Relationships between Activators and Inhibitors of Plasminogen, and the Progression of Small Abdominal Aortic Aneurysms

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Objective: plasmin is a common activator of the known proteolytic systems involved in the aneurysmal degradation, and is reported to be associated with the expansion of abdominal aortic aneurysms (AAA). The aim of this study was to study the activating pathways of plasminogen as predictors of the progression of AAA.

Materials and Methods: one hundred and twelve of 122 male patients with a small AAA (def.: 3 cm) were interviewed, examined, had blood samples taken at diagnosis, and scanned annually for 1–5 years (mean 3.5 years), and referred for surgery if the AAA exceeded 5 cm in diameter.

A random sample of 70 of the 112 cases had plasma levels of urokinase-like-plasminogen activator (uPA), tissue-type-plasminogen activator (tPA), plasminogen-activator-inhibitor-1 (PAI-1), macrophage inhibiting factor (MIF), tumour-growth-factor-β1 (TGF-β1), homocysteine, and serum levels of IgA-antibodies against Chlamydia pneumoniae (IgA-CP) and Cotinine (a nicotine metabolite) measured. Spearman’s correlation analysis was used for statistics.

Results: the annual expansion rate correlated positively with tPA, IgA-CP and S-Cotinine; r = 0.37 (p = 0.002), 0.29 (p = 0.006) and 0.24 (p = 0.038), while PAI1, uPA, TGF-β1, homocysteine, and MIF did not. S-Cotinine did also correlate positively with tPA, r = 0.24 (p = 0.049).

Conclusion: the aortic matrix degradation in AAA may be partly caused by an activation of plasminogen by tPA, but apparently not by uPA, which usually dominates matrix degradation. Smoking seems to be a factor for this pathway, while the pathways of IgA-CP and MIF, a new marker of aneurysmal progression, seem different. The latter observations suggest that other proteolytic pathways are involved in the aortic wall degradation in AAA.

Key Words: Plasmin; Expansion; Pathogenesis; Abdominal aortic aneurysm; Plasminogen; tPA; uPA.

Introduction

The identification of predictors of the expansion of small abdominal aortic aneurysms (AAA) may become important for managing this type of patients with regard to operative intervention or other therapies, and may also help to understand the pathogenesis of AAA.

Three proteolytic systems seem to be involved in the degradation of aorta causing AAA:

1. The serine-dependent proteases. The levels of elastase have been found elevated in circulation and aneurysmal walls compared with aortic walls of ouclusive atherosclerosis.1–3

2. The cysteine-dependent proteases. Circulating levels of Cystatin C – the major inhibitor of cysteine proteases – have been reported decreased in aneurysmal cases compared with a sex- and age-matched control group,4 and negatively correlated to aneurysmal expansion rate.5

3. The metallo-dependent proteases. Levels of various metallo-dependent proteases especially MMP2 and MMP9 have been found elevated in aneurysmal aortic walls compared with aortic walls of occlusive atherosclerosis.6,7 The plasma level of MMP9 has also been correlated with the expansion of small AAA.8

Besides its fibrinolytic function in plasma, plasmin also plays a central role in the activation of the degenerative processes in tissues. Thus, plasmin is a common activator of the mentioned proteolytic systems,9–11 and could be involved in the pathogenesis of AAA.
Recently, we demonstrated the plasma concentrations of plasmin–antiplasmin complex (PAP) correlates with aneurysmal expansion and are predictive for cases expanding to operation recommendable sizes in a cohort of male AAA-patients diagnosed by ultrasonographic screening.12 Years ago, Reilly et al. claimed that uPA and especially tPA is expressed more in AAA than in atherosclerotic occlusive aortae.13 Consequently, this study was performed in order to study potential prognostic markers for aortic expansion in this plasmin activation. As potential activators of tPA or uPA, smoking, homocysteine, IgA–CP, MIF, and TGF-β1 were also studied.

Material and Methods

In 1994, half (4404) of all 65–73 year-old males in Viborg County, Denmark, were invited to B-mode-ultrasonographic screening for AAA at their regional hospital.15 An AAA was defined as an aortic diameter of 30 mm or more. The participants were informed, interviewed, and examined, including a rescan, by the trial doctor. An AAA was defined as an infrarenal aortic diameter of 30 mm or more, and AAA >50 mm were referred for surgery. AAA of 30–49 mm was offered yearly follow-up examinations to check for any expansion.

The expansion was calculated as the change in the anterior–posterior (AP) diameter during the whole observation period, transformed to annual units.

All the patients with an AAA consulted the trial doctor for information, examination, and a rescan. Two observers were used. Their arimetric inter-observer variation (2s.d.) of the measurements was 1.4 mm.14

In order to reduce the round the clock variations of the various serological parameters, all samples were taken between 9.00 a.m. and 12.00 noon by the screening team.15 It was impossible to do this during the daily screening sessions, so sample days were arranged within ten days of the initial scan. Only subjects living less than 30 km from the hospital were asked to attend blood sampling, and two of these refused sampling. In all, 13 had no blood samples taken. Their age, AAA size, and systemic blood pressures did not differ from those who had blood samples taken. The plasma samples were stabilized with EDTA and left at 18 C in 45 min before centrifugation. Hereafter serum and plasma samples were stored in multiple aliquots at −70 C until analysis. Due to limited economical resources, a random sample had plasma levels of urokinase-like-plasminogen activator (uPA), tissue-type-plasminogen activator (tPA), plasminogen-activator-inhibitor-1 (PAI-1), macrophage inhibiting factor (MIF), tumour-growth-factor-β1 (TGF-β1), homocysteine, and serum levels of IgA-antibodies against Chlamydia pneumoniae (IgA-CP) and Cotinine (a nicotine metabolite) measured. All parameters were measured in all patients.

The randomization was performed with the utility in EPI-info 6. P-PAI-1 was measured with the ELISA “Imulyse PAI-1” from Biopool Int., Umeå, Sweden. The within- and between-assay coefficient of variation is 5 and 9%, respectively. It detects latent and active PAI-1, but poorly complexes with uPA and tPA.16

Similarly, the P-tPA was measured with the ELISA “Imulyse tPA” from Biopool Int., Umeå. The within- and between-assay coefficient of variation is 8 and 10%, respectively. It detects free single and two-chain tPA and tPA in complex with its inhibitors.17

MIF levels in serum were measured blindly with a routine MIF-specific sandwich ELISA using recombinant human MIF as standard.19,20 The coefficient of variation was 18.5%.

Serum TGF-β1 levels were determined using TGF-β1 ELISA kit according to the manufacturer (BioSource International, Camarillo, CA). The coefficient of variation was 28%.

P-total homocysteine (P-tHcy) were analysed using gas chromatography–mass spectrometry after reduction with dithiothreitole. Deuterated homocysteine was used as internal standard.21 The inter- and intra-assay coefficient of variation of the P-tHcy measurements was 5 and 3%, respectively, as assessed by internal and external quality assessment.21

IgA antibodies against C. pneumoniae measured by means of microimmunofluorescence (MIF) tests.22

S-Cotinine were determined by a commercial radioimmunoassay (Diagnostic Products Corp., LA, U.S.A.) modified as suggested by Perkins et al. The analytical coefficient of variation was 5.4%. The trial was approved by the national board of health, local scientific ethics committee and reported to the data protection authorities.

Wilcoxon’s non-parametric test for unpaired data was used to compare selected and non-selected cases from the population. Spearmann’s correlation analyses were used to correlate the parameters initially with expansion rate, and secondary, significant findings with tPA and uPA. To correct for the possibility of chance findings, the p-values in primary and
secondary correlations were multiplied with the number of tests performed, respectively.
Finally, logarithmic transformation of S-Cotinine were performed in order to make a multiple regression analysis concerning tPA and expansion rate adjusting for smoking.

**Results**

Of the 4404 men invited to screening, 3344 (76%) attended, and an AAA was diagnosed in 141 of these (4.2%). Nineteen AAA were over 5 cm in diameter, and these patients were soon referred for surgery; the remaining 122 cases were offered annual control scans and referred for surgery if the AAA expanded to more than 5 cm in diameter. Ten cases were lost to follow up due to death or severe illness during the first year, and the rest have been followed for one to five years, in average 3.5 years.

The medians and interquartiles of the parameters among the selected and non-selected cases from the population are shown in Table 1. There were no differences between the selected and non-selected cases concerning age, initial AAA diameter, systolic and diastolic blood pressure, and mean annual expansion rate.

The annual expansion rate correlated positively with tPA, IgA±CP and S-Cotinine; $r = 0.37$ ($p = 0.002$, Fig. 1), $0.29$ ($p = 0.006$) and $0.23$ ($p = 0.038$), while MIF ($r = 0.22$, $p = 0.061$), PAI-1 ($r = 0.015$, $p = 0.015$), uPA ($r = 0.001$, $p = 0.993$, Fig. 2), TGF-$\beta1$ ($r = 0.001$, $p = 0.999$), and Homocysteine ($r = 0.063$, $p = 0.535$) did not. Adjustment for the risk of chance findings removed the significant finding of S-Cotinine, while tPA and IgA–CP were still significantly associated with expansion rate.

S-Cotinine did also correlate positively with tPA, $r = 0.24$ ($p = 0.049$) negatively significantly with uPA ($r = -0.345$, $p = 0.006$), while IgA–CP did not correlate with tPA or uPA (Table 2). Logarithmic transformation of S-Cotinine and tPA normalised their distributions acceptable. In multiple linear regression analyses adjusting for S-Cotinine, the correlation between tPA and expansion rate remained significantly correlated, while S-Cotinine failed to reach significance and *vice versa* if the sequence of independent variables was reversed.

**Discussion**

The study showed significantly positive correlations between tPA, S-Cotinine, and aneurysmal progression but not with uPA, which usually dominates matrix degradation. The latter may be due to the fact that the binding of uPA to their specific receptors on the membrane of the cells is quite stable and long lasting in order to cause local matrix degradation. It may prevent inactivation but maybe also relevant for systemic detection. This mechanism and the unavoidable pollution of the systemic measurement of uPA originating from non-aortic locations, could explain the missing correlation with aneurysmal expansion.

The optimal study design would of course have been also to have aortic samples tested, but this was obviously not possible in this prospective study. Nevertheless, the present study shows that systemic measurement of uPA is not prognostic for the progression of AAA.

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**Table 1. Medians and interquartiles of the various analysed parameters compared with the non-selected part of the population of screening diagnosed small abdominal aortic aneurysms. p-values from a non-parametric comparison of these two subgroups are shown in the right column.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Selected population ($n = 70$)</th>
<th>Non-selected population ($n = 52,\ast$)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>65</td>
<td>66</td>
<td>0.23</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>80</td>
<td>85</td>
<td>0.84</td>
</tr>
<tr>
<td>AAA-size (mm)</td>
<td>31</td>
<td>30</td>
<td>0.48</td>
</tr>
<tr>
<td>Expansion rate (mm/year)</td>
<td>0.92</td>
<td>0.74</td>
<td>0.83</td>
</tr>
<tr>
<td>tPA (ng/ml)</td>
<td>6.80</td>
<td>0.74</td>
<td>0.83</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>22.5</td>
<td>31.5</td>
<td>40.0</td>
</tr>
<tr>
<td>uPA (ng/ml)</td>
<td>1.53</td>
<td>1.80</td>
<td>2.40</td>
</tr>
<tr>
<td>IG-A–CP (ie/l)</td>
<td>11.8</td>
<td>14.2</td>
<td>18.0</td>
</tr>
<tr>
<td>Homocysteine (mmol/l)</td>
<td>11.8</td>
<td>14.2</td>
<td>18.0</td>
</tr>
<tr>
<td>MIF (ng/ml)</td>
<td>0.00</td>
<td>2.30</td>
<td>3.85</td>
</tr>
<tr>
<td>TGF-$\beta1$ (ng/ml)</td>
<td>445</td>
<td>527</td>
<td>598</td>
</tr>
<tr>
<td>Cotinine (ng/ml)</td>
<td>0.00</td>
<td>165</td>
<td>340</td>
</tr>
</tbody>
</table>

\*n = 42 concerning expansion data.
They also did an immunohistochemistry study, which showed that tPA in normal aortas only are present in the intima, while tPA is diffusely present in the intima and media of AAA-walls. UPA was only present in the monocellular cells in the infiltrate associated with the adventitia in AAA-walls. Thus the role of uPA in AAA remains unsolved. The described localization in the adventitia could indicate a role in the neo-angiogenesis as suggested by Scheiderman et al.27

TPA is binding to the surface of the matrix in order to cause matrix degradation and avoid inactivation from PAI. The inactivation is very strong and fast when tPA leaves their receptors. However, in spite of this and the potential pollution with tPA from non-aortic locations, a strong correlation between tPA and aneurysmal expansion rate was noticed indicating that the cell and matrix plasmin activation through the tPA pathway could play a major role in the progression of small AAA. One could argue, that the events going on in the AAA wall probably has nothing or very little to do with the systemic changes as we were measuring. However, AAA is associated with structural changes in the other parts of the major and even minor arterial vessels without any sign of dilatation yet. These laboratory reports are complementary to the clinical knowledge that AAA is associated with arterial dilatations and aneurysms at other locations. Thus, we may be able to demonstrate the association between arterial wall degradation and tPA, otherwise the role of tPA in the AAA is so strong that it overcomes the pollution of tPA from other locations. The design of this present study can never discriminate between these two possibilities.

Again, the optimal study design would of cause have been also to have aortic samples tested. Nevertheless, the present study shows that systemic measurement of tPA may have a potential of being a clinically useful prognostic marker. However, a larger sample size, longer follow up time, and perhaps repeated measurements are needed before any clinical recommendations can be made.

In all, we believe this prospective study supports Reillys suggestion, that plasminogen activators participate in the pathogenesis of AAA, and that this participation is unique because it is mainly performed by tPA.

Genetic polymorphic variations in PAI-1 have been associated with family AAA.26 However, it did not correlate with the expansion rate suggesting a minor, if any role, in the progression of AAA. Smoking and hyperhomocysteinemia has been associated with AAA, and is known to influence

**Table 2. Non-parametric correlation matrix between activators and inhibitors of plasminogen, and the size and progression of small abdominal aortic aneurysms. Spearman’s correlation coefficients, p-values in parenthesis.**

<table>
<thead>
<tr>
<th></th>
<th>tPA</th>
<th>uPA</th>
<th>Cotinine</th>
<th>IgA-CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expansion rate</td>
<td>0.368*</td>
<td>0.001</td>
<td>0.234†</td>
<td>0.290**</td>
</tr>
<tr>
<td>(mm/yr)</td>
<td>(0.002)</td>
<td>(0.993)</td>
<td>(0.038)</td>
<td>(0.006)</td>
</tr>
<tr>
<td>tPA (ng/ml)</td>
<td>0.125</td>
<td>0.238†</td>
<td>0.135</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.308)</td>
<td>(0.049)</td>
<td>(0.252)</td>
<td></td>
</tr>
<tr>
<td>uPA (ng/ml)</td>
<td>-0.345*</td>
<td>-0.053</td>
<td>0.006</td>
<td>0.690</td>
</tr>
</tbody>
</table>

* p < 0.05.
** p < 0.05 after correction for mass significance.
relevant matrix proteases. TGF-β1,32 MIF33 and IgA–CP34 are also reported associated with AAA but their pathway is partly unknown. Consequently, they were studied in order to be potential activators of tPA or uPA.

The positive correlations between S-Cotinine, tPA and expansion rate suggests that smoking is participating in this pathway, and smoking has been reported associated with AAA and aneurysmal progression.38–40

This is in accordance with the confounding which seemed to be present in the performed multiple linear regression analyses since the significant correlations between expansion rate, tPA and S-Cotinine, tPA disappeared. However, the association between S-Cotinine and expansion rate was relatively weak, and failed to reach significance when adjusted for the risk of chance findings.

The lack of significant correlations between tPA, IgA–CP and MIF, two other markers of aneurysmal progression,33,34 suggests additional proteolytic pathways must be involved. MIF levels did not correlate with expansion rate (p = 0.06). However, it was recently reported to correlate positively with aneurysmal expansion.35,36 The negative result in this study may be due to the relatively low numbers studied, a relatively high frequency of cases with MIF-levels below the detection border and a relatively high CV.

TGF-β1 is suspected to activate tPA and PAI-110 but it did not correlate with expansion rate. However, the high variation of the measurements of TGF-β1, the interobserver variation of the aortic measurements, and the pollution of TGF-β1 originating from non-aortic locations could hide a weak correlation.

The role of hyperhomocysteinaemia in atherosclerosis and AAA remains controversial; it has been reported increased in AAA cases in a case-control study,31 and is known to influence the matrix proteases. Consequently, we analysed it in this study, and failed to reach significance when adjusted for the risk of chance findings.

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