# Elevation of Factor XIa–$\alpha_1$-Antitrypsin Complex Levels in NIDDM Patients With Diabetic Nephropathy

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**Excess activated FXIa in plasma indicates hypercoagulability in the early contact phase. We have already developed methods for detecting the hypercoagulable state in clinical samples by our ELISA for complexed FXIa and $\alpha_1$AT, which has been confirmed to be the predominant inhibitor of FXIa. In diabetes, whether the activation of FXI is associated with the development of vascular complications remains unknown, although various hemostatic abnormalities have been described. We tested the complexed FXIa-$\alpha_1$AT level in 45 NIDDM patients, who were divided into three groups according to the development of diabetic nephropathy, as assessed by UAE. Normoalbuminuria was defined as UAE <15 μg/min, microalbuminuria as UAE in the range of 15–200 μg/min, and albuminuria as UAE >200 μg/min. In the patients as a whole, FXIa-$\alpha_1$AT and TAT levels were significantly increased compared with these levels in age-matched control subjects (17.3 ± 5.7 vs. 12.4 ± 2.4 ng/ml and 2.67 ± 1.23 vs. 1.93 ± 0.45 ng/ml, respectively). No significant difference was observed between FXIa-$\alpha_1$AT levels in the control subjects and in the normoalbuminuric group (13.0 ± 2.1 ng/ml; *n* = 19). However, in the microalbuminuric (17.9 ± 3.9 ng/ml; *n* = 16) and albuminuric (24.1 ± 5.4 ng/ml; *n* = 10) groups, FXIa-$\alpha_1$AT levels were significantly increased compared with those in the control and normoalbuminuric group. The TAT level was not correlated with FXIa-$\alpha_1$AT, and no significant differences in its levels were found among these diabetic groups. We agree with the widely held belief that diabetic nephropathy is strongly related to widespread microangiopathy, and suggest that the FXIa-$\alpha_1$AT level reflects vascular complications that occur concomitantly with such nephropathy. Therefore, we conclude that plasma FXIa-$\alpha_1$AT levels may be of potential pathophysiological or clinical importance for detecting early diabetic microangiopathy. *Diabetes* 42:233–38, 1993**

FXI, which is activated by factor XIIa or thrombin in the early phase of blood coagulation, catalyzes the activation of factor IX in the presence of calcium (1,2). It seems that high levels of FXIa indicate hypercoagulability in the contact phase. $\alpha_1$AT has been confirmed as the predominant inhibitor of FXIa in plasma (3,4). We previously developed a method for detecting the hypercoagulable state in clinical samples by our ELISA for FXIa as a recognized activated coagulation factor–inhibitor complex, FXIa-$\alpha_1$AT (5). In addition, we reported that the measurement of plasma FXIa-$\alpha_1$AT levels was useful not only for the early diagnosis of disseminated intravascular coagulation (5,6), but that it also had possible applications in the evaluation of atherosclerosis (7).

In recent years, various hemostatic abnormalities have been described in diabetes, for example, hypercoagulability, hypofibrinolysis, and platelet dysfunction, some of which may be caused by the pathogenesis of diabetic microangiopathy (8–10). However, it is not clear whether FXI activation occurs in diabetic patients. In this study, we investigated whether plasma FXIa-$\alpha_1$AT levels were related to the development of diabetic nephropathy in NIDDM patients, and whether the determination of these levels could be of value in assessing diabetic vascular complications.

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FXIa, factor XIa; FXI, factor XI; $\alpha_1$AT, $\alpha_1$-antitrypsin; FXIa-$\alpha_1$AT, complexed factor XIa and $\alpha_1$-antitrypsin; ELISA, enzyme-linked immunosorbent assay; NIDDM, non-insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; UAE, urinary albumin excretion; TAT, thrombin-antithrombin III; BMI, body mass index; BP, blood pressure; sBP, systolic blood pressure; dBP, diastolic blood pressure; CV, coefficient of variation; PT, prothrombin time; APTT, activated partial thromboplastin time; ANOVA, analysis of variance; FPA, fibrinopeptide A; NS, no significance.

## RESEARCH DESIGN AND METHODS

We studied 45 NIDDM patients (40–69 yr of age; 24 men and 21 women), who regularly attended our outpatient clinic from April 1990 to January 1991. All these patients had more than three blood examinations during the observation period, and their glycemic control was stable, as assessed by $HbA_{1c}$ levels. No patients had clinical evidence of congestive heart failure and renal insufficiency (serum creatinine >130 μM). Duration of diabetes, BMI, BP, smoking habits (smokers were defined as individuals who had smoked cigarettes for >1 yr or who still did smoke), type of antidiabetic therapy, and cardiovascular complications were recorded. Retinal status was examined by an ophthalmologist and was classified as normal, background, or proliferative retinopathy.

Fasting blood samples for the measurement of FXIa-$\alpha_1$AT and TAT were drawn from an antecubital vein with a plastic syringe containing 10% volumes of 0.13 M trisodium citrate and were centrifuged for 15 min at 1500 *g* at 4°C. Benzamidine hydrochloride (1 M, 1% vol/vol) was then added to the supernatant plasma, and aliquots were stored at −80°C.

An ELISA, described previously (5,6), was used for FXIa-$\alpha_1$AT determination. TAT level was assayed by ELISA with a commercially available kit (Behringwerke, Marburg, Germany; 11). The intra- and interassay CVs for the assays of plasma pools prepared from patients' samples are as follows: FXIa-$\alpha_1$AT, 6.2 and 7.6%; TAT, 4.5 and 6.0%. Glucose concentration was measured by a glucose oxidase method. $HbA_{1c}$ was determined by high-performance liquid chromatography (HLC-723GHb, TOSOH, Tokyo, Japan; 12). Plasma insulin levels were assayed by double-antibody radioimmunoassay (13). Total insulin was measured for non-insulin-treated diabetic patients, and free insulin was measured for insulin-treated patients (14). Serum creatinine was measured by a method based on reaction-rate kinetic principles, which eliminated pseudocreatinines (15). Serum cholesterol and triglyceride were determined enzymatically (Determiner TC-S, TG-S555, Kyowa-Medex, Tokyo, Japan). PT, APTT, and plasma fibrinogen levels were assessed by a clot turbidity assay conducted on a Coagmaster (Hitachi, Tokyo, Japan).

The FXIa-$\alpha_1$AT levels in randomly selected normal, nondiabetic subjects were determined in our laboratory. Blood samples were drawn from 87 healthy volunteers on our hospital staff (20–69 yr of age [mean ± SD, 48 ± 12 yr]; 48 men and 39 women). The mean FXIa-$\alpha_1$AT level in these normal subjects was 11.0 ± 4.1 ng/ml, but because age is slightly correlated with FXIa-$\alpha_1$AT (6), subjects ($n$ = 20) who were age-matched to the NIDDM patients were selected as control subjects. For the small intraindividual variations in the FXIa-$\alpha_1$AT levels in patients occurring within the observation period, data were averaged.

The UAE, assayed by a nephelometric method (16) with interassay CV of <9%, was expressed as micrograms per minute. The UAE determinations were performed twice (at the first visit and at the conclusion of the visit in the observation period). The subjects were instructed to avoid strenuous exercise and to complete overnight urine collection at home. The urine volume was recorded, and aliquots were stored at −80°C. Sterility of urine was checked by urinalysis. Patients were divided into the following three subgroups: normoalbuminuria defined as UAE <15 μg/min, microalbuminuria as UAE in the range of 15–200 μg/min, and albuminuria as UAE >200 μg/min (17). The patients in whom two UAE allocated in the split group were excluded. The urine of patients who had persistent proteinuria, which was detected with a urine test strip (Albustix; Boehringer, Mannheim, Germany), was not subjected to UAE determination, and these patients were placed in the albuminuric group. Although UAE was not performed in normal subjects, Albustix tests on urine samples were all negative.

Statistical significance was determined by ANOVA and linear regression analysis. Data are means ± SD. In our laboratory, FXIa-$\alpha_1$AT and TAT levels in normal subjects showed a normal distribution. The UAE values are logarithmically distributed (16); therefore, a logarithmic transformation for the linear regression analysis toward FXIa-$\alpha_1$AT was used.

## RESULTS

Table 1 shows the clinical data of the NIDDM patients, who were divided into three groups according to the development of nephropathy, as assessed by UAE. The three groups did not differ significantly in age, duration of diabetes, BMI, or frequency of smokers. Compared with normo- and microalbuminuric patients, albuminuric patients had a tendency to relatively severe retinopathy and higher incidence of coronary heart disease, and were more often on insulin therapy. No significant differences were found in sBP, dBP, PT, APTT, serum cholesterol, triglyceride, glucose, and $HbA_{1c}$ levels among these groups.

The mean UAE in the normoalbuminuric and microalbuminuric groups was 6.0 (range 1.1–13.0) and 44.3 (range 15.2–171.9) μg/min, respectively; in the albuminuric group, it was >200 μg/min (not determined for those patients with persistent proteinuria). UAE was positively correlated with sBP ($r$ = 0.33; $P$ < 0.05), but not with dBP.

In patients as a whole, FXIa-$\alpha_1$AT levels were significantly elevated (17.3 ± 5.7 ng/ml; $n$ = 45) compared with those in the age-matched control group (12.4 ± 2.4 ng/ml; $n$ = 20); the levels in the patients were not correlated with sex, age, duration of diabetes, and BMI and were not influenced by either glucose or $HbA_{1c}$ levels. Smoking did not influence the FXIa-$\alpha_1$AT level.

Linear regression analysis in patients as a whole showed that the FXIa-$\alpha_1$AT level was significantly correlated with the UAE ($r$ = 0.77; $P$ < 0.01; Fig. 1), and FXIa-$\alpha_1$AT level was significantly correlated with the sBP ($r$ = 0.32; $P$ < 0.05) but not with dBP. The FXIa-$\alpha_1$AT level was not correlated with plasma insulin level ($r$ = −0.11). In addition, in the patients receiving insulin incorporation, the FXIa-$\alpha_1$AT level was not correlated with their insulin dose.

The TAT levels in the diabetic patients as a whole

TABLE 1
Clinical characteristics of NIDDM patients

| | NIDDM patient groups | | |
|---|---|---|---|
| | Normoalbuminuria | Microalbuminuria | Albuminuria |
| *n* (M/W) | 19 (7/12) | 16 (12/4) | 10 (5/5) |
| Age (yr) | 62 ± 8 | 63 ± 5 | 64 ± 4 |
| Duration of diabetes (yr) | 8 ± 6 | 9 ± 5 | 14 ± 7 |
| BMI (kg/m$^2$) | 24.3 ± 3.4 | 24.1 ± 3.6 | 25.0 ± 3.2 |
| Smoker (*n*) | 5 | 7 | 3 |
| Retinopathy (*n*)* | 14/3/2 | 11/3/2 | 3/2/5 |
| Coronary heart disease (*n*) | 4 | 7 | 5 |
| Antidiabetic treatment (*n*)† | 6/11/2 | 5/8/3 | 2/3/5 |
| sBP (mmHg) | 129 ± 14 | 134 ± 20 | 143 ± 15 |
| dBP (mmHg) | 75 ± 8 | 71 ± 14 | 73 ± 6 |
| PT (s) | 12.2 ± 0.7 | 12.3 ± 0.6 | 12.1 ± 0.8 |
| APTT (s) | 26.1 ± 2.8 | 25.9 ± 3.5 | 27.0 ± 2.0 |
| Plasma fibrinogen (μM) | 7.18 ± 1.32 | 8.06 ± 1.85 | 11.18 ± 3.12‡ |
| Serum creatinine (μM) | 86 ± 14 | 94 ± 18 | 117 ± 16‡ |
| Cholesterol (mM) | 5.25 ± 0.88 | 4.99 ± 1.14 | 5.94 ± 1.16 |
| Triglyceride (mM) | 2.14 ± 1.14 | 2.20 ± 2.69 | 2.00 ± 0.63 |
| Plasma insulin (pM) | 136 ± 171 | 120 ± 115 | 130 ± 92 |
| Serum glucose (mM) | 10.3 ± 2.83 | 9.94 ± 2.22 | 10.6 ± 3.39 |
| $HbA_{1c}$ (%) | 7.6 ± 1.4 | 7.4 ± 1.5 | 7.7 ± 2.1 |

Values are means ± SD.
*Retinopathy is shown as normal retina/background retinopathy/proliferative retinopathy.
†Antidiabetic treatment is shown as diet only/oral hypoglycemic agent/insulin.
‡$P < 0.01$ versus normoalbuminuria and microalbuminuria.

(2.67 ± 1.23 ng/ml) were significantly increased compared with those in the control subjects (1.93 ± 0.45 ng/ml). Nevertheless, no correlation existed between FXIa-$\alpha_1$AT and TAT levels (Fig. 2).

Figure 3 shows the FXIa-$\alpha_1$AT levels in the control subjects and in the three groups of diabetic patients. No significant difference was observed in the FXIa-$\alpha_1$AT levels between the control and the normoalbuminuric group (13.0 ± 2.1 ng/ml; $n = 19$). However, the FXIa-$\alpha_1$AT levels in the microalbuminuric (17.9 ± 3.9 ng/ml; $n = 16$) and albuminuric (24.1 ± 5.4 ng/ml; $n = 10$) groups were significantly increased compared with those in both the control and the normoalbuminuric groups. In contrast, no significant differences were noted in TAT level among the diabetic subgroups (data not shown).

## DISCUSSION

Microalbuminuria, which is defined as subclinical elevation of UAE, predicts the development of diabetic nephropathy in IDDM patients (18,19). In contrast, in NIDDM it may be a less predictive marker because some differences occur in the natural course of disease compared with IDDM: e.g., renal failure is a much less common consequence (20), and the major cause of the excess morbidity and mortality is cardiovascular disease (21,22). Additionally, for IDDM patients with microalbuminuria, renal dysfunction would appear to be potentially reversible because appropriate treatment could delay or even prevent the development of clinical nephropathy (23), but it remains ambiguous whether microalbuminuria may be similarly predictive in NIDDM. Nevertheless, microalbuminuria, which is defined as incipient nephrop-

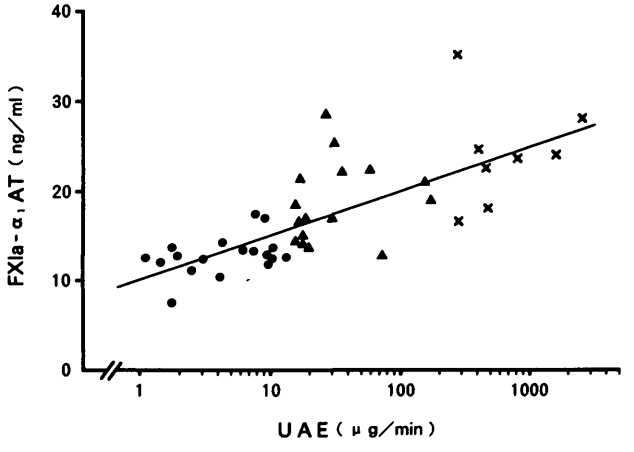


**FIG. 1. Relationship between plasma levels of FXIa-$\alpha_1$AT and UAE rates in NIDDM patients. Normoalbuminuric (●), microalbuminuric (▲), and albuminuric (×) patients $r = 0.77$; $P < 0.01$.**

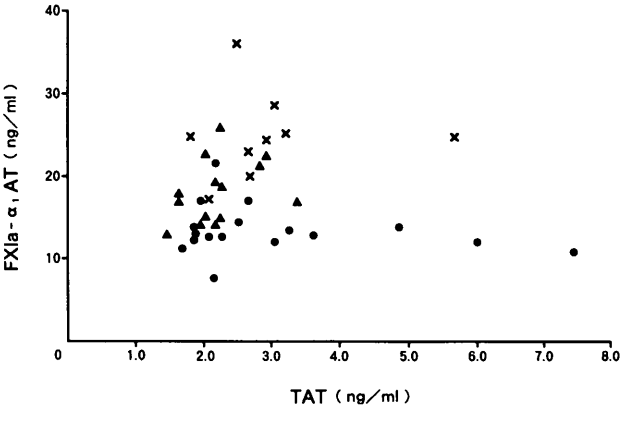


**FIG. 2. Relationship between plasma levels of FXIa-$\alpha_1$AT and TAT in NIDDM patients. Normoalbuminuric (●), microalbuminuric (▲), albuminuric (×) patients $r = -0.017$; NS.**

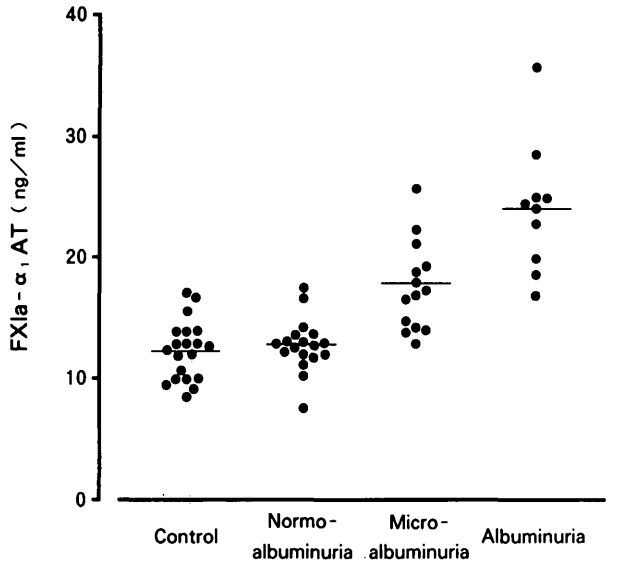


**FIG. 3. Plasma levels of FXIa-$\alpha_1$AT in NIDDM patients with different UAE rates compared with FXIa-$\alpha_1$AT levels in control subjects. Normoalbuminuria was defined as UAE <15 μg/min, microalbuminuria as UAE of 15–200 μg/min, and albuminuria as UAE <200 μg/min or persistent proteinuria detected with Albustix. Horizontal lines show mean values. For control versus normoalbuminuric groups, NS; $P < 0.01$ for all other comparisons.**

athy in NIDDM, predicts the development of clinical proteinuria (24) and is significantly related to all causes of mortality (22,24–26). Thus, we looked for a new sensitive indicator, such as UAE, to aid in the diagnosis of early diabetic nephropathy.

As reported previously, plasma levels of thrombomodulin or von Willebrand factor, parameters of vascular endothelial origin, showed a significant increase in patients with diabetic nephropathy (27,28). However, it is not known whether the activation of coagulation factors is related to diabetic complications that occur concomitantly with diabetic nephropathy. In this study, our hypothesis is that FXI activation reflects the generalized vascular damage that is observed as diabetes develops, and that therefore determination of the FXIa-$\alpha_1$AT level can provide evidence of vascular complications in patients at the microalbuminuric stage.

Interestingly, we found that FXIa-$\alpha_1$AT levels were not only significantly elevated in NIDDM patients but also correlated with UAE. Thus, the increased FXIa-$\alpha_1$AT may be only associated with the progression of renal insufficiency rather than diabetic nephropathy. Our study design obscured the impact of renal insufficiency by eliminating diabetic patients in whom the serum creatinine is >130 μM. However, the increased FXIa-$\alpha_1$AT level, even in microalbuminuric patients in whom glomerular filtration rate is considered normal (20), can exclude such a possibility. Because the control data on nondiabetic populations with microalbuminuria and early renal insufficiency have not been provided, whether our findings are essentially characteristic phenomena in diabetes remains to be determined. However, excess activation of FXI in diabetic patients may result from not only glucose intolerance and the following impaired metabolic control, but also the whole of abnormalities associated frequently with diabetes, such as systemic micro- and macroangiopathy.

Although it is uncertain whether diabetic nephropathy parallels systemic vascular complications, generalized atherosclerosis is associated with the development of diabetes and, especially in proteinuric patients, increases the incidence and causes the high morbidity of vascular complications such as cardiovascular accident and stroke (29–31). Morphological studies have also demonstrated the widespread micro- and macrovascular abnormalities found in diabetic patients (32,33). Thus, we agree that diabetic nephropathy is strongly related to widespread vascular damage (34). Furthermore, previous reports have suggested a significant correlation between arterial BP and UAE in diabetic (35) and nondiabetic subjects (36). In our study, sBP was slightly correlated with UAE, but dBP failed. The three subgroups did not differ in either sBP or dBP; nevertheless, a slight but significant correlation was observed between FXIa-$\alpha_1$AT and sBP. Because systolic hypertension, so frequently observed in NIDDM patients, adds to the increased risk of atherosclerosis (37), the increased FXIa-$\alpha_1$AT level may partly be attributable to hypertension, which is associated with diabetic nephropathy.

On the other hand, it is a widely held belief that a relationship exists between hypercoagulability and vascular endothelial dysfunction, and this appears to involve the activation of coagulation factors, e.g., thrombin formation or factor IX or X activation on endothelial cells, as has been shown in the culture of these cells (38–40). Furthermore, we have demonstrated accelerated FXI turnover and high levels of distribution of FXI-associated products in atherosclerotic lesions in Watanabe hereditary hyperlipidemic rabbits (7). Thus, our results provide presumptive evidence that FXI, located in the early contact phase, was activated according to the development of diabetic vascular complications.

The increased levels of coagulation factors, e.g., factors X, VIII, VII, V, and fibrinogen, in diabetes mellitus, which has been reported by many investigators (41–43), alone indicate no immediate evidence of the hypercoagulable state, because the presence of large amounts of coagulation factors does not necessarily indicate enhanced activation of any particular coagulation factor. Only a few studies have explored the relationship between plasma levels of activated coagulation factors and diabetes mellitus. van Wersch et al. (44) reported that TAT levels were not significantly enhanced in IDDM. In contrast, we found a significant increase of TAT levels in NIDDM patients compared with the control subjects in this study, similar to results of a study done by Takahashi et al. (45). Although we cannot explain why the TAT level was not correlated with FXIa-$\alpha_1$AT in diabetes, from the point of view of the coagulation cascade, it appears likely that increased FXIa should lead to or accompany abundant thrombin formation. Moreover, the plasma level of FPA, which is a sensitive marker for thrombin action on fibrinogen, has been shown to increase in diabetes (46–49), but these reports of the relationship between FPA and vascular complications are confusing. Although we could not infer whether FPA is correlated with FXIa-

$\alpha_1$AT or is related to diabetic nephropathy, it is not inconceivable that large amounts of thrombin could be produced, but not detected as TAT in plasma. Further studies are needed to clarify the apparent discrepancy between the FXIa-$\alpha_1$AT and TAT.

In this regard, Ceriello et al. investigated the influence of hyperglycemia on TAT levels in diabetic patients and normal subjects (47). They found that the TAT levels were significantly reduced in the diabetic patients, and that levels in both diabetic patients and normal subjects decreased during hyperglycemia induced by glucose infusion, but recovered to the basal levels during euglycemia. They also found that factor X activation was reduced in diabetic patients, and they concluded that these phenomena were caused by a hyperglycemia-dependent decrease of antithrombin III (50). In this study, in contrast, FXIa-$\alpha_1$AT levels were not affected by glucose and $HbA_{1c}$ levels. We also examined the changes in the trypsin-inhibitor activity of $\alpha_1$AT incubated with various glucose concentrations in vitro; no alteration was seen in this activity when $\alpha_1$AT was incubated with up to 20 mM glucose for 72 h (unpublished observations). Although it is uncertain whether the level of $\alpha_1$AT fluctuates physiologically in hyperglycemic conditions in vivo, these findings indicate that the FXIa-$\alpha_1$AT levels might not depend on short-term glycemic control.

In conclusion, we found that the FXIa-$\alpha_1$AT levels were elevated in diabetic patients with developed nephropathy. Although further longitudinal follow-up study of the relationship between long-term diabetic vascular complications and FXIa-$\alpha_1$AT levels in individual diabetic patients is required, we suggest that the FXIa-$\alpha_1$AT level reflects the vascular complications that occur concomitantly with diabetic nephropathy. Whether the screening measurement of the FXIa-$\alpha_1$AT level in NIDDM patients can detect the microalbuminuric stage or can predict the progression of diabetic nephropathy is beyond the scope of our study. We emphasize that plasma FXIa-$\alpha_1$AT levels may be of potential pathophysiological or clinical importance for detecting early diabetic microangiopathy.

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