

# Cerebrospinal fluid activity of tissue plasminogen activator in patients with neurological diseases

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## Abstract

**Aim**—To study cerebrospinal fluid (CSF) activity of tissue plasminogen activator (tPA) in patients with neurological diseases.

**Methods**—CSF tPA and urokinase (uPA) activities were studied using an immunocapture assay and zymography in 44 patients with neurological disease and 20 reference subjects. The patient group comprised three patients with meningitis, 21 with encephalitis, nine with acute lymphoblastic (n = 7) and myeloid (n = 2) leukaemia, seven with multiple sclerosis, three with facial paresis, and one with polyradiculitis.

**Results**—Raised tPA activities were observed in patients with multiple sclerosis, leukaemia and encephalitis. In contrast, there were no differences in the mean activities of tPA in patients with meningitis or other diseases compared with the reference subjects. The highest tPA activities were found in patients with multiple sclerosis. The mean activity in patients with leukaemia was higher than in those with meningitis and polyradiculitis, but not encephalitis and facial paresis. Although the CSF tPA activity correlated positively with age in reference subjects, no correlation was observed in patients. Samples were qualitatively screened for both tPA and uPA activity by zymography and positive samples were quantitated. Some of the samples had quantifiable levels of uPA activity: three of seven multiple sclerosis samples, 10 of 21 samples from patients with encephalitis and five of nine leukaemic samples. The highest activities were recorded in patients with leukaemia. uPA was not detected in the CSF of the patients with meningitis, facial paresis or polyradiculitis.

**Conclusions**—Plasminogen activator activity can be measured reliably in CSF and the assessment of tPA activity may be useful for studying the pathogenesis of neurological diseases.

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**Keywords:** cerebrospinal fluid, neurological diseases, plasminogen activators.

Plasminogen activators, tissue-type (tPA) and urokinase-type (uPA), are serine proteases, the most well characterised function of which is the conversion of the inactive zymogen plas-

minogen into the active protease plasmin. Plasminogen activators have been implicated in a number of biological processes, including fibrinolysis and thrombolysis, cell migration, tissue invasion by both normal and malignant cells, and tissue remodelling.<sup>1,2</sup> Altered plasminogen activation has been reported in patients with diabetes<sup>3,4</sup> and rheumatoid arthritis.<sup>5</sup> The latter study also recorded raised plasmin concentrations in synovial fluid.<sup>5</sup> Similarly, high plasma plasminogen activator concentrations have been observed in patients with acute non-lymphoblastic leukaemia.<sup>6</sup> Ridker *et al*<sup>7</sup> reported that in prospectively collected blood samples, high concentrations of endogenous tPA, the primary mediator of intravascular fibrinolysis, in apparently healthy men are strongly associated with the risk of future myocardial infarction. Similarly, high concentrations of tPA antigen in apparently healthy men have been reported to be independently associated with a high risk of stroke.<sup>8</sup> tPA antigen concentrations have also been found to correlate with the presence of carotid atherosclerosis.<sup>9</sup> Lipoprotein (a) has been reported to exert an atherogenic effect by inhibiting plasminogen activation.<sup>10-12</sup> Moreover, the roles of plasminogen activators and plasminogen activation inhibitors (PAI-1 and PAI-2) in the pathogenesis of neurological diseases and tumour development have been reported.<sup>13-16</sup> The tPA gene is expressed in the developing brain<sup>17-19</sup> and in human tumour cells of neuroectodermal origin.<sup>20</sup>

Here, we report the activity of plasminogen activators in cerebrospinal fluid (CSF) of patients with multiple sclerosis, encephalitis, meningitis, polyradiculitis, facial paresis, and leukaemia in comparison with reference subjects without neurological disease.

## Methods

### STUDY POPULATION/CHOICE OF SAMPLES

The procedures followed were in accord with the Helsinki Declaration of 1975 as revised in 1983 for human experimentation. This preliminary study initially involved 200 subjects undergoing spinal taps for clinical reasons, from whom about 1 ml of CSF was drawn into plain plastic containers. Samples were accepted for this study if the difference in time in days between specimen collection and analysis was less than or equal to 10. The final diagnosis was confirmed by the clinician. Samples which had been kept frozen for more than 10 days were excluded. Selection of samples became necessary when we observed that the

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tPA activity correlated negatively with storage time. Samples from 64 subjects (from newborn to 77 years of age), with a storage time of less than 10 days, were included in this study. Subjects with no neurological disease ( $n = 20$ ) served as controls. The patient group comprised three patients with meningitis, 21 with encephalitis, seven with multiple sclerosis, three with facial paresis, one with polyradiculitis, seven with acute lymphoblastic leukaemia, and two with acute myeloid leukaemia.

#### ZYMOGRAPHY

Zymography was used for the qualitative detection of plasminogen activator activity in CSF. Briefly, samples were run on an 8% polyacrylamide gel in the presence of SDS under non-reducing conditions.<sup>21</sup> The molecular weights of the lysed bands were estimated by comparison with pre-stained low molecular weight marker proteins (Pharmacia, Uppsala, Sweden). Activity standards of uPA (Calbiochem, La Jolla, California, USA) and tPA (American Diagnostica, Greenwich, Connecticut, USA) were also included. Before zymography, SDS was removed by washing the gel for six hours with phosphate buffered saline (PBS), pH 7.4, containing 2.5% Triton X-100. An agarose gel containing casein and 1.7 µg/ml plasminogen was then placed over the polyacrylamide gel, and the overlay incubated for 24–48 hours at 37°C in a humidified chamber. To characterise the protease present further, 10 µg/ml of an anticatalytic monoclonal antibody directed against uPA (catalogue number 394; American Diagnostica) was added to the polyacrylamide gel before incubation with the casein-agarose gel.

#### IMMUNOCAPTURE ASSAY FOR tPA AND uPA

The uPA and tPA activities of CSF were measured by an immunocapture assay as reported previously.<sup>22, 23</sup> Pro-uPA was activated by the addition of purified human plasminogen containing traces of plasmin. Thus, PAI-1 and pro-uPA can be present together in the sample without interference. tPA was assayed by a slightly modified procedure using 20 µg/ml poly-D-lysine in the assay buffer. Briefly, polystyrene microplate wells (Nunc, Roskilde, Denmark) were coated overnight at 37°C with 50 µl of a solution of rabbit or goat antihuman IgG antibodies directed against either human uPA (10 µg/ml) or tPA (2.5 µg/ml) (catalogue numbers 389 and 387, respectively; American Diagnostica). The plates were washed three times with PBS, pH 7.2, containing 0.05% Tween 20. Fifty microlitres of CSF were then dispensed and allowed to bind for two hours at room temperature. After binding, the wells were washed again (three times with PBS/Tween), and human plasminogen containing traces of plasmin was added to assay the proenzyme activity bound to the wells (2 µg in 50 µl uPA assay buffer consisting of 50 mM glycine pH 7.8, 0.1% Triton X-100, 0.1% gelatin, and 10 mM 6-aminocaproic acid). Plasminogen was purified from fresh human plasma by affinity chromatography on lysine-Sepharose.<sup>24</sup> The reaction with plasminogen

Table 1 Cerebrospinal fluid activities of plasminogen activators and their *p* values in comparison with the reference subjects (mean (SEM))

Subject group	tPA (µIU/ml)	uPA (µIU/ml)
Reference subjects ( $n = 20$ )	43.7 (6)	0
Multiple sclerosis ( $n = 7$ )	497 (23)†	14.0 (2)
Leukaemia ( $n = 9$ )	275 (48)***	55.0 (15)
Encephalitis ( $n = 21$ )	245 (25)†	28.0 (8)
Meningitis ( $n = 3$ )	63.5 (25)	0
Facial paresis ( $n = 3$ )	199 (0)	0
Polyradiculitis ( $n = 1$ )	51.7	0

\*\*\* $p < 0.001$  and † $p < 0.0001$  v reference subjects.

was allowed to proceed for 45 minutes at 37°C and the plasmin produced was assayed by its thioesterase activity.<sup>25</sup> To estimate the amount of enzyme produced, high molecular weight two-chain uPA (80 000 IU/mg; American Diagnostica) and two-chain tPA (650 000 IU/mg; American Diagnostica) were used as standards.

#### STATISTICS

Results are reported as mean (SEM). The Student's *t* test was used for the comparison of means. Correlation coefficients were evaluated according to Pearson. Results were considered significant when  $p \leq 0.05$ .

#### Results

Samples were qualitatively screened for both uPA and tPA activity by zymography and positive samples were quantitated by the immunocapture assay. Positive samples were seen as lytic bands of tPA at molecular weight of 70 kilodaltons and uPA at molecular weight of 50 kilodaltons (data not shown).

We observed significantly higher mean tPA activities in patients with multiple sclerosis ( $p < 0.0001$ ), leukaemia ( $p < 0.001$ ) and encephalitis ( $p < 0.0001$ ) in comparison with the reference subjects (table 1).

In contrast, there were no statistically significant differences in the mean activities of tPA in patients with meningitis, facial paresis and polyradiculitis when compared with the reference subjects (table 1). The mean tPA activity in patients with multiple sclerosis was very increased compared with the mean activities in each of the other patient groups. The mean activity in patients with leukaemia was higher than the mean activities in those with meningitis and polyradiculitis, but not in those with encephalitis and facial paresis.

The CSF tPA activity correlated positively with age in the reference subjects ( $r = 0.52$ ;  $p = 0.018$ ). No correlation was observed in patients with leukaemia, encephalitis and multiple sclerosis.

Some of the samples had quantifiable levels of uPA activity: three of seven samples from patients with multiple sclerosis, 10 of 21 from those with encephalitis and five of nine from patients with leukaemia. The highest activities were recorded in patients with leukaemia (table 1).

#### Discussion

tPA activity in patients with multiple sclerosis was raised consistently and was significantly higher than in any of the other groups studied.

Multiple sclerosis is a chronic disease of the central nervous system of largely unknown aetiology, affecting young and middle aged adults. The myelin sheaths surrounding nerves in the brain and spinal cord are damaged, which affects the function of the nerves involved. The disease affects different parts of the brain and spinal cord, resulting in typically scattered symptoms. The involvement of plasminogen activator induction in the pathogenesis of multiple sclerosis and encephalitis has been suggested by studies of peripheral blood lymphocytes.<sup>26</sup> The authors also reported the disappearance of plasminogen activator induction in association with resolution of neurological symptoms.<sup>26</sup>

It is also worth considering activation of the vascular endothelium, as this is thought to have an important role in the pathogenesis of inflammation, thrombosis and vasculitis. Endothelial activation plays a central role in the pathogenesis of multiple sclerosis and encephalitis. Furthermore, proteases and cytokines have also been implicated in these diseases.<sup>26-31</sup> Activation of endothelial cells in patients with encephalitis by vasoactive amines and proteases induces vasospasm and breakdown of the blood-brain barrier.<sup>28</sup> The damage to the blood-brain barrier leads to biochemical changes in the CSF as previously impermeable substances can pass through the barrier more freely.<sup>32</sup> The damage to the blood-brain barrier perhaps explains the raised tPA activity observed in patients with encephalitis and normal activity in those with meningitis. The same mechanism of tissue destruction is thought to be active in autoimmune encephalomyelitis and multiple sclerosis.<sup>27</sup>

In contrast, the tPA activities observed in patients with meningitis, facial paresis and polyradiculitis were very similar to those observed in the reference subjects. The CSF tPA activity correlated positively with age in the reference subjects; no correlation was observed in any of the other groups. The tPA activity also correlated negatively with the interval between sample collection and analysis; samples were stable for up to 10 days when stored frozen. This was not the case for uPA, which may be the result of the very low levels of uPA activity detected in these samples.

The significance of tPA gene expression in the developing brain is not understood clearly.<sup>17-19</sup> According to Carroll *et al*<sup>17</sup> in the adult rat brain, transcription of the tPA gene is an immediate early response in the hippocampus following the induction of neuronal plasticity. The results they obtained supported the concept that tPA plays a role in neurogenesis and morphogenesis, and identified the promoter region that directs transcriptional regulation, both during development and in the adult central nervous system. tPA is the main plasminogen activator associated with the rat brain growth cones, suggesting that it is required for neurite growth.<sup>18</sup> tPA expression during embryogenesis has been confirmed by *in situ* hybridisation.<sup>19</sup> The results indicate strongest expression in the basal midbrain and hindbrain, continuing posteriorly into the neu-

ral canal. The expression coincides with extensive cell migration and proliferation, and tissue remodelling in these regions. It seems that tPA is expressed by a number of different cell types in the developing nervous system and may also play a role in cell migration and tissue remodelling in later life.

Some of the samples had quantifiable levels of uPA activity. The highest uPA activity was recorded in samples from patients with leukaemia. Myeloid leukemia cells have been found to produce either tPA or uPA in culture.<sup>33</sup> Cell surface bound uPA activity has been detected in patients with acute leukaemia, whereas plasminogen activator activity was not detected in mononuclear cells from either healthy controls or patients with chronic lymphoid leukaemia.<sup>34</sup>

Zymography was more suitable for the detection of uPA than tPA. The latter could only be detected when a thinner casein-milk gel was used as the overlay. The uPA activities detected in the present study were so low that the immunocapture assay was probably not the most appropriate quantitative assay. Some samples which had detectable uPA activity on zymography, had no measurable activity in the immunocapture assay. Such results can occur if the uPA is non-covalently bound either to an inhibitor or to another protein inhibiting the binding of this serine protease to the immunocapture plate.<sup>35</sup>

Samples from patients with leukaemia had high tPA activities and most had quantifiable uPA activity. Earlier studies of patients with myeloid leukaemia have shown that cells from these patients produce either tPA or uPA in culture. The type of plasminogen activator produced was found to have prognostic significance: patients whose cells secreted tPA failed to respond to chemotherapy, whereas 80% of those whose cells secreted uPA achieved remission.<sup>33</sup> Wilson *et al*<sup>36</sup> again reported that at the earlier stages of differentiation, the precursors of myeloid cells mainly produced tPA.

Further studies involving CSF are required to elucidate the role of plasminogen activators in the diagnosis and pathogenesis of the above clinical conditions, especially in multiple sclerosis, leukaemia and encephalitis, and possibly also in facial paresis. There will also be the need to study PAI-1 in the CSF of these patients, in comparison with concentrations present in CSF of normal subjects.<sup>37</sup>

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