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. 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 (FVIIa:Ag) ratios. Among the elderly, arterial cardiovascular disease was more common in a high-FVIIa than 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 A: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

Factor VII (FVII) is a vitamin K-dependent glycoprotein in plasma that plays an important role in the initiation of tissue factor-induced coagulation. The Northwick Park Heart Study has found that FVII coagulant activity (FVIIc) is an independent risk factor for coronary artery disease and cardiac death and is more significant than total cholesterol in the first 5 years after screening. Although coagulation data obtained in the Prospective Cardiovascular Münster Study (PROCAM) also supported the significance of FVIIc as a risk factor, the data from other reports disagree or point to certain discrepancies. The Progetto Lombardo Anti-Trombosi (PLAT) study, a prospective study of 953 patients with preexisting arterial cardiovascular disease, did not confirm the significance of FVIIc. In addition, other studies have shown that high levels of FVIIc are observed during normal aging and menopause as well as in atherosclerotic diseases, such as coronary artery disease, acute ischemic stroke, and peripheral vascular disease. It remains uncertain whether the high FVIIc levels detected in these conditions are due to an increase in activated FVII (FVIIia), an increase in the concentration of FVII mass expressed as FVII antigen (FVIIa:Ag) levels or FVII amidolytic activity (FVIIam), or both of the above. Furthermore, some authors have not found any increase in plasma levels of FVIIc, FVIIam, and/or FVIIa:Ag in coronary artery disease. Another problem is that plasma FVII levels always show a positive correlation with total serum cholesterol and/or triglycerides, but the mechanism of this correlation is unclear. Some authors have suggested that FVII binds to very-low-density lipoprotein (VLDL) and prolongs its half-life and that small amounts of native FVII are converted to FVIIa through contact surface activation on VLDL. It has also been suggested that these differing results might be partly due to differences in the detection of FVIIc and FVII mass by various assays.

FVII is a single-chain zymogen of the serine protease FVIIa. In vitro, FVII is converted to two-chain FVIIa by various coagulation proteases, including FXa, FIXa, FXIIa, thrombin, and FVIIa. At sites of vascular injury and on exposure of the subendothelium to circulating blood, an integral membrane glycoprotein known as tissue factor comes in contact with circulating FVII to form a bimolecular complex. The formation of this complex is widely believed to be the initial event in the extrinsic blood coagulation pathway. The activation of FVII--tissue factor complex by FVIIa has recently been discovered to be involved in the FVII autoactivation mechanism, and autoactivation has been argued to be much more important than FXa-catalyzed activation. However, it is still an open question as to what represents the most important pathway of FVII activation. Normal individuals have trace levels of circulating FVIIa (1% of the total FVIIa:Ag level), suggesting...
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 FXa-catalyzed activation of FVII but retains the ability to promote FVIIa-catalyzed activation of FX. 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.12-13 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, Kumanoto, Japan, and Novo Nordisk Japan, Tokyo, Japan, respectively. The FVIIa concentration was determined by using a 1% extinction coefficient at 280 nm of 14.934 and a calibration curve.1-3 Liquid human plasma FVIIa was prepared by mixing 1 mL 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 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-bufffered 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 Chromoimol, Behringwerke AG), a human placentacal calculated thromboplastic reagent (Chromotrain, Behringwerke 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-Fi, Dade). Plasma levels of FVIII and FX were determined by chromogenic assays with Testzyme VIII and Testzyme FX (Chromogenex AB), respectively. The plasma antithrombin III level was determined by a chromogenic assay with Berichrom antithrombin III (Behringwerke AG). Protein C amidolytic 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.

Procedures

Serum levels of albumin, pseudocholinesterase, and uric acid were also measured with enzymatic procedures. Serum total cholesterol and triglyceride levels were determined with enzymatic 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 enzymatic 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.

Subjects

Forty-two healthy young control subjects (18 men and 24 women; mean age, 29 years; range, 13 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 an

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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 (leukocyte 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 IU/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, 65-93 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 chronic cerebral infarction, 23 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 antplatelet 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 3000g 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.30,32 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 in the fluorogenic activated factor VII (FVIIa) assay. Varying amounts of plasma-derived FVIIa were added to FVII-deficient plasma in the presence of phospholipid and recombinant human soluble tissue factor. The reaction was started by adding calcium chloride and the fluorogenic thrombin substrate N-tert-butoxycarbonyl-Val-Pro-Arg-4-methylcoumar-7-amide, and the increase in relative fluorescence was measured as described in “Methods.” The fluorescence of 0.1 mmol/L 7-amino-4-methylcoumarin was assigned a value of 1.0. Numbers 1 through 6 show the reaction curves obtained with 20, 10, 2, 1, 0.2, and 0 ng FVIIa/mL, respectively. FVIIa activity was expressed by the reaction time, defined as the time elapsed to reach a relative fluorescence of 0.05 (dashed line).

Fig 1. Reaction curves obtained in the fluorogenic activated factor VII (FVIIa) assay. Varying amounts of plasma-derived FVIIa were added to FVII-deficient plasma in the presence of phospholipid and recombinant human soluble tissue factor. The reaction was started by adding calcium chloride and the fluorogenic thrombin substrate N-tert-butoxycarbonyl-Val-Pro-Arg-4-methylcoumar-7-amide, and the increase in relative fluorescence was measured as described in “Methods.” The fluorescence of 0.1 mmol/L 7-amino-4-methylcoumarin was assigned a value of 1.0. Numbers 1 through 6 show the reaction curves obtained with 20, 10, 2, 1, 0.2, and 0 ng FVIIa/mL, respectively. FvIIa activity was expressed by the reaction time, defined as the time elapsed to reach a relative fluorescence of 0.05 (dashed line).
ected. 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.31 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.31 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.1,38 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. A
Factor VIIa Levels and Cardiovascular Diseases

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 (•), sample 2 from an 87-year-old hypertensive man with a prior myocardial infarction (○), 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.²¹ 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.

Then 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-FVIIa 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 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 disease was in the high-FVIIa group.

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

<table>
<thead>
<tr>
<th></th>
<th>Healthy Young Subjects</th>
<th>Healthy Elderly Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men (n=18)</td>
<td>28±7</td>
<td>77±7</td>
</tr>
<tr>
<td>Women (n=24)</td>
<td>29±7</td>
<td>75±8</td>
</tr>
<tr>
<td>FVIIa, ng/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men (n=35)</td>
<td>2.2±0.4</td>
<td>2.6±0.6*</td>
</tr>
<tr>
<td>Women (n=33)</td>
<td>2.1±0.4</td>
<td>2.9±0.8†</td>
</tr>
<tr>
<td>FVIIc, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men (n=35)</td>
<td>89±18</td>
<td>109±23†</td>
</tr>
<tr>
<td>Women (n=33)</td>
<td>97±26</td>
<td>135±22‡</td>
</tr>
<tr>
<td>FVII:Ag, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men (n=35)</td>
<td>95±18</td>
<td>100±22</td>
</tr>
<tr>
<td>Women (n=33)</td>
<td>99±17</td>
<td>128±28§</td>
</tr>
<tr>
<td>FVIIa/FVIIc</td>
<td>0.93±0.15</td>
<td>1.12±0.21‡</td>
</tr>
<tr>
<td>FVIIa/FVII:Ag</td>
<td>1.08±0.24</td>
<td>1.35±0.40†</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<.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</td>
<td>No. of men/women</td>
<td>35/33</td>
<td>15/14</td>
<td>3/10</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±0.8†</td>
</tr>
<tr>
<td>FVIIc, %</td>
<td>122±26</td>
<td>126±24</td>
<td>133±30</td>
<td>120±30</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>1.06±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.31 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. Scatterplots showing correlations between activated factor VII (FVIIa) and VII 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; †, 46 subjects with coronary artery disease or chronic cerebral infarction; and x, 6 subjects with both coronary artery disease and chronic cerebral infarction.
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>-0.220t</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>0.2724</td>
<td>-0.141</td>
<td>0.027</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.167</td>
<td>0.252†</td>
<td>0.3246</td>
<td>-0.145</td>
<td>-0.132</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.253‡</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>0.313¾</td>
<td>0.257‡</td>
<td>-0.2351</td>
<td>0.012</td>
</tr>
<tr>
<td>Albumin</td>
<td>-0.236t</td>
<td>0.111</td>
<td>0.009</td>
<td>-0.258‡</td>
<td>-0.091</td>
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<tr>
<td>Glucose</td>
<td>0.086</td>
<td>0.101</td>
<td>0.110</td>
<td>-0.012</td>
<td>-0.032</td>
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<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>
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<tr>
<td>Fibrinogen</td>
<td>0.253†</td>
<td>0.219†</td>
<td>0.127</td>
<td>0.117</td>
<td>0.096</td>
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<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.12,13,18 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 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.30,31 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,30,31 our method was somewhat insensitive. However, all samples from healthy subjects and patients with various diseases had values exceeding 1 ng FVIIa/mL plasma, 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.31

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.30,31 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 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. The resulting FVIIa-tissue factor complex would start to dissociate as soon as filtration started. The complex would interfere with the accurate measurement of FVIIa. In addition, the FVIIc-to-FVII:Ag ratio, previously used as an indirect indicator of FVIIa, did not show any correlation with plasma FVIIa levels (r=0.19). This was unexpected. We can only speculate on the possible mechanisms concerning this point. The ratio method requires the measurement of FVIIc and uses tissue thromboplastin as a tissue factor. Tissue factor is known to serve as a protein cofactor for FVIIa, and the resulting FVIIa–tissue factor complex catalyzes FX activation. This means that FVIIa–tissue factor complex–catalyzed FVII activation would occur in the course of the FVIIa assay. We speculate that newly formed FVIIa during incubation of the FVIIa assay would interfere with the accurate measurement of plasma FVIIa, resulting in a lack of significant correlation between the level of plasma FVIIa and the FVIIc-to-FVII:Ag ratio. We used human thromboplastin for the FVIIa assay. There are several reports that bovine thromboplastin is the most sensitive to the presence of FVIIa.26,27 FVIIc values obtained from the assay with bovine thromboplastin might correlate with FVIIa levels determined by the direct FVIIa assay.

In addition to aging and cardiovascular disease, another factor that modulates the FVIIa level in elderly subjects appears to be malnutrition (Table 3). In adults, body mass index has been shown to have a positive correlation with plasma FVIIa levels in a population study. However, our previous study of subjects older than 60 years did not show any correlation between body mass index and either FVIIc or FVII:Ag levels. In the present study, we confirmed this finding in elderly subjects and also found a negative correlation between body mass index and serum albumin level with the FVIIa level and the FVIIc-to-FVII:Ag ratio. Considering that plasma FVIIa levels and inhibitors such as heparin cofactor II and antithrombin III are decreased in elderly subjects with malnutrition, this condition can promote a tendency to thrombosis in the elderly.
It has been reported that high levels of FVIIc and fibrinogen were associated with an increased risk of cardiovascular disease.\textsuperscript{24-44} 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 (\textit{r}=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{5-20} 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.

Acknowledgments

This study was supported in part by grants-in-aid from the Foundation for the Development of the Community, Tottori; the Japanese Medical Association, Hyogo; and the Japanese Ship Building Industry Foundation, Tokyo, Japan.

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K Kario, T Miyata, T Sakata, T Matsuo and H Kato

doi: 10.1161/01.ATV.14.2.265
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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