FVIIa level and cardiovascular disease. Elderly subjects with arterial cardiovascular disease showed significantly higher FVIIa levels (3.3 ng FVIIa/mL) than did healthy subjects (2.7 ng FVIIa/mL) (Table 2), and the FVIIa-to-FVII:Ag ratio also tended to be increased in the former group. In addition, plasma FVIIa levels were increased in hypertensive or diabetic subjects. When the elderly individuals were divided into high- and low-FVIIa groups, arterial cardiovascular disease was far more common in the high-FVIIa group than the low-FVIIa group. These results suggest a close relation between arterial cardiovascular disease and FVII activation in elderly Japanese individuals. Thus, it may be important to directly measure plasma FVIIa levels in patients with cardiovascular disease, although it is not clear where and how the activation of FVII takes place in vivo.

FVIIa levels were positively correlated with both FVIIc and FVII:Ag levels (Fig 5), but both correlation coefficients were smaller than that between FVIIc and FVII:Ag. In addition, the FVIIc-to-FVII:Ag ratio, previously used as an indirect indicator of FVIIa,

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It has been reported that high levels of FVIIc and fibrinogen were associated with an increased risk of cardiovascular disease.\textsuperscript{2,4,44} Morrissey et al\textsuperscript{51} have reported that FVIIa levels are not significantly correlated with plasma fibrinogen in normal subjects (nonsmokers and women not using oral contraceptives; mean age of 41.9 years for men and 40.2 years for women). However, our data obtained from elderly subjects showed a weak but significant correlation between FVIIa levels and fibrinogen (r = 0.253 in Table 3). This discrepancy might be explained by the age of the subjects.

It has been reported that plasma FVIIc and/or FVII:Ag levels exhibit a positive correlation with serum levels of total cholesterol and/or triglycerides,\textsuperscript{16-20} and the present study also confirms these findings (Table 3). However, plasma FVIIa levels were not independently correlated with total serum cholesterol or triglyceride levels. The plasma FVIIc and FVII:Ag levels were correlated with lipids, pseudocholinesterase (an indicator of hepatic protein synthesis), vitamin K-dependent coagulation factors (FX and protein C), and antithrombin III, all of which are synthesized by the liver. This indicates that the FVIIc and FVII:Ag levels may change in accordance with liver function, whereas the FVIIa level is not influenced by hepatic synthetic activity.

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References


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