Tissue plasminogen activator in brain tissues infected with transmissible spongiform encephalopathies

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This article is dedicated to the memory of Professor Stelios Orphanoudakis.

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Introduction

Prions are proposed to be propagated through transmission of a disease-related isoform of the prion protein (Prusiner, 1982). PrP C is a glycoprotein, mainly located in neuronal tissue, but detectable in other tissues as well (Manson et al., 1992; Moser et al., 1995). The physiological role of prion protein has not been defined yet.

Prion propagation involves conversion of host PrP C to a disease-related isoform, PrP Sc, which accumulates during disease and is the principal component of the transmissible agent. PrPSc formation seems to play an important role in PrP metabolism. Plasminogen, a serine protease precursor, has been shown to interact with PrPSc. Plasminogen can be proteolytically activated by tissue plasminogen activator (tPA). Recent reports imply a crosstalk between tPA-mediated plasmin activation and PrP. In our study, both tPA activity and tPA gene expression were found elevated in TSE-infected brains as compared to their normal counterparts. Furthermore, it was proved that PrPSc, in contrast to PrP C, could not be degraded by plasmin. In addition, it was observed that TSE symptoms and subsequent death of plasminogen-deficient and tPA-deficient scrapie challenged mice preceded that of wild-type controls. Our data imply that enhanced tPA activity observed in prion infected brains may reflect a neuro-protective response.

There are some indications it may act as a cellular receptor, as a protein receptor (Martins et al., 1997) and as a protein involved in cell signaling (Massimino et al., 2002). There is evidence about an active role of prion protein in the metabolism of copper (Brown et al., 1997; Pauly and Harris, 1998) and furthermore, it is suggested that it has a protective role for the cell against oxidative stress (Brown et al., 1999, 2001; Ellis et al., 2002; Klamt et al., 2001).

Human tPA is an approximately 68 kDa molecular weight serine protease composed of 527–530 amino acids. It has two kringle domains involved in its binding to fibrin and possibly in its binding to a prion–plasminogen complex (Ryou et al., 2003). It is secreted mainly from vascular endothelial cells and in the nervous system from neurons and microglia (Tsirka et al., 1997). It can be detected in most biological fluids; its concentration in plasma is 50 \( \mu \)g/l. tPA is primarily involved in the activation of the inactive zymogen plasminogen to the active protease plasmin by hydrolyzing the peptidic bond between residues R561 and V562. Plasmin is involved in a variety of biological processes, such as cell migration, growth, inflammation and tumor invasion, but its primary function is to dissolve blood clots in the vasculature. It has been reported that tPA is secreted in response to mental stress (Jern et al., 1989) and that it plays a role in neurodegeneration (Qian et al., 1993) and neuronal plasticity (Baranes et al., 1998; Qian et al., 1993). It was recently shown that tPA can cross the blood–brain barrier via interaction with one of its cell surface receptors, the LDL receptor-related protein (Yepes et al., 2003).

Plasminogen has been reported to have high affinity for PrPSc but not for PrP C (Fischer et al., 2000; Maissen et al., 2001). However, more recent studies proved that plasminogen can be bound to human recombinant PrP, which resembles the normal isoform of PrP (PrP 0) (Ellis et al., 2002; Kornblatt et al., 2003; Praus et al., 2003). The thermodynamics and the kinetics of the complex formation between...
human recombinant PrP and plasminogen have been studied by Cuccioloni et al. (2004), whereas Kornblatt et al. (2004) have studied the pressure and thermal sensitivity of the complex between recombinant sheep PrP and human plasminogen.

The biological role of this interaction has not yet been elucidated, but given the complex formation, plasminogen has been considered to be a possible carrier of PrPSc to the central nervous system, through the blood circulation (Fischer et al., 2000). As far as plasmin-mediated PrP proteolysis is concerned, it has been shown that soluble recombinant PrP is cleaved by plasmin (Kornblatt et al., 2003; Praus et al., 2003) and that it can stimulate tPA activity (Ellis et al., 2002; Praus et al., 2003). Ellis et al. reported that the plasminogen activation system is stimulated by the presence of apo-PrP, i.e. PrP not bound to Cu2+, whereas Praus et al. found that stimulation occurs through its NH2-terminal fragment (PrP23–110) (Praus et al., 2003). The latter is in accordance with an earlier finding that tPA-mediated plasminogen activation can be stimulated by partially denatured proteins as well (Machovich and Owen, 1997).

In light of these findings, we assessed tPA activity in TSE-infected tissue and evaluated tPA gene expression. We also verified PrPSc stability towards plasmin-mediated proteolytic degradation and challenged tPA and plasminogen-deficient mice with scrapie agents. As expected, PrPSc contrary to PrPC proved to be protease-resistant. Interestingly, tPA activity was found elevated, in accordance to elevated tPA gene expression, while the tPA and the plasminogen-deficient mice appeared to be more susceptible to TSEs than their wild-type counterparts.

Materials and methods

Tissue homogenates and specific reagents

All the tissues in the study originated from animals of the same age, gender and clinical stage, with similar living conditions. Following sacrifice, tissues were stored at −70°C, and subsequent thawing was accomplished by placing them in an ice bucket for the necessary amount of time. Hamster scrapie 263K strain was kindly provided by Dr. R. Gabizon, Hadassah University Hospital, Jerusalem, Israel. Scrapie strains ME7, 22A and 79A were kindly provided by Dr. M. Groschup, Federal Research Centre for Virus Diseases of Animals, Institute for Novel and Emerging Infectious Diseases, Riems, Germany. Brains from 301V BSE mice were obtained from Dr. M. Dawson, VLA Weybridge. Native scrapie and normal sheep autopsy material was kindly provided by Dr. P. Tourmazos, Veterinary Services Laboratory, Cyprus.

For the tPA assay, 10% (w/v) homogenates were prepared using a motor-driven homogenizer (Polytron Kinematica, Switzerland). Two homogenization runs were performed (setting 4, 6 s each). The homogenization buffer consisted of 0.5% w/v sodium deoxycholate (DOC Fluka, Germany) and Igepal CA-630 0.5% v/v (Sigma, St. Louis, MO) in PBS (phosphate-buffered saline, pH 7.4). For PrPSc immunoprecipitation and quantitative isolation of PrPSc, 10% homogenates were prepared using a glass pestle and mortar. The homogenization buffer was the same as above, the only difference being the addition of Protease Inhibitors Cocktail Mix at a final concentration of 0.1 v/v (Sigma, St. Louis, MO) in the buffer used for the immunoprecipitation experiments.

Specific materials used for the tPA assay described below include tPA and plasminogen, both of which were purchased from Sigma, St. Louis, MO and the chromogenic substrate d-Ile-Pro-Arg-p-nitroanilide dihydrochloride, which was acquired from Chromogenix, Italy or Sigma, St. Louis, MO.

tPA assay

300 mg wet brain tissue (cerebella or brain stems for sheep and cortices for mice and hamsters) was used to prepare homogenates. Upon homogenization, the total protein content in each homogenate was assayed, using the BCA protein assay kit (Pierce, Rockford, IL). The homogenates were aliquoted and stored at −70°C. The aliquots used for this study were not thawed more than once.

Quantitative isolation of PrPSc was performed for every sample (Polymenidou et al., 2002), and the presence of PrPSc was confirmed by Western blotting.

The tPA activity assay was carried out in 96-well ELISA plates (Greiner, Longwood, Fl). To each well were added: 30 μl distilled water, 130 μl 0.1 M Tris–HCl, pH 8.0 (Sigma, St. Louis, MO), 0.1% v/v Tween 80 (Sigma, St. Louis, MO), 10 μl 8.4 μM plasminogen, 10 μl 10% brain homogenate [diluted in 0.25% v/v Triton X-100 (Sigma, St. Louis, MO) in order to contain 500 μg brain equivalent] and 20 μl 3 mM substrate d-Ile-Pro-Arg-p-nitroanilide dihydrochloride. The assay was performed for each sample in triplicates. Plates were incubated at room temperature for 3 h, and readings at 405 nm were performed using an ELISA plate reader (MWG, Germany). Samples containing known amounts (0–50 mIU) of tPA were also assayed. The OD680 readings of those samples were used to plot a standard curve and thus, to convert the OD reads of the samples into specific tPA activity, expressed in International Units (IU). Blank samples, containing water instead of brain homogenates, were used as negative controls. The final results were expressed as milli International Units of tPA per μg of total protein (mIU/μg).

In order to optimize the assay, the kinetics of the reaction and the effect of pH were investigated. For the kinetics study, the tPA activity of a sample containing 500 μg sheep brain equivalent was measured every 5 min for 210 min and then after 24 h. As a control, the tPA activity of the same sample was estimated in a reaction mixture which contained no plasminogen. For the pH-effect study, the pH of the reaction mixtures was adjusted with NaOH or HCl to a variety of pH values, ranging from 6.0 to approximately 12.0, and then the OD680 was assessed after a 3 h incubation period at room temperature.

RNA isolation

Total RNA was isolated from C57Bl mouse brains infected with the 79A scrapie strain and normal C57Bl mouse brains, using a commercially available kit (Promega, Madison, WI). All mice were sacrificed at the same age of about 6 months, when the scrapie-infected animals were in the final clinical stages of the disease. The starting material for each RNA isolation was 50 mg cortex, homogenized in 600 μl of denaturing buffer. The isolation was performed following the manufacturer’s instructions. Isolated RNA was resuspended in 60 μl RNase-free H2O and then treated with RQ1 RNase-free DNase (Promega, Madison, WI) to remove co-isolated DNA. Briefly, 6 μl of the RQ1 DNase and 7 μl of the appropriate 10× reaction buffer were added to the RNA suspension, and the mixture was incubated for 30 min at 37°C. RQ1 DNase was inactivated by addition of 7 μl of stop solution.
containing 20 mM ethylene glycol-bis[\(\beta\)-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA) pH 8.0 and incubation for 10 min at 65°C. To remove all compounds added for the DNase treatment, RNA was purified on a Nucleospin RNA II column (Macherey-Nagel, Germany), and it was finally eluted in 60 μl of RNase-free H₂O. The purified RNA was quantified and qualified spectrophotometrically.

To assess the integrity of the isolated RNA, cDNA synthesis was performed. PCR primers tPA1 and tPA2 were used (tPA1: 5'-CCA CCT GTG GCC TGA GGC AGT ACA A-3'; tPA2: 5'-ATG CCT CAT GCT TGC CGT AGC CAG A-3'). These primers amplify a 473 bp fragment from the mouse tPA gene, when cDNA serves as template. When genomic DNA is used as template, the amplification product has a size of 750 bp because the genomic sequence includes two introns in the genomic sequence (Siao et al., 2004). In brief, 10% brain homogenates were diluted 1:3500 in PBST, or anti-PrP mAb 6H4, diluted 1:5000 in PBST; and 6H4 (1.00 mg/ml, Prionics, Switzerland), which recognizes an epitope located at the C-terminal PrP domain (144–152), diluted 1:5000 in PBST. Both 4F2 and 12F10 antibodies were kindly provided by Prof. Walter Bodemer, Goettingen, Germany (Krasemann et al., 1996). The membranes were incubated with rabbit anti-mouse alkaline phosphatase conjugated secondary antibody (Pierce, Rockford, IL) and developed with the CDP star reagent (NEB, Beverly, MA) following the manufacturer’s instructions.

**Real-time PCR**

cDNA was synthesized following a standard RT-PCR protocol, using random hexamers as primers (Promega, Madison, WI) and AMV reverse transcriptase (Promega, Madison, WI). Total RNA (4 μg) was first denatured for 5 min at 70°C and then hybridized with 1 μg of the random hexamers for 5 min on ice. cDNA was synthesized for 1 h at 42°C by addition of the RT-PCR master mix containing 5 U AMV, 50 mM Tris–HCl pH 8.3, 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, 10 mM DTT and 1 mM of each dNTP in a final reaction volume of 20 μl. The AMV reverse transcriptase was inactivated by heating at 99°C for 5 min. For the amplification of murine tPA and β-actin gene fragments, the following primers were designed: mtPA117as: 5'-GCC AGA ACA TAC AGG GTG GT-3'; mtPA117as: 5'-CTG CAG TAA TGC GAT GTC GT-3'; moACT139s: 5'-TGT TAC CAA CTG GGA CGA CA-3'; moACT139as: 5'-CTG GTG CAT CTT TTC ACG GT-3'. The tPA primers amplify a 117 bp DNA fragment, while the primers for β-actin amplify a 139 bp DNA fragment. Real-time PCR was carried out in the Opticon DNA Engine II (MJ-Research, Reno, NV) as outlined: after initial denaturation for 3 min at 94°C, the following cycle was repeated 40 times: 60 s at 94°C, 20 s at 62°C and 15 s at 72°C with a final extension of 3 min at 72°C. SYBR Green I emission of the accumulating PCR product was measured at the end of the extension step after each cycle. Melting curve analysis was performed by measuring the emission of SYBR Green I at 515 nm from 60°C to 90°C every 1°C after a 10 s hold at each temperature. The PCR reaction mixtures contained cDNA corresponding to 1.6 μg of RNA, 2 U of the DyNaZyme (Finzymes, Finland), Mg²⁺-free buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl 0.1% Triton X-100), 0.2 mM of each dNTP and SYBR Green I (Sigma, St. Louis, MO) at a final concentration of 0.25×. PCR amplification products were visualized after separation on a 1.5% TBE agarose gel subsequently stained with ethidium bromide.

**PrP⁰⁰⁺ quantitative isolation and tPA/plasminogen treatment**

10% w/v brain homogenates, prepared as previously described, were centrifuged at 24,000 × g for 10 min. The supernatants were used for PrP⁰⁰⁺ isolation, as described elsewhere (Polyminidou et al., 2002). After the addition of PMSF, the samples were kept overnight at 4°C. The resulting pellets were resuspended in 20 μl of PBS and treated with 4.9 nM tPA and 0.28 μM plasminogen for 4 h at 37°C with agitation. The reactions were stopped by adding SDS-PAGE sample buffer, and the samples were boiled for 10 min before loading on an 18% SDS-PAGE gel. Electro-transfer and Western blotting of the proteins with either anti-PrP mAb 4F2, which recognizes an epitope on the N-terminal domain of PrP (51–90), diluted 1:3500 in PBST; 12F10 (0.88 mg/ml), which recognizes an epitope on the C-terminus domain of PrP (142–160), diluted 1:3500 in PBST; and 6H4 (1.00 mg/ml, Prionics, Switzerland), which recognizes an epitope located at the C-terminal PrP domain (144–152), diluted 1:5000 in PBST. Both 4F2 and 12F10 antibodies were kindly provided by Prof. Walter Bodemer, Goettingen, Germany (Kräsemann et al., 1996). The membranes were incubated with rabbit anti-mouse alkaline phosphatase conjugated secondary antibody (Pierce, Rockford, IL) and developed with the CDP star reagent (NEB, Beverly, MA) following the manufacturer’s instructions.

**In vivo experiments**

The wild-type C57Bl6 mice used were purchased originally from Jackson Laboratories and bred at SUNY Stony Brook. The tPA-deficient (tPA⁻⁻) animals (Carmeliet et al., 1994) were generated on a 129 background and have subsequently been backcrossed to the C57Bl6 background for 12 generations. The plasminogen-deficient (plg⁻⁻) mice (Bugge et al., 1995) originally purchased from Jackson Labs, and bred in SUNY Stony Brook are also in the C57Bl6 background.

Groups of 4 adult mice of either sex, C57Bl wild type, tPA⁻⁻ or plg⁻⁻, weighing approximately 25 g, were injected intraper-
measurements were taken (data not shown). Since the substrate is the tPA activity in TSE-infected brain tissue. We studied the tPA activity in infected brain homogenates, relative to appropriate control homogenates using an indirect chromogenic assay based on the proteolytic activity of plasmin. Plasmin is produced by the hydrolysis of the plasminogen and it cleaves a peptidic bond (Arg-p-nitroanilide) of the substrate D-Ile-Pro-Arg-p-nitroanilide producing p-nitroaniline. p-Nitroaniline is soluble and exhibits a strong absorption at 405 nm that is linear with increasing tPA concentrations over a broad working range.

Our preliminary work indicated that at least 500 µg brain equivalents are required for linear kinetics. The time course of tPA activity is sigmoid with a lag period of 40–45 min. The reaction is linear for the time period from 45 up to 180 min and reaches a plateau after approximately 3 h. All subsequent measurements were made after 180 min. Interestingly enough, in control (blank) reactions, containing plasminogen but no tPA enzyme source, the OD increases linearly with time, even after 3 h, indicating that the substrate is degraded. On the other hand, blank samples, which contained no exogenous plasminogen, did not exhibit enzymatic activity over the time course that the measurements were taken (data not shown). Since the substrate cannot be hydrolyzed by tPA or other proteolytic enzymes which may be present in the homogenate and it is stable throughout the time period the readings were performed, the observed OD increase in reactions containing only plasminogen should be attributed to spontaneous hydrolysis of plasminogen to plasmin. Hence, as the addition of plasminogen is mandatory in order to achieve the desired results, the OD readings must be performed within 2 and 3 h in order to rule out the effect of the spontaneous hydrolysis of plasminogen.

tPA and plasmin are serine proteases, and as such, their activity is pH-dependent. After a detailed investigation, we concluded that the tPA-mediated plasmin reaction is pH-sensitive and that, although significant enzymatic activity is obtained at various pH settings, ranging from 7.5 to 9.0 without significant effects on tPA activity, the optimal pH value setting is 8.0 (data not shown).

To assess the possibility of inactivation of tPA during the process of sample preparation, two homogenization methods were evaluated, the one using a motor-driven homogenizer and the other using a glass pestle and mortar. The homogenization buffer in both procedures was the same. When the OD values were normalized per microgram of protein, both protocols revealed similar tPA activity, and the specific activity of tPA was not affected, independent of the employed method. Clearly, both methods are reliable and efficient and both of them can be used, depending on the experimental needs and tissue availability. Homogenization with the pestle and mortar is more versatile and suitable for smaller amounts of tissue.

Significant elevation of tPA activity in different species, including hamsters (experimentally infected with scrapie), mice (experimentally infected with scrapie and BSE) and naturally occurring scrapie sheep, was observed, compared to the respective normal animals (Fig. 1). Preliminary data also imply elevated tPA activity in mice experimentally infected with BSE (301V BSE mice, data not shown).

Variation of tPA activity in sheep scrapie clinical cases can be attributed to different stages of the disease, age, brain region, tissue processing or handling. An interesting observation was that hamster tissue has significantly lower tPA activity than the other species.

Results

tPA assay

There is mounting evidence for an interaction between prion protein, plasminogen and tPA (Fischer et al., 2000; Maissen et al., 2001; Praus et al., 2003; Ryou et al., 2003), while urokinase plasminogen activator does not seem to associate with PrP (Ellis et al., 2002). Prompted by these observations, we investigated the tPA activity in TSE-infected brain tissue. We studied the tPA activity in infected brain homogenates, relative to appropriate control homogenates using an indirect chromogenic assay based on the proteolytic activity of plasmin. Plasmin is produced by the hydrolysis of the plasminogen and it cleaves a peptidic bond (Arg-p-nitroanilide) of the substrate D-Ile-Pro-Arg-p-nitroanilide producing p-nitroaniline. p-Nitroaniline is soluble and exhibits a strong absorption at 405 nm that is linear with increasing tPA concentrations over a broad working range.

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prion protein in vitro. Immunoprecipitated prion protein originating from normal sheep brain homogenates was precipitated with anti-PrP mAb 66.94b4 (manuscript in preparation). The isolated PrPC was incubated with exogenous tPA and plasminogen at concentrations of 4.9 nM and 0.28 \( \mu \text{M} \) respectively. Previous reports demonstrated that, when recombinant prion protein was treated with tPA and plasminogen, PrP was cleaved at residue 110, resulting in an N-terminal (aa 23–110) and a C-terminal fragment (aa 111–231). The N-terminal fragment was shown to accelerate plasminogen activation, suggesting that this mechanism could represent a regulatory mechanism of pericellular proteolysis (Kornblatt et al., 2003; Praus et al., 2003). Following the experimental protocol, immunoprecipitated PrP C from normal sheep brain homogenates was digested with exogenous tPA and plasminogen. Using antibodies that recognize different epitopes of prion protein, we detected an N-terminal fragment with anti-PrP mAb 4F2 (Fig. 3, lanes 1 and 2; arrow indicates the expected fragment) and three C-terminal fragments, resulting from the digestion of double-, mono- and non-glycosylated PrP\(^\text{C} \) (Fig. 3, lanes 3 to 6). The expected molecular weights, indicated by arrows, are 26 kDa, 22 kDa and 16.5 kDa respectively. These fragments do not result from the cleavage of anti-PrP mAb 66.94b4 that was used for PrP\(^\text{C} \) immunoprecipitation, as the antibody remains undigested after treatment with tPA and plasminogen (data not shown).

PrP\(^\text{Sc} \) enriched preparations were similarly treated with exogenous tPA and plasminogen to determine if the pathological isoform was similarly digestable. PrP\(^\text{Sc} \) quantitative isolation was performed from scrapie-infected sheep brain homogenates in the presence or absence of proteinase K. The epitope recognized by anti-PrP mAb 4F2 is destroyed during treatment with proteinase K, and this antibody should not detect the remaining proteolytic-resistant fragments of prion protein (Fig. 4, lanes 3 and 4), while the epitope recognized by anti-PrP mAb 6H4 is unaffected by proteinase K treatment. The isolated PrP\(^\text{Sc} \) was not cleaved in the presence of tPA and plasminogen, as no truncated forms of PrP\(^\text{Sc} \) were observed with either of the antibodies (4F2 and 6H4) used for the detection of smaller fragments (Fig. 4).

In vivo experiments: inoculation of wild-type, \( \text{tPA-} \)deficient and \( \text{plasminogen-deficient} \) mice with PrP\(^\text{Sc} \) brain homogenates

Since it was reported that the misfolded PrP\(^\text{Sc} \), but not the normal cellular protein PrP\(^\text{C} \), could bind to plasminogen (Fischer et al., 2000), we decided to assess in vivo whether plasminogen could actively participate in pathogenesis, hypothesizing that it might aid PrP\(^\text{C} \) to misfold into PrP\(^\text{Sc} \). If that were the case, we would expect that mice deficient in plasminogen would also be deficient in the conversion of PrP\(^\text{C} \) to PrP\(^\text{Sc} \) and would not develop disease or show a significant delay in the progression of the disease and the appearance of clinical symptoms.

**Fig. 1. Comparison of tPA activity in TSE and uninfected brain tissue from different species.** tPA activity, expressed as mIU of tPA per microgram of total protein, was measured as described in Materials and methods. The sheep samples are from the brain stem and cerebellum of animals naturally infected with scrapie or control sheep. tPA activity of mice and hamster samples was measured from whole brain homogenates of animals experimentally infected with TSEs and control animals. The data are analyzed by a mixed effects model with the healthy-scrapie factor a fixed effect and the animal factor, which is nested within the healthy-scrapie factor, a random effect. The ANOVA statistics indicates that there is a significant difference (\( P < 0.05 \)) between the scrapie and the healthy animals for the hamster and the sheep brain stem samples. \( P \) values in mice and sheep cerebellum are 0.13 and 0.16 respectively indicating similar pattern.

**Fig. 2. Real-time PCR data.** (A) RT-PCR amplification products of the tPA gene using the primers tPA1 and tPA2. Lane 1: template RNA was not RQ1 RNase-free DNase treated. Lane 2: RT-PCR amplification product of RQ1 RNase-free DNase treated total RNA, as described in the Materials and methods section. (B) Real-time PCR amplification product of RQ1 RNase-free DNase treated total RNA, as described in the Materials and methods section. (B) Real-time PCR amplification products representative for all reactions. Lane 1: \( \beta \)-actin fragment. Lane 2: tPA fragment. (C) Relative expression of the tPA gene resulted from real-time PCR data analysis, normalized data using \( \beta \)-actin as reference gene. tPA gene expression in normal animals (control) compared to 79A mouse scrapie-infected animals at the terminal stage of the disease. Mean values of three normal and three scrapie samples, each one in triplicate. Bars represent the standard deviation of the samples. Relative expression was estimated by using the REST software (Pfaffl et al., 2002).
Three different strains of mice were challenged: C57Bl wild-type, tPA-deficient and plasminogen-deficient. Four mice from each genotype were injected with ME7 and 22A brain homogenates (Figs. 5A and B) respectively. Mice were then monitored for the progression of disease, evaluating the time of appearance of clinical symptoms, as well as the time of death. The clinical symptoms observed included muscle strength weakness resulting in ataxia, hunched posture, ocular secretion and eventually death. Routine immunohistochemistry was performed for the detection of PrPSc deposition and vacuolization (data not shown). As it is demonstrated in Fig. 5, the plasminogen-deficient mice developed symptoms of disease and succumbed to it faster than the tPA-deficient or wild-type animals, irrespective of the strain of the infectious scrapie material. All the strains of mice injected with ME7 showed a faster progression of disease in general, but well within the timing reported in the literature (Walsh et al., 2000).

Mice of all three genotypes injected with normal (uninfected) brain homogenate did not show any clinical symptoms, thus ruling out technical or non-specific problems with the transgenic animals.

**Discussion**

tPA is a multifunctional molecule, directly involved in normal functioning of the nervous system (Strickland, 2001) but also related to neuronal cell death (Tsirka, 1997; Tsirka et al., 1996). Relation of tPA to prion diseases (Ellis et al., 2002; Fischer et al., 2000; Kornblatt et al., 2003; Maissen et al., 2001; Praus et al., 2003) and other neurodegenerative diseases (Ledesma et al., 2000; Lu et al., 2002) has previously been reported. Little notice, though, has been given to the regulation of tPA activity during the course of prion diseases. In our study, an upregulation of tPA levels is demonstrated in parallel with the occurrence of prion diseases. Furthermore, it was found that transgenic mice, lacking the tPA or the plasminogen gene, are more susceptible to the TSEs than their wild-type counterparts. Our data therefore imply a neuroprotective rather than a neurotoxic role for tPA.

Although age and gender do not seem to affect tPA activity significantly (Eliasson et al., 1993), a serious effort was made to use age- and sex-matched animals in the tPA activity and gene expression studies. The observed increase in tPA activity is
consistent with the real-time PCR results, where tPA mRNA in 79A scrapie terminally ill mice is found to be expressed at a level about 10% higher than in normal mice. The upregulation of such a molecule could be used as an additional biochemical marker in coordination with more studies to further analyze its role and its involvement in TSE pathogenesis and other neurodegenerative diseases.

Furthermore, we tried to identify the function of elevated tPA activity in prion diseases by studying the impact of tPA-generated plasmin on prion protein. Several studies for the role of the plasminogen system on the metabolism of prion protein have been published (Ellis et al., 2002; Kombholt et al., 2003; Praus et al., 2003), but all were based on use of recombinant PrP. In our study, immunoprecipitated PrPSc from normal sheep brain homogenates was used, as well as PrPSc quantitatively isolated from scrapie-infected sheep brain homogenates. Our experiments show cleavage of PrPSc, in accordance with the reported digestion of recombinant PrP (Kombholt et al., 2003; Praus et al., 2003), whereas cleavage of PrPSc under similar reaction conditions failed or at least was not detectable under our experimental conditions.

It is already known that PrPSc stimulates tPA-mediated plasminogen generation (Praus et al., 2003). A possible explanation for this phenomenon could be the binding of both proteases on PrP via their kringle domains (Ryou et al., 2003). It is further known that PrPSc binds tightly to plasminogen (Fischer et al., 2000; Maissen et al., 2001). The reported failure of tPA-generated plasmin to cleave PrPSc could be explained by possible inaccessibility of plasmin due to the supramolecular conformational changes of PrPSc.

Given the in vitro interaction between the misfolded prion protein and plasminogen, a unique handle was offered to look at the molecular mechanisms that follow prion misfolding by using mice deficient in plasminogen or tissue plasminogen activator. To address the question if and how the tPA/plasminogen system is implicated in prion diseases, we performed a bioassay by inoculating tPA- or plasminogen-deficient mice with two different mouse scrapie strains. Our in vivo results with tPA- and plasminogen-deficient animals demonstrated a direct impact on the course of the disease, suggesting that a potential assignment to plasminogen, as implied by Fischer et al. (2000), would not be appropriate since plasminogen-deficient animals did develop symptoms and eventually died. To the contrary, incubation period in plasminogen-deficient mice was significantly shorter than their tPA-deficient or wild-type counterparts. The elevated tPA activity and tPA gene expression in combination with our in vivo results may argue in favor of a neuroprotective action of tPA, similar to the one described for Alzheimer disease, where the elevated tPA activity seems to correlate with the plasmin cleavage of beta-aggregates (Ledesma et al., 2000). Our results from the in vitro incubation of PrPSc with tPA/plasminogen, however, rule out the possibility that tPA or plasminogen (via their kringle domains) may act to bind and remove misfolded PrPSc from its sites of accumulation during early stages of disease.

The elevated tPA activity observed in our study may be linked to neuroprotection against TSEs through proteolytic processing of the PrPSc, which was found in vitro to be sensitive to the effects of the tPA/plasminogen system. The primary structure of the prion protein is well conserved over many mammalian species (van Rheede et al., 2003), and the 106−126 PrP fragment has been shown to be necessary for the conversion of PrPSc to PrPSc (Tagliavini et al., 1993). Since PrP cleaved at position 110 is a less suitable substrate for PrPSc replication, tPA/plasminogen-mediated cleavage of PrPSc at position 110 could act protectively against the conversion of PrPSc to the pathological isoform [also discussed in Praus et al. (2003)].

Another hypothesis is that tPA may provide nonproteolytic neuroprotection. It has been shown that tPA attenuates oxidative-stress-related neuronal death and that this action is independent of plasminogen activation (Kim et al., 1999). Taking into consideration that oxidative stress is a key event in the neuropathology of prion diseases (Budka, 2003; Guentchev et al., 2002; Hur et al., 2002), tPA overexpression in tissues infected with TSEs might represent a response to this stress factor. However, since plasminogen-deficient mice seem to be more susceptible to TSEs than their tPA-deficient counterparts, tPA’s nonproteolytic neuroprotection mechanisms should be considered to be of lesser importance.

Up to this point, our results, including elevated tPA activity during TSE pathogenesis and significant reduction in TSE incubation time in tPA-deficient mice, indicate that tPA has a protective rather than a neurodegenerative role. Of course, we cannot rule out the possibility that a more complex phenomenon is taking place including other molecules with a simultaneous protective and destructive role of the tPA/plasminogen system.

We strongly believe that more detailed studies will contribute significantly to a better understanding of the precise relationship between the tPA/plasminogen system and TSE pathogenesis, yielding further knowledge of these pathogens that will lead in the development of novel therapeutic approaches.
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Appendix A. Supplementary data


References


