Increased Plasminogen Activator Inhibitor Type 1 in Coronary Artery Atherectomy Specimens From Type 2 Diabetic Compared With Nondiabetic Patients
A Potential Factor Predisposing to Thrombosis and Its Persistence

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Background—Inhibition of fibrinolysis attributable to elevated concentrations of plasminogen activator inhibitor type 1 (PAI-1) in blood is associated with insulin resistance, hyperinsulinemia, and type 2 diabetes mellitus. Because we have shown that insulin can stimulate PAI-1 synthesis in vivo and because accelerated vascular disease is common in such patients as well, we hypothesized that increased PAI-1, potentially predisposing to thrombosis, acute occlusion, and accelerating atherosclerosis because of thrombus-associated mitogens, would be present in excess in atheroma from type 2 diabetic subjects.

Methods and Results—Samples acquired by directional coronary atherectomy from 25 patients with type 2 diabetes and 18 patients without diabetes were characterized qualitatively histologically for cellularity and by immunohistochemistry visually and quantitatively and by quantitative image analysis for assessment of urokinase-type plasminogen activator (u-PA) and PAI-1. Patients with and without diabetes were similar with respect to demographic features and the distribution and severity of coronary artery disease. Substantially more PAI-1 and substantially less u-PA were present in the atherectomy samples from subjects with diabetes.

Conclusions—The disproportionate elevation of PAI-1 compared with u-PA observed in atheromatous material extracted from vessels of diabetic subjects is consistent with increased gene expression of PAI-1 in vessels as well as the known increase of PAI-1 in blood, presumably reflecting increased synthesis. The increased PAI-1 detected in the atheroma may contribute in vivo to accelerated or persistent thrombosis underlying acute occlusion and to vasculopathy exacerbated by clot-associated mitogens in the vessel wall. Because the changes were observed to be associated with insulin resistance and type 2 diabetes mellitus, they may be modifiable by reduction of insulin resistance with insulin sensitizers and stringent control of hyperglycemia. (Circulation. 1998;97:2213-2221.)

Key Words: diabetes mellitus ■ coronary disease ■ fibrinolysis ■ insulin ■ thrombosis

The prevalence of coronary artery disease in type 2 diabetic subjects is ≥4-fold greater than that in nondiabetic subjects.1 Even with stringent control, an increased risk for macrovascular disease in type 2 diabetes persists. Accordingly, the possibility exists that the combined hyperproinsulinemia and hyperinsulinemia typical of type 2 diabetes may exert adverse influences on the vessel wall independent of hyperglycemia and other derangements of intermediary metabolism typically present in both type 1 (insulinopenic) and type 2 (hyperinsulinemic and dysinsulinemic) diabetes.2-5

We and others6-8 have observed consistent elevations of concentrations in blood of PAI-1 reflected by reduced fibrinolysis in response to venous occlusion, a phenomenon consistent with intermittent persistence of microthrombi in vessels with consequent adverse effects of clot-associated mitogens on the vessel wall accelerating macrovascular disease.

Subjects with type 2 diabetes who undergo percutaneous transluminal coronary angioplasty exhibit 4-fold higher 5-year mortality than do nondiabetic subjects treated comparably and with similar lesions.9,10 The increase appears to be attributable largely to restenosis. As judged from analysis of human and porcine vessels, early atherosclerotic lesions and complex plaques are characterized by disproportionate increases in PAI-1 within the vessel wall compared with concentrations of u-PA and t-PA.11 Furthermore, rats genetically predisposed to insulin resistance, postprandial hyperglycemia, and macroangiopathy late in life exhibit changes in the...
walls of vessels analogous to those seen in type 2 diabetic human subjects.\textsuperscript{12} Explants of vascular smooth muscle cells from vessels of the animals predisposed to atherosclerosis exhibit higher rates of proliferation in vitro than do cells from controls, even when the explants have been obtained many months before the time of appearance, in vivo, of macroangiopathy.\textsuperscript{13}

These observations led us to hypothesize that one of the factors contributing to macroangiopathy in type 2 diabetes and to the accelerated restenosis compromising the long-term efficacy of angioplasty in diabetic subjects is a disproportionate elevation of PAI-1 in vascular wall components and atheroma. Such an elevation would be likely to predispose to thrombosis and its persistence, thereby increasing the risk of acute coronary events and possibly restenosis potentiated by clot-associated mitogens.

Methods

Patients Studied

Twenty-five patients with type 2 diabetes and 18 without clinical evidence of diabetes were studied between April 1996 and March 1997 at the time of DCA mandated by clinical indications (performed by H.G. at the Massachusetts General Hospital). The two groups of patients (diabetic and nondiabetic) were similar with respect to clinical indications for DCA, use of heparin during all groups of patients (diabetic and nondiabetic) were similar with respect to clinical indications for DCA, use of heparin during all procedures, absence of gross thrombus detected angiographically, initial success of the procedure, and other characteristics, including age and sex (Tables 1 and 2) and the nature (primary or restenotic) and distribution of culprit coronary arterial lesions (Table 3).

Coronary atherectomy samples were excised selectively and retrieved from coronary atherosclerotic lesions by conventionally performed DCA.

Medications

Each patient was pretreated with aspirin 325 mg/d and dipyridamole 50 mg every 6 hours initiated 24 hours before DCA and continued after hospital discharge. Heparin was administered as an initial bolus of 10 000 U IV after insertion of an arterial sheath and as additional 2500-U boluses every 30 minutes as needed to maintain the activated clotting time between 250 and 300 seconds throughout the DCA procedure.

DCA was successful in each case. There was no associated mortality. The extent of residual stenosis was reduced to <50\% of the initial stenosis universally.

Characterization of Atherectomy Specimens

Atherectomy samples were assayed for u-PA and PAI-1 by immunohistochemistry (immunoperoxidase staining).\textsuperscript{14} In addition, the material was assayed for cellularity. Analysis was performed as follows.

Qualitative Assessments

A visual grading scale (+ to ++++) was used for the assessment of the intensity of immunohistochemical staining as shown in Figures 1 and 2. An analogous grading scale (+ to ++++) was used for assessment of cellularity as shown in Figure 3. Sections from the DCA-extracted material were characterized by comparison of the sections with the grading scale sections by two observers who were blinded to the characteristics of the patients. Each observer evaluated a minimum of three sections per block (selected randomly and independently), evaluated three to five fields per section (again selected randomly and independently), and scored each field on a 0 to 4+ scale for intensity and a 0 to 4+ scale for the extent of distribution of positive staining to yield an average result for each

TABLE 1. Biochemical Variables in Specific Categories of Patients

<table>
<thead>
<tr>
<th>Categories of Patients</th>
<th>Age, y</th>
<th>u-PA, AU†</th>
<th>IA–u-PA Pixel Intensity</th>
<th>PAI-1, AU†</th>
<th>IA–PAI-1, Pixel Intensity</th>
<th>Cellularity, AU†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects with type II diabetes (n=25)</td>
<td>64.0±2.1</td>
<td>1.28±0.16*</td>
<td>15.9±1.5*</td>
<td>2.32±0.19*</td>
<td>28.8±2.3*</td>
<td>1.76±0.20</td>
</tr>
<tr>
<td>Subjects without diabetes (n=18)</td>
<td>60.2±2.6</td>
<td>2.62±0.26</td>
<td>50.2±8.7</td>
<td>1.55±0.17</td>
<td>14.2±2.2</td>
<td>2.02±0.21</td>
</tr>
<tr>
<td>Subjects with type II diabetes treated with insulin+ diet, sulfonylurea, or both (n=14)</td>
<td>63.1±3.4</td>
<td>1.50±0.23*</td>
<td>18.0±2.0*</td>
<td>2.57±0.24*</td>
<td>32.5±3.4*</td>
<td>1.64±0.24</td>
</tr>
<tr>
<td>Subjects without diabetes (n=18)</td>
<td>60.2±2.6</td>
<td>2.62±0.26</td>
<td>50.2±8.7</td>
<td>1.55±0.17</td>
<td>14.2±2.2</td>
<td>2.02±0.21</td>
</tr>
<tr>
<td>Subjects with type II diabetes treated with diet+ exercise alone (n=11)</td>
<td>65.1±2.2</td>
<td>1.00±0.22*</td>
<td>12.9±2.3*</td>
<td>2.0±0.29</td>
<td>24.0±2.5*</td>
<td>1.90±0.34</td>
</tr>
<tr>
<td>Subjects without diabetes (n=18)</td>
<td>60.2±2.6</td>
<td>2.62±0.26</td>
<td>50.2±8.7</td>
<td>1.55±0.17</td>
<td>14.2±2.2</td>
<td>2.02±0.21</td>
</tr>
<tr>
<td>Subjects with type II diabetes with primary lesions (n=18)</td>
<td>64.8±2.3</td>
<td>1.28±0.20*</td>
<td>14.0±1.6†</td>
<td>2.36±0.22</td>
<td>30.1±2.8†</td>
<td>1.50±0.24</td>
</tr>
<tr>
<td>Subjects without diabetes with primary lesions (n=8)</td>
<td>58.9±3.7</td>
<td>3.00±0.31</td>
<td>67.6±12.3</td>
<td>1.56±0.34</td>
<td>17.1±4.2</td>
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<td>Subjects with type II diabetes with restenotic lesions (n=7)</td>
<td>61.7±5.1</td>
<td>1.28±0.33†</td>
<td>20.0±3.3</td>
<td>2.21±0.42</td>
<td>25.3±4.4†</td>
<td>2.43±0.14</td>
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<tr>
<td>Subjects without diabetes with restenotic lesions (n=10)</td>
<td>61.3±4.0</td>
<td>2.36±0.38</td>
<td>32.8±6.8</td>
<td>1.55±0.18</td>
<td>11.7±1.8</td>
<td>2.25±0.21</td>
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</table>

†The grading scales used provide a range of values from 0 (minimum) to 4+ (maximum intensity and distribution).

\begin{flushleft}†P<0.05 for differences between subjects with and without diabetes.

\begin{flushleft}‡P<0.01 for differences between subjects with and without diabetes.

\begin{flushleft}AU indicates arbitrary units; IA, quantitative image analysis. Mean±SE scores for immunohistochemically detected PAI-1 and u-PA (assessed with reference to the semiquantitative scales depicted in Figures 1 and 2); mean pixel intensity based on quantitative image analysis of immunohistochemically detected PAI-1 and u-PA described in Methods; and cellularity assessed with reference to the semiquantitative visual scoring system depicted in Figure 3.

\end{flushleft}
TABLE 2. Patient Characteristics

<table>
<thead>
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<th>Patient</th>
<th>Sex</th>
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<th>Treatment of Type II Diabetes</th>
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<td>M</td>
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<tr>
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<td>F</td>
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Patients with type II diabetes

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<td>M</td>
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<td>M</td>
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<td>F</td>
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<td>F</td>
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</tr>
<tr>
<td>25</td>
<td>F</td>
<td>76</td>
<td>Diet</td>
</tr>
</tbody>
</table>

NA indicates not applicable.

*All treatments with oral agents were with sulfonylureas.

Quantitative Assessments

Quantitative image analysis of the sections was performed by observers blinded with respect to the patients from whom samples were obtained, as follows: immunoperoxidase-stained slides were viewed with an Olympus BX-50 upright light microscope. Digital gray-scale images were acquired with an attached Sony DXC-960MD/LLP charged coupled device camera connected via an RS-170 cable to a frame grabber board in a Sun SPARCstation 5 computer. Image collection and analysis was performed with IMIX/IMAGIST Version 8 software (Princeton Gamma Tech). Data for all images of each type were collected with identical camera settings, followed by selection of regions of interest on the digitized image. Gray-scale values (pixel intensities) within the regions of interest were plotted as histograms, and minimum, maximum, and mean pixel intensity values were calculated with conventional software. These values were used to compare intensity of immunoperoxidase reaction products in vessels from diabetic and nondiabetic subjects. Data are expressed as intensity units above values with mouse IgG used as a control for comparison. To verify the representative nature of computed values used for comparisons between groups, coefficients of variation were evaluated for repeated readings of the same field, values from multiple fields within the same section, and multiple sections within a block from a given sample (6 to 10 values for each). Results were 0.26%, 2.3%, and 3.6%. The low values of these coefficients of variation are consistent with the validity of between-group comparisons of values obtained within each group.

Preparation of Specimens for Analysis

The DCA samples were fixed in ethanol for a minimum of 48 hours and processed conventionally for histochemical analysis. Sections 6 μm thick were prepared and stained with antibodies specific for each protein of interest as described below. Three sections from each tissue sample were assayed for each component, and results were averaged to obtain a score for each variable in each DCA sample.

Immunoperoxidase Staining

Immunoperoxidase staining with the antibodies selected was performed as follows. Tissue sections were deparaffinized through two incubations in xylene, 5 minutes each, followed by successive incubations in ethanol (100%, 95%, 75%, and 50%) for 3 minutes each and subsequently in PBS for 3 minutes. All incubations were performed at room temperature. Sections were treated with 0.25% trypsin for 15 minutes at ambient temperature, followed by two washes in PBS. They were then placed in 0.75% H₂O₂/75% methanol for 30 minutes, washed twice in PBS, and maintained in a humidified chamber in 3% BSA in PBS for 30 minutes at room temperature. Subsequently, they were treated with 10% normal goat serum for 30 minutes, followed by exposure to primary antibody in a humidified chamber at 37°C for 30 minutes.

The primary antibodies used were monoclonal antihuman PAI-1 (product No. 3785) (10 μg/mL); monoclonal antihuman u-PA (product No. 3689) (10 μg/mL); monoclonal anti–smooth muscle α-actin (from Sigma Chemical Co) as a positive control; anti-CD44 (acquired from R & D Systems); and normal mouse IgG (from Sigma Chemical Co) as a negative control. The anti–human PAI-1 antibody recognizes both free PAI-1 and PAI-1 complexed to plasminogen activators. The anti–human u-PA antibody recognizes free u-PA and receptor-bound u-PA. It also recognizes u-PA complexed with PAI-1 as verified in our laboratory and shown in Western blots.

After incubation at 37°C, sections were washed three times in PBS and treated with secondary antibody (DAKO Envision System, a horseradish peroxidase polymer system conjugated with secondary antibodies) that reacted with mouse primary antibodies (DAKO). Again, the sections were incubated at 37°C for 30 minutes, washed three times in 0.05 mol/L Tris-hydroxymethylaminomethane (Tris), pH 7.6, and treated with a substrate solution consisting of 25 mg DAB (Sigma Chemical Co) and 17.5 μL 30% H₂O₂ in 50 mL Tris for 5 minutes at room temperature followed by three washes in PBS.

For conventional histology, counterstaining was performed with Mayer’s hematoxylin solution (Sigma Chemical Co) for 3 minutes. The sections were then washed three times in PBS and dehydrated through 50%, 75%, 95%, and 100% ethanol. They were washed twice in xylene before being mounted under coverslips with Permount.
### TABLE 3. Characteristics of Coronary Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site of Culprit</th>
<th>Nature of Lesion</th>
<th>Interval to Restenosis, d</th>
<th>Use of Stent</th>
<th>Indication for Procedure</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>RCA</td>
<td>Primary</td>
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</tr>
<tr>
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<td>LAD</td>
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<td>AMI</td>
</tr>
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<td>NA</td>
<td>—</td>
<td>UAP</td>
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<tr>
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<td>—</td>
<td>UAP</td>
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<td>NQMI</td>
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<td>UAP</td>
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<td>UAP</td>
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<td>Restenotic</td>
<td>1 d</td>
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<td>UAP</td>
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</tbody>
</table>

RCA indicates right coronary artery; LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; UAP, unstable angina pectoris; AMI, acute myocardial infarction; NQMI, non–Q-wave myocardial infarction; and SEA, stable effort-induced angina.
Grading of Immunohistochemical Staining

Intensity and Cellularity

Grading scales are shown in Figures 1 through 3. The absence of staining for PAI-1 and/or u-PA in negative controls is shown in Figure 4. This figure also demonstrates the colocalization of PAI-1 with smooth muscle α-actin–positive cells, indicating that the PAI-1 seen was present primarily in smooth muscle cells. Values in samples were based on the intensity of staining and the percentage of tissue stained, both referenced to the sections in the grading scale used to define the 4-point grading scale as judged independently by two observers evaluating independently and randomly identified fields (three to five per section) and blinded with respect to the characteristics of the patients from whom the sections had been obtained.

Statistical Analysis

Comparisons between groups were performed with two-tailed Student’s t tests for unpaired samples. Differences with values of $P \leq 0.05$ were considered to be significant.

Results

Patients with and without diabetes did not differ with respect to age (Table 1) or other demographic features (Table 2). The
nature (primary or restenotic) and distribution of coronary lesions were generally similar in the two groups (Table 3). As shown in a representative example of a photomicrograph of immunostained material in Figure 5 and in Table 1, atherectomy samples from subjects with diabetes appeared to exhibit less u-PA than those from subjects without diabetes (1.28 ± 0.16 [SE] arbitrary units compared with 2.62 ± 0.26), \( P < 0.01 \), a difference consistent with decreased atheroma-associated cell surface-mediated proteolytic system capacity. By contrast, tissue from diabetic subjects appeared to exhibit significantly more PAI-1 (2.32 ± 0.19) than tissue obtained from nondiabetic subjects (1.55 ± 0.17, \( P < 0.01 \)), as shown in the example in Figure 6, and by results in Table 1. Results with automated image analysis were consistent with the qualitative differences observed visually (Table 1). Thus, as shown in Table 1 and Figure 7, samples from patients with diabetes exhibited consistently more PAI-1 and consistently less u-PA than samples from patients without diabetes. These differences were consistent in subsets of patients with primary and restenotic lesions (Table 1) and evident regardless of the nature of the treatment of diabetes (Table 3).

Cellularity scores assessed visually and hence qualitatively did not differ significantly for tissues from the two groups of

Figure 3. Grading scale used to score each sample with respect to cellularity and applied in a fashion analogous to that used with grading scales noted in legends to Figures 1 and 2. Sections were stained with hematoxylin, which stains nuclei blue. Arrows point to regions with cellularity of increasing extent in A through D. Magnification \( \times 100 \). Note: These samples were also stained for PAI-1.

Figure 4. Concordant localization of immunostained PAI-1 and \( \alpha \)-actin indicative of vascular smooth muscle cells. Serial sections stained with PAI-1 antibody (A) and \( \alpha \)-actin antibody (B). PAI-1 was detected virtually exclusively in vascular smooth muscle cells characterized by presence of \( \alpha \)-actin. C and D show two negative controls illustrating lack of staining with normal mouse IgG used instead of primary antibody compared with results in A and B, respectively. As noted in text, other negative controls were obtained routinely to verify specificity of immunostaining with antibody against PAI-1, u-PA, or \( \alpha \)-actin.
patients (Table 1) (1.76±0.20 [SE] arbitrary units compared with 2.02±0.21 in nondiabetic subjects). These results are not inconsistent with proliferation of vascular smooth muscle cells within the arterial wall of the vessels from diabetic subjects in situ in view of the nature of the coronary atherectomy procedure used, which extracted material not necessarily representative of cellularity within the entire vessel wall but more likely to be indicative of overall cellularity in complex plaques.

Discussion

Results in the present study are consistent with the hypothesis that one of the contributors to the development of accelerated macroangiopathy in type 2 diabetes mellitus is altered tissue...
expression of components of the fibrinolytic system within atheroma and presumably therefore arterial walls; specifically, disproportionate elevations of concentrations of PAI-1 with respect to concentrations of u-PA. The results support the possibility that inhibition of the fibrinolytic system activity in situ in syndromes of insulin resistance and hyperinsulinemia, including type 2 diabetes, predisposes to thrombosis and its persistence, setting the stage for thrombotic coronary artery occlusion and accelerated atherosclerosis or restenosis in response to clot-associated mitogens. The present results demonstrate an increase in total PAI-1 (free plus plasminogen activator–complexed PAI-1) and a decrease in total u-PA (free and receptor bound) in the specimens from the diabetic patients. Thus, even if some of the PAI-1 detected is complexed with some of the u-PA (an interaction possible with the two-chain species), the amount of free PAI-1 appears to be increased. Accordingly, the results indicate that immunohistochemically detectable PAI-1 is increased and immunohistochemically detectable u-PA is decreased in the specimens from the diabetic patients. Thus, even if some of the PAI-1 detected is complexed with some of the u-PA (an interaction possible with the two-chain species), the amount of free PAI-1 appears to be increased.

Concentrations of PAI-1 in blood are elevated in association with diverse states of insulin resistance. A salient example is the genetically obese mouse (ob/ob) as reported initially by Samad and Loskutoff.14 The same laboratory demonstrated high concentrations of PAI-1 in murine adipose tissue in vivo and its augmentation in response to tumor necrosis factor-α and tumor necrosis factor-ß.15 Consistent with these observations, elevations of PAI-1 have been seen in human subjects with obesity, type 2 diabetes mellitus, and the polycystic ovary syndrome.6,16–20 We recently demonstrated that weight reduction induced by modest caloric restriction in elderly obese subjects leads to a decline in prevailing concentrations of PAI-1 in blood paralleled by augmentation of functional activity of the fibrinolytic system.20 When we administered troglitazone, a thiazolidinedione known to enhance insulin sensitivity, to patients with the polycystic ovary syndrome in whom concentrations of PAI-1 in blood are markedly elevated, concentrations of PAI-1 in blood declined markedly.19 If such changes are paralleled by altered expression of PAI-1 within atheroma and/or vessel walls, as appears likely judged from the result in the present study, augmentation of insulin sensitivity may prove to be effective in ameliorating disproportionate elevation of vascular wall PAI-1 and its potential pathogenetic impact on thrombosis and the evolution of macroangiopathy.

The present results underscore the potential pathogenetic importance of derangements in expression of fibrinolytic system proteins in tissues as well as in blood. Insulin augments expression of PAI-1 in HepG2 cells (a human hepatoma cell line). Both insulin and its precursor, proinsulin, in concentrations consistent with those prevailing in blood in subjects with type 2 diabetes mellitus, augment elaboration of PAI-1 from these cells.8 Furthermore, insulin augments PAI-1 elaboration by endothelial cells in the presence of cocultured vascular smooth muscle cells, again in concentrations consistent with those seen in blood in subjects with type 2 diabetes.21 Administration of either insulin or proinsulin to experimental animals maintained under euglycemic conditions leads not only to augmented concentrations of PAI-1 in blood but also to augmented concentrations of PAI-1 protein and PAI-1 mRNA in vessel walls.22 Although administration of insulin to human subjects with type 2 diabetes has not been shown to elevate PAI-1 in blood, possibly because of the
concomitant reduction in proinsulin secretion as noted by Iain et al,21 we have recently found it to do so in human cells in a setting in which the metabolic milieu simulates that associated with type 2 diabetes.22 Taken together with the present observations, these findings suggest that vascular wall PAI-1 synthesis is a potentially useful target for measures designed to reduce the acceleration of macroangiopathy typical of type 2 diabetes and to reduce the risk of thrombotic coronary occlusion underlying many acute coronary events.

Conclusions

The results obtained in this study are consistent with the hypothesis that a disproportionate elevation of PAI-1 not only in blood but also in extracted atheroma and presumably vessel walls is characteristic of and perhaps a direct consequence of hyperinsulinemia and hyperproinsulinemia typical of insulin-resistant states, including type 2 diabetes mellitus. Accordingly, reduction of insulin through diet and exercise programs, optimal control of hyperglycemia, and perhaps concomitant use of insulin sensitizers, such as thiazolidinediones, offers particular promise for attenuating progression of macrovascular disease exacerbated by thrombosis in insulin-resistant states, including type 2 diabetes mellitus.

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References

Increased Plasminogen Activator Inhibitor Type 1 in Coronary Artery Atherectomy Specimens From Type 2 Diabetic Compared With Nondiabetic Patients: A Potential Factor Predisposing to Thrombosis and Its Persistence

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