The aim of the study was to investigate the long-term chronic effects of smoking on the fibrinolytic enzyme system by comparing two groups of healthy male volunteers (aged 30 to 40 years). One group consisted of 15 habitual smokers who consumed 20 or more cigarettes a day; the other consisted of 15 nonsmokers. Fibrinolysis was studied at rest (baseline) and after infusion of 1-desamino-8-D-arginine vasopressin (DDAVP; 0.4 µg/kg body weight). Smokers had significantly lower baseline blood fibrinolytic activity as determined by the overall assays: dilute blood clot lysis ($p < 0.05$) and euglobulin-fibrin plate assay ($p < 0.05$). Further analysis showed that these low activities could be attributed to a lower baseline level of extrinsic tissue-type plasminogen activator (t-PA) activity ($p < 0.05$) in the smokers. There were no significant differences between the groups in various fibrinolytic inhibitors or in the intrinsic fibrinolytic activation pathways. The increased levels of t-PA activity and factor VIII R:Ag in response to DDAVP were also reduced in the smokers ($p < 0.01$). The relative increase (ratio of post-DDAVP activity/baseline activity) for these parameters was not significantly different for the two groups. Smokers also had significantly higher levels of the acute phase reactants, α1-antitrypsin ($p < 0.002$) and plasminogen ($p < 0.02$) and C-reactive protein ($p < 0.01$). We suggest that in smokers the endothelial cells show a normal responsiveness to DDAVP stimulation, but produce less t-PA activity and factor VIII R:Ag. This may be significant for the risk of cardiovascular disease in smokers. (Arteriosclerosis 5:443-450, September/October 1985)
Methods

Study Subjects

This research was carried out according to the principles of the Declaration of Helsinki; informed consent was obtained from each of the volunteers. Healthy male Caucasian volunteers between the ages of 30 and 40 were recruited into the study and divided into two groups. The smoking group consisted of men who inhaled cigarette smoke and who had consumed 20 or more cigarettes per day for a minimum of 5 years. The nonsmoking group consisted of men who had been nonsmokers for a minimum of 5 years. All subjects had not taken aspirin for at least 1 week or other medications for 1 month. Other exclusion criteria were: 1) relative weight, according to the formula height (cm) − 100/weight (kg) > 1.10; 2) diastolic blood pressure > 90 mm Hg; 3) regular alcohol intake of 0.5 liter of beer (or equivalent) per day; 4) serum cholesterol level ≥ 7.2 mmol/liter; and 5) serum triglyceride level ≥ 2.0 mmol/liter. Volunteers were obtained by advertisement, and the first 15 in each group who fulfilled the criteria were selected for study. In the group of nonsmokers, four volunteers were excluded; in the group of smokers, five were excluded.

Each volunteer completed an extensive questionnaire to establish general parameters. This included a detailed medical history, drinking (arbitrary grades 0–3) and smoking habits, and work activity (arbitrary scores: 1 = sedentary; 2 = standing/walking; 3 = manual). The volunteers were studied at 9 A.M. after fasting for 12 hours. The smokers were asked to refrain from smoking for 12 hours or, if they were unable to do so, for at least 1 hour before the start of the investigation. Blood samples were taken by means of an indwelling catheter according to the scheme shown in Figure 1; the patency of the needle was maintained by a slow saline infusion, with the volunteers in the supine position. The blood sample after a 30-minute rest period (i.e., at t = 0) was used for studying the fibrinolytic baseline value in accordance with previous studies. Immediately after the sampling at time 0, infusion with 1-desamino-8-D-arginine-vasopressin; Minirin (DDAVP) was started. DDAVP (0.4 µg per kg body weight) was added to 50 ml sterile saline and infused over a 10-minute period. Blood samples were collected at 30 minutes and in some volunteers at 10, 20, 30, 40, and 50 minutes after the start of the infusion (Figure 1).

Plasma Collection

Platelet-poor plasma was prepared from citrated blood (1 vol 0.11 mol/liter citrate to 9 vol blood) by centrifugation for 30 minutes at 2500 g at 4°C. Pooled normal plasma was prepared as earlier described; values were expressed in percent relative to this pool. The group of donors for the pool was composed of equal numbers of males and females, whereas the study concerned males only and resulted in mean values (Table 1) deviating from 100% for some factors. Aliquots of 5 ml of blood for serum samples to study fibrin degradation products were collected in 0.1 ml Michaelis buffer containing aprotonin (25 KIU/ml), 2-amino caproic acid (25 mg/ml), and human cerebral thromboplastin (10 µl/ml). This was allowed to clot for 2 hours at 37°C.

General Assays

Levels of triglyceride, total cholesterol, and HDL cholesterol were determined in serum. The HDL fraction was isolated and its cholesterol content was determined by the same method used for total cholesterol. The hematocrit was measured at t = 0 in heparinized capillary tubes by using a Hawksley microhematocrit centrifuge and reader (Lancing, England). Carboxyhemoglobin was determined at t = 0 as a percentage of the total hemoglo-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Fibrinogen (%)</td>
<td>106±19</td>
<td>101±23</td>
</tr>
<tr>
<td>Factor XII-dependent activator activity (BAU/ml)</td>
<td>42±15</td>
<td>41±9</td>
</tr>
<tr>
<td>Plasma UK activity (BAU/ml)</td>
<td>47±10</td>
<td>47±17</td>
</tr>
<tr>
<td>Factor XII (%)</td>
<td>73±13</td>
<td>77±15</td>
</tr>
<tr>
<td>α2-antiplasmin (α2-AP) (%)</td>
<td>97±6</td>
<td>100±6</td>
</tr>
<tr>
<td>Fibrin-bound α2-AP (%)</td>
<td>26±5</td>
<td>30±5</td>
</tr>
<tr>
<td>Histidine-rich glycoprotein (%)</td>
<td>100±17</td>
<td>90±22</td>
</tr>
<tr>
<td>Clot lysis t-PA inhibition (%)</td>
<td>89±9</td>
<td>92±8</td>
</tr>
<tr>
<td>Fast-acting t-PA inhibition (%)</td>
<td>82±44</td>
<td>110±82</td>
</tr>
<tr>
<td>α2-macroglobulin (%)</td>
<td>90±12</td>
<td>99±29</td>
</tr>
</tbody>
</table>

Mean values plus standard deviations are given in percentage of pooled normal plasma.

*Percentage of total α2-antiplasmin in pooled plasma.
bin.36 M-Partigen plates (Behringerwerke AG, Marburg, West Germany) were used to assay for α1-antitrypsin and the results were expressed as a percentage of the content of pooled normal plasma. C-reactive protein was assayed by the Mancini method36 and the results were expressed as a percentage relative to a commercial plasma standard (100% = 76 mg/ml; Behringerwerke A.G. Marburg, West Germany).

**Hemostatic Assays**

The Laurell method36 was used to determine fibrinogen (antiserum from Dr. W. Nieuwenhuizen of our institute); histidine-rich glycoprotein (antiserum kindly supplied by Dr. N. Heimbürger, Behringerwerke AG); factor VIII R:Ag (antiserum from the Central Laboratory, Dutch Blood Transfusion Service, Amsterdam); and factor XII (antiserum from Nordic, Tilburg, The Netherlands). M-Partigen plates (Behringerwerke AG, Marburg, West Germany) were used for the α2-macroglobulin assays. Fibrinogen degradation products (FDP) were kindly determined by Dr. L.H. van Hulsteyn, Academic Hospital, Leiden, by the Laurell method36 using antihuman fibrinogen; the values were always below the detection limit.

An immediate plasmin inhibition assay using S 2251 determined the α2-antiplasmin activity in plasma and serum (AB KABI, Stockholm, Sweden) as previously described.36 The amount of α2-antiplasmin activity bound to fibrin by the action of factor XIII during clotting was calculated as the difference between the citrated plasma value and the serum value (divided by 1.2 to account for the citrate dilution) and was expressed in percentages of the plasma α2-antiplasmin activity of the pooled normal plasma. Plasminogen was assayed by means of the streptokinase method and S 2251 determined the α2-antiplasmin activity in plasma that was not strongly inhibited by the Laurell method36 using antihuman fibrinogen; the values were always below the detection limit.

The dilute blood-clot lysis time (DBCLT) method was performed on fresh blood at t = 0 using a 10% dilution as described earlier.41 Normal euglobulin fractions (NEF) were prepared from plasma by using a 1:10 dilution with pH 5.9.32 NEF activity was determined on standard plasminogen-rich fibrin plates,42 and lysis zones were recorded after 17 hours at 37°C. The factor XII-dependent and plasma-urokinase activator activities were determined in the dextran sulphate euglobulin fractions of plasma.43 The fraction of this activity that could be quenched by excess antibodies to urinary urokinase raised in rabbits (purified on protein A Sepharose and kindly provided by Dr. G. Wijngaards of our institute) was attributed to urinary urokinase activator activity.43 The amount of activator in the normal euglobulin fraction was recorded by the Mancini method (antiserum from Nordic, Tilburg, The Netherlands) and was not strongly variable nor different between nonsmokers and smokers (19% ± 3% and 18% ± 3% (mean ± sd) of pooled plasma, respectively). The inhibition of t-PA activity was assayed by a fibrin clot-lysis method previously described36 that uses relatively high concentrations of t-PA (about 40 IU/ml plasma). The fast-acting t-PA inhibitor activity of plasma was determined specifically by titration with purified two-chain melanoma t-PA and measurement of remaining activity as described previously,46 inhibition was expressed relative to pooled normal plasma.

**Statistical Analysis**

Statistical analysis used the Mann-Whitney "U" test for nonparametric data.

**Results**

As shown in Table 2, there were no significant differences in general parameters between the two groups in characteristics known to affect fibrinolytic activity.

**Baseline Blood Fibrinolysis**

The smoking group had a significantly lower (p < 0.05) baseline level (t = 0) of blood fibrinolytic activity as shown in the two overall methods, i.e., the dilute blood clot lysis time method and the normal euglobulin fraction activity assayed on fibrin plates.

**Table 2. General and Lipid Parameters of the Two Groups of 15 Nonsmokers and Smokers**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>33.9 ± 3.0</td>
<td>33.2 ± 2.2</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.81 ± 0.09</td>
<td>1.81 ± 0.04</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>73.1 ± 9.8</td>
<td>74.3 ± 8.2</td>
</tr>
<tr>
<td>Height (cm) - 100/weight (kg)(^{-})</td>
<td>0.90 ± 0.08</td>
<td>0.92 ± 0.09</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>122.0 ± 8.8</td>
<td>130.0 ± 19.1</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>79.0 ± 6.3</td>
<td>79.7 ± 7.7</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>61.1 ± 9.2</td>
<td>63.3 ± 10.6</td>
</tr>
<tr>
<td>Alcohol use score</td>
<td>1.1 ± 0.6</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>Work activity score</td>
<td>1.6 ± 0.6</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>43.4 ± 2.1</td>
<td>44.1 ± 2.5</td>
</tr>
<tr>
<td>Blood glucose (mmol/liter)</td>
<td>3.7 ± 0.5</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>Carboxyhemoglobin (%)</td>
<td>1.7 ± 0.4</td>
<td>4.4 ± 2.1*</td>
</tr>
<tr>
<td>Triglycerides (mmol/liter)</td>
<td>0.86 ± 0.32</td>
<td>1.16 ± 0.31*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/liter)</td>
<td>5.3 ± 1.1</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/liter)</td>
<td>1.4 ± 0.3</td>
<td>1.1 ± 0.3†</td>
</tr>
</tbody>
</table>

Mean values plus standard deviations are given.

* p < 0.002, statistically significant difference between the two groups.

† p < 0.02, statistically significant difference.
(Figure 2). Results of the two overall methods correlated significantly within each group ($r = 0.51; p \leq 0.05$) and in the two groups together ($r = 0.59; p \leq 0.001$), which suggests a common factor responsible for the variations found in plasma. Further analysis of specific components and activities of fibrinolysis revealed no significant differences between the two groups for fibrinogen, FDP, factor XII-dependent activator activity, plasma urokinase activity, factor XII, $\alpha_2$-antiplasmin, fibrin-bound $\alpha_2$-antiplasmin, histidine-rich glycoprotein, clot lysis t-PA inhibition, fast-acting t-PA inhibition (Figure 3) and $\alpha_1$-macroglobulin (see Table 1). The only significant differences were found in plasminogen and t-PA activity (Figures 3 and 4). The t-PA activity in each group correlated significantly with the activity found by the overall methods, i.e., with euglobulin activity ($r = 0.75; p \leq 0.005$) and with DBCLT ($r = 0.55; p \leq 0.05$). This suggests that t-PA activity is a major factor responsible for baseline blood fibrinolytic activity (as obtained with the overall methods) and also for the difference between nonsmokers and smokers.

Response to DDAVP

Figure 5 shows the time course of the DDAVP-induced t-PA release in subgroups of smokers and nonsmokers. In both groups, the maximum response occurred at about 20 to 30 minutes after the start of the infusion. As shown in Figure 3, the peak of the DDAVP-induced t-PA release ($t = 30$) was significantly lower in the smokers than in the nonsmokers ($p \leq 0.01$). Thus, both the baseline- and DDAVP-induced t-PA activities were lower in smokers. The response to DDAVP expressed as the ratio of post- and pre-DDAVP t-PA activity, however, was not significantly different between the groups. This ratio, or response factor, for the two groups was: nonsmokers, $10.8 \pm 8.9$ (SD); smokers, $8.2 \pm 6.0$ (SD) (Figure 3). Additionally, for both groups, there was a significant correlation between pre- and post-DDAVP values for t-PA activity ($r = 0.61; p \leq 0.02$). We concluded that nonsmokers and smokers show a similar responsiveness to DDAVP (expressed as the response factor) but that smokers show less of an absolute rise in t-PA activity at a generally lower level of t-PA activity. Fast-acting t-PA inhibition was reduced after DDAVP in all subjects; in the majority of cases (21 of 30) it was reduced to nil. In accordance with the lower post-DDAVP values in the smokers’ group, more subjects showed residual t-PA inhibition after DDAVP (n = 7; mean = 41% $\pm$ 40% (SD) compared to the nonsmokers’ group (n = 2; mean = 45% $\pm$ 25% (SD) (differences $= ns$).

Factor VIII R:Ag blood levels also respond to DDAVP-infusion and were studied for comparison. As shown in Figure 6, the baseline factor VIII R:Ag levels were significantly lower in smokers than nonsmokers. The factor VIII R:Ag levels ($t = 0$ vs $t = 30$) were significantly increased in both groups by the DDAVP infusion; however, the peak levels were significantly lower in smokers than in nonsmokers ($p \leq 0.001$). The ratio, or response factors (factor VIII R:Ag at $t = 30/t = 0$), however, were not different: for nonsmokers, $2.01 \pm 0.48$ (SD) and, for smokers, $1.92 \pm 0.50$. We concluded that for factor VIII R:Ag nonsmokers and smokers have a similar responsiveness to DDAVP, but in smokers the absolute levels remain lower. This situation is similar to that with t-PA.

Acute Phase Reaction

Among potential acute phase reactants (C-reactive protein, $\alpha_2$-antitrypsin, fibrinogen, plasminogen, $\alpha_1$-macroglobulin, $\alpha_2$-antiplasmin, t-PA inhibition, Cl-inactivator, and factor VIII R:Ag), $\alpha_2$-antitrypsin, plasminogen, and C-reactive protein had significantly higher values in smokers (Figure 4). In the smoking group there were weak correlations between C-reactive protein and fibrinogen ($r = 0.54; p \leq 0.05$), between $\alpha_1$-antitrypsin and $\alpha_2$-antiplasmin ($r = 0.51; p \leq 0.05$) and between fibrinogen and t-PA inhibition ($r = 0.57; p \leq 0.01$). This suggests a weak, chronic, acute phase process in the smoking group.
Figure 3. Comparison of assay values for smokers and nonsmokers for t-PA activity before (t = 0) and after (t = 30) DDAVP infusion and fast-acting t-PA inhibition before DDAVP infusion. The means are indicated by horizontal bars and the standard deviations by dotted lines. The t-PA activity is expressed in blood activator units (BAU), and fast-acting t-PA inhibition, in percent of the value in normal pooled plasma. The individual responses in t-PA activity are shown by the ratios in t-PA activity after and before DDAVP infusion.

Figure 4. Comparison of assay values for smokers and nonsmokers for α1-antitrypsin, plasminogen, and C-reactive protein. The means are indicated by horizontal bars and the standard deviations, by dotted lines.
Figure 5. Fibrinolytic time-response curves of smokers and nonsmokers after DDAVP infusion. The t-PA activity was determined in the normal euglobulin fraction as Cl-Inactivator resistant activator activity and is expressed in blood-activator units (BAU).

Discussion

In this study of two well-matched groups of apparently healthy volunteers, we confirmed a number of differences between smokers and nonsmokers that have been reported in the literature. The smokers had a significantly higher carboxyhemoglobin level \( p \leq 0.002 \), as would be expected. A lower baseline level of factor VIII R:Ag in smokers has been reported.\(^{46} \) The results of the lipid screen confirmed that the smokers had a higher triglyceride level \( p \leq 0.002 \)\(^{8,14,18} \) and a lower HDL cholesterol level \( p \leq 0.02 \).\(^{15,16} \) Smokers had a higher cholesterol level, but the difference, 5.45 compared with 5.27 mmol/liter, was not significant, in contrast to other studies.\(^{8,10,14} \) The elevation in triglyceride levels was not sufficiently high to interfere with fibrinolytic parameters according to the results of Brommer et al.\(^{31} \)

Our observation of reduced fibrinolytic activity in smokers is in accord with the literature.\(^{6,24,26} \) The intrinsic fibrinolytic pathways, as well as factor XII itself, showed no significant differences between the groups. Notably, the primary plasmin inhibitor showed no significant differences between the groups nor was its factor XII-mediated binding to fibrin affected by smoking. In the group of smokers, plasminogen originating from the liver\(^{47} \) and a potential acute phase reactant\(^{48} \) was significantly (± 10%) higher than in nonsmokers. This difference cannot explain the main differences in the fibrinolytic system found with the overall methods for blood fibrinolytic activity. This was evident from negative correlations between plasminogen levels and activity. If there was a causal relation in vitro, a positive correlation would be expected. The main difference between nonsmokers and smokers concerns the extrinsic system of fibrinolysis which involves the extrinsic (tissue-type) plasminogen activator (t-PA).

Figure 6. Comparison of assay values for smokers and nonsmokers for factor VIII R:Ag before (t = 0) and after (t = 30) DDAVP infusion. The means are indicated by horizontal bars and the standard deviations, by dotted lines. Factor VIII R:Ag is expressed in percent of the value in normal pooled plasma. The individual responses are shown by the ratios in factor VIII R:Ag after and before DDAVP infusion.
The results of t-PA activity assays and of the overall methods correlated within the groups. Thus, smokers had a significantly lower baseline activity of t-PA in the blood. Likewise, the lower peak level of t-PA activity after stimulation with DDAVP suggests a reduced release potential of the extrinsic system. Since both t-PA activity and factor VIII R:Ag levels were significantly different between nonsmokers and smokers in both the baseline condition and after DDAVP stimulation, it appears that smoking affects the endothelial cell, resulting in lowered endothelial function. This lowered function apparently does not affect responsiveness to DDAVP, since for both t-PA and factor VIII R:Ag the response factor to DDAVP was not significantly different between the two groups. The generally lower level at which the system operates may be due directly to endothelial functioning (e.g., reduction in healthy cells or reduced synthesis), but may also be mediated through a regulation center elsewhere (e.g., in the brain) similar to the effect of smoking on the prolactin of lactating women.49

Recently a specific inhibitor for t-PA has been discovered in plasma,46 and this inhibitor can modulate t-PA activity. Determined by a specific method, the level is not significantly different between smokers and nonsmokers. The t-PA inhibitor occurs at very low concentrations and its effect is only expressed to a minor degree in the clot lysis t-PA inhibition assay (which primarily expresses α2-antiplasmin- and α1-macroglobulin-inhibiting activity); also this assay shows no significant differences between smokers and nonsmokers.

It is not known what constituents in cigarette smoke are responsible for the observed effects on fibrinolysis. Nicotine and carbon monoxide are two potential candidates that have been singled out from the thousands of constituents of cigarette smoke. We have previously demonstrated that when two cigarettes are smoked in 15 minutes, there is an increase in the activity of circulating t-PA (acute effect).30 A possible mediator for the activator release is nicotine, either directly or via adrenaline or vasopressin.30 Exhaustion of the extrinsic fibrinolytic system in smokers by this route may occur. Carbon monoxide is another constituent of cigarette smoke that could be responsible for the observed differences between smokers and nonsmokers. Moderate carbon monoxide levels have been shown to cause endothelial cell damage.10,22 and a damaged endothelial cell population could explain the lower baseline levels of plasminogen activator and the lower level in response to DDAVP. This possibility is supported by findings of elevated levels of acute phase reactants and mutual correlations exclusively in the group of smokers.

The main difference between nonsmokers and smokers recorded by us is a reduced t-PA activity and a reduced factor VIII R:Ag level, opposite changes in the balance between coagulation and fibrinolysis; the resultant effect on hemostasis may tend to balance out. However, a mere subtraction of the two effects on hemostasis may be too simple a concept apart from the possibility that the impacts on the venous and arterial situations may be very different. It should be noted that it has been claimed paradoxically that smoking protects against thromboembolic complications after myocardial infarction,50,51 and also protects against the development of deep venous thrombosis.52,53 In these situations, the lowered levels of factor VIII R:Ag may be more important than the accompanying reduction in fibrinolytic activity.

Acknowledgments

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References


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R A Allen, C Kluft and E J Brommer

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