Expression of PAI-1 and PN-1 in human astrocytes; response to injury-related factors

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ABSTRACT

Astrocytes play a diverse role in central nervous system (CNS) injury. Production of the serine protease inhibitors (serpins) plasminogen activator inhibitor-1 (PAI-1) and protease nexin-1 (PN-1) by astrocytes may counterbalance excessive serine protease activity associated with CNS pathologies such as ischemic stroke. As knowledge regarding the regulation of these genes in the brain is limited, the objective of the present study was to characterize the effects of injury-related factors on serpin expression in human astrocytes. Native human astrocytes were exposed to hypoxia or cytokines, including interleukin-6 (IL-6), IL-1β, tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), transforming growth factor-α (TGF-α) and TGF-β for 0 to 20 h. Serpin mRNA expression and protein secretion was determined by real-time RT-PCR and ELISA, respectively. Localization of PAI-1 and PN-1 in human brain tissue was examined by immunohistochemistry. Hypoxia and all assayed cytokines induced a significant increase in PAI-1 expression, while prolonged treatment with IL-1β or TNF-α resulted in a significant down-regulation. The most pronounced induction of both PAI-1 and PN-1 was observed following early treatment with TGF-α. In contrast to PAI-1, the PN-1 gene did not respond to hypoxia. Positive immunoreactivity for PAI-1 in human brain tissue was demonstrated in reactive astrocytes within gliotic areas of temporal cortex. We here show that human astrocytes express PAI-1 and PN-1, and demonstrate that this astrocytic expression is regulated in a dynamic manner by injury-related factors.

Key words: Gene expression, Astroglia, Cytokines, Hypoxia

INTRODUCTION

It is becoming increasingly recognized that astrocytes play an important neuroprotective role in the brain. Astrocytes are effective sensors of the brain microenvironment and provide neurons with metabolic support. In the event of an acute insult they can also limit the extent of injury to the surrounding brain tissue, for example by secreting potentially neuroprotective molecules (Takano et al. 2009). In recent years, the role for astrocytes as regulators of the balance between serine proteases and their inhibitors (serpins) has gained increasing attention, as serine protease activity has been identified as a possible determinant for the outcome of central nervous system (CNS) disorders, such as ischemic stroke (Yepes et al. 2008).

Tissue-type plasminogen activator (t-PA) is one of the most well-characterized serine proteases in the CNS, where neurons and microglia are believed to be its major source (Gravanis and Tsirka 2005). t-PA participates in several aspects of neuronal plasticity (Calabresi et al. 2000). However, following an ischemic stroke or brain trauma, excessive microglial activation and neuronal depolarization results in accumulation of t-PA in the extracellular space, which can potentiate neurodegeneration (Tsirka et al. 1997) and induce break-down of the blood-brain barrier (Yepes et al. 2003). Altered serine protease activity has also been associated with neurological disorders, such as multiple sclerosis (Gveric et al. 2001) and Alzheimer´s disease (Cacquevel et al. 2007) (Fabbro and Seeds 2009). The negative effects of excessive serine protease activity in CNS pathology can be reduced by serine protease inhibitors (serpins), such as plasminogen activator inhibitor type-1 (PAI-1), protease nexin-1 (PN-1) and neuroserpin. PAI-1 and neuroserpin act by forming a complex with the catalytic residue on t-PA, thus inhibiting t-PA activity (Chmielewska et al. 1988) (Hastings et al. 1997). PN-1 is most potent as a thrombin inhibitor but can also form complexes with other serine proteases, including plasmin, trypsin, urokinase plasminogen activator (u-PA), and to a lesser extent with t-PA (Scott et al. 1985). PAI-1 is predominantly produced by astrocytes (Gravanis and Tsirka 2005), whereas PN-1 expression also has been demonstrated in neurons and microvascular endothelial cells (Choi et al. 1990). Neurons are considered as the primary source of neuroserpin (Hastings et al. 1997) (Teesalu et al. 2004), although its expression has also been observed in mouse astrocytes *in vitro* (Docagne et al. 1999).

Investigations of human brain tissue have demonstrated an increased expression of PAI-1 (Dietzmann et al. 2000) and PN-1 (Wu et al. 2008) following traumatic brain injury or ischemic stroke. As regards the neuroprotective properties of serpins, astrocyte-derived PAI-1 has been shown to protect neurons against the deleterious effects exerted by t-PA during excitotoxicity (Docagne et al. 1999). Moreover, administration of neuroserpin after experimental ischemic stroke prevents neuronal death (Yepes et al. 2000). Thus, an increased expression and release of serpins may serve as an important endogenous mechanism to reduce the deleterious effects of excessive serine protease activity within the CNS. Among the most potent factors known to induce PAI-1 expression in various cell types *in vitro* are inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), as well as low oxygen tension (i.e. hypoxia) (Kruithof 2008) (Nagamine 2008).

Despite a documented effect of ischemic insult or brain trauma on serpin expression in the CNS, little is known about how the serpin genes are regulated in astrocytes. As the regulation of these genes may not only be cell-type specific, but also species-dependent (Dimova and Kietzmann 2008), we have chosen to focus this study on cells of human origin. Thus, aim of the present study was to investigate the time-dependent effects of various injury-related factors, including hypoxia and cytokines, on serpin expression in human astrocytes.

MATERIAL AND METHODS

Native human astrocytes (#CC-2565), astrocyte growth medium (AGM) and trypsin/EDTA were purchased from Clonetics (Walkersville, MD, USA). Dulbecco´s modified eagle medium (DMEM) (low glucose, 1g/l) was purchased from Invitrogen (Carlsbad, CA, USA). Human recombinant IL-6, IL-1β, TNF-α, interleukin-10 (IL-10), TGF-α and TGF-β were purchased from Sigma-Aldrich (St Louis, MO, USA). Primary mouse antibody directed against PAI-1 was kindly provided by Prof. P. J. Declerck and Prof. A. Gils (Katholieke Universiteit Leuven, Belgium). Primary rabbit antibody against PN-1 was kindly provided by Prof. P. Andreasen (University of Aarhus, Denmark), and goat anti-PN-1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary rabbit antibody directed against glial fibrillary acid protein (GFAP) was from Dako (Glostrup, Denmark) and mouse anti-GFAP antibody was from Sigma-Aldrich. Rabbit anti-neuron specific enolase antibody was from AbD Serotec (Dusseldorf, Germany). Secondary Alexa Fluor 488-conjugated donkey anti-mouse antibody, Alexa Fluor 488-conjugated donkey anti-rabbit antibody, Alexa Fluor 555-conjugated donkey anti-mouse antibody, Alexa Fluor 594-conjugated donkey anti-rabbit antibody and TOPRO-3 were from Molecular Probes (Leiden, The Netherlands).

Stock solutions of IL-6 (10 μg/ml), IL-1β (0.5 μg/ml), TNF-α (10 μg/ml), IL-10 (2 μg/ml), TGF-α (0.1 mg/ml) and TGF-β (50 μg/ml) were prepared in distilled water and stored at -20°C. Prior to experiments, stock solutions of the various agents were diluted in AGM to the indicated concentrations.

Cell culture

Astrocytes were cultured in AGM supplemented with 3% fetal bovine serum at 37°C in a CO_2 atmosphere. The media was replaced every 2-3 days. Subcultures of astrocytes were obtained by trypsin treatment (trypsin/EDTA, 0.25 mg/ml) of semi-confluent monolayers at a split ratio of 1:4. Astrocyte cultures were >95% GFAP positive. All experiments were performed at passages 2-4.

Gene expression studies

Astrocytes were seeded in 12-well dishes and allowed to reach 100% confluency. Cells were incubated in AGM containing IL-6 (10 ng/ml), IL-1β (10 ng/ml), TNF-α (5 ng/ml), IL-10 (10 ng/ml), TGF-α (1 ng/ml) or TGF-β (1 ng/ml), for 3, 6, 14 and 20 h. In control cultures (i.e. untreated cells), media was replaced at the indicated time periods. Each treatment was performed in three different wells on two separate occasions (n = 6). Cells in each experimental series were derived from no more than three individuals.

Prior to hypoxia treatment, astrocytes were washed twice with PBS and refed with serum-free DMEM, after which cells were transferred to a hypoxic chamber (Proox C21, Biospherix, Redfield, NY, USA), and maintained under hypoxic conditions (1% O_2, 5% CO_2 and 94% N_2) for the indicated time periods. The O_2-level was selected based on previous reports on hypoxia-induced PAI-1 gene expression in human cells *in vitro,* demonstrating most profound up-regulation at oxygen concentrations below 2% (Fink et al. 2002). Control cultures were refed with serum-free DMEM and kept under standard cell culture conditions (95% air, 5% CO_2) for the indicated time periods.

After treatment, the conditioned media was collected and stored at -20°C until analyzed. Cells were lysed by the addition of lysis buffer (Buffer RLT; QIAGEN, Hilden, Germany), and lysates were stored at -70°C until preparation of total RNA.

mRNA levels of PAI-1, PN-1 and neuroserpin

Total cellular RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) and mRNA was converted to cDNA with GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). Target mRNA was quantified by real-time RT-PCR and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA according to a standardized protocol (Wolf et al. 2005) (Ulfhammer et al. 2005), with the modification that the PCR was performed in a 384-well format on a ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) in a total volume of 10 µl. The oligonucleotide primers and TaqMan probes for quantification of t-PA, PAI-1 and GAPDH mRNA were as follows: t-PA forward primer (fp): 5′-GGCCTTGTCTCCTTTCTATTCG-3′, t-PA reverse primer (rp): 5′-AGCGGCTGGATGGGTACAG-3′, t-PA probe (pr): 5′-(FAM)TGACATGAGCCTCCTT-CAGCCGCT(TAMRA)-3′, PAI-1 (fp) 5′-GGCTGACTTCACGAGTCTTTCA-3′, PAI-1 (rp) 5′-TTCACTTTCTGCAGCGCCT-3′, PAI-1 (pr) 5′-(FAM)ACCAAGAGCCTCTCCACGTC-GCG(TAMRA)-3′, GAPDH (fp) 5′-CCACATCGCTCAGACACCAT-3′, GAPDH (rp) 5′-CCAGGCGCCCAATACG-3′, and GAPDH (pr) 5′-(FAM)AAGGTGAAGGTCGGAGTCA-ACGGATTTG(TAMRA)-3′. For hypoxia experiments, target mRNA was normalized to 18S mRNA (Applied Biosystems Assay-on-demand Hs99999901_s1). For amplification of PN-1 and neuroserpin, pre-designed primers and probes were used (Applied Biosystems Assay-on-demand Hs00299953_m1 and Hs00192380_m1, respectively). Relative quantification of gene expression was analyzed as a treatment-to-control expression ratio using the comparative C_T method. Each sample was analyzed in triplicate for both target and control gene.

Protein release of PAI-1

The concentration of PAI-1 antigen in the cell culture media from treated astrocytes and control cultures was determined by ELISA (TintElize®PAI; Biopool® International, Umeå, Sweden) according to the manufacturer's protocol. This assay detects both free and complex-bound PAI-1 protein with high affinity. All samples were assayed in duplicate. Mean intra-assay coefficient of variation (CV) was 2.7%.

Immunohistochemistry for PAI-1 and PN-1

Immunohistochemistry for PAI-1 and PN-1 was performed on brain tissue resected from three patients undergoing surgery for epilepsy. The patients (one male and two females) all had a long history of temporal lobe epilepsy with mesial sclerosis, and all three underwent anterior temporal lobe resection (two left, one right). The specimens used in this study consisted of neocortex from the left middle temporal gyrus. The tissue was fixed in 4% paraformaldehyde overnight and immersed in 30% sucrose. Free floating cryosections of 30 µm thickness were pre-incubated in phosphate buffered saline (PBS) containing 0.5% Triton-X and 1% bovine serum albumin (BSA). The following primary antibodies were used: mouse anti-PAI-1 antibody, rabbit anti-GFAP antibody, rabbit anti-neuron specific enolase, rabbit and goat anti-PN-1 antibodies and mouse anti-GFAP antibody. After washing, the sections were incubated with the appropriate Alexa Fluor-conjugated secondary antibodies. Nuclei were visualized by adding TOPRO-3 to the last incubation step. Sections were mounted on slides with Mowiol containing 2.5% DABCO (Sigma-Aldrich, St Louis, MO, USA) and examined by a laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany).

Statistical analysis

Values are presented as mean and standard error of the mean (SEM) and a p-value of 0.05 or less was considered statistically significant. Changes in mRNA levels in response to treatment were evaluated by one-way analysis of variance (ANOVA). When ANOVA indicated a significant overall effect, post-hoc analysis was performed by Tukey's test. SPSS (SPSS Inc., Chicago, IL, USA) for the Macintosh system was used for all statistical calculations.

RESULTS

Hypoxia induces an early up-regulation of PAI-1 expression

Exposure to hypoxia induced a 2.2-fold increase in PAI-1 mRNA expression at 6 h compared to control cultures (Figure I). At the protein level, a similar although delayed response was observed (Table III, Appendix). PN-1 mRNA levels remained essentially unchanged following exposure to hypoxia (Figure I).

Early up-regulation of PAI-1 expression by cytokines

Treatment with the pro-inflammatory cytokines IL-6 or TNF-α resulted in an approximate 2-fold increase in PAI-1 mRNA levels after 3 h, whereas IL-1β only induced a slight but significant increase (Table I). Prolonged treatment with IL-1β or TNF-α suppressed PAI-1 expression, causing about a 40% and 20% reduction at 20 h, respectively, compared to control-treated cultures (Table I). Following treatment with the anti-inflammatory cytokine IL-10, an approximate 2-fold induction was observed after 3 h (Figure II). A pronounced induction of PAI-1 gene expression was also observed following treatment with TGF-α or TGF-β for 3 h, with an approximate 2-fold increase in PAI-1 mRNA levels compared to control cultures (Figure III) and (Table I), respectively. Following all treatments, changes at the protein level mirrored those observed at the mRNA level (Table III, Appendix).

PN-1 mRNA expression is induced by cytokines

Treatment with TGF-α induced an early up-regulation of PN-1 expression, reaching 3.1-fold after 3 h compared to control cultures (Figure III). A similar PN-1 response was observed following IL-10 treatment (Figure II). A slight but significant increase in PN-1 mRNA levels was also demonstrated after treatment with TGF-β, IL-6, IL-1β, or TNF-α (Table II).

Neuroserpin mRNA expression is unaffected by injury-related factors

A low expression of neuroserpin mRNA was detected. However, the basal level of expression was only around the threshold of detection and showed no significant response to treatment (data not shown). In agreement with this, immunohistochemical examinations of both adult human and murine brain tissue suggest that neurons are the primary source of neuroserpin *in vivo* (Teesalu et al. 2004) (Hastings et al. 1997). Staining for neuroserpin was thus excluded from the subsequent immunohistochemical analysis.

Reactive astrocytes stain positively for PAI-1

Widespread PAI-1 immunoreactivity was observed in astrocytes and in cerebral endothelial cells. The most pronounced PAI-1 staining was observed in reactive astrocytes within gliotic areas of human temporal cortex (Figure IV D-F). Double labeling with antibodies against astrocyte intermediate filament protein GFAP show that the two proteins are co-expressed in the same astroglial cell (Figure IV C, F, I). PAI-1 immunoreactivity was most prominent in astrocytic processes and perivascular endfeet. The immunohistochemical staining patterns of PAI-1 were similar in all samples from the three patients (Figure V). In addition, tissue sections were double labeled for PAI-1 and neuron-specific enolase. Only a small number of neurons displayed positive staining for PAI-1 (data not shown). As regards PN-1 staining, although two different antibodies against PN-1 were used, no clear immunoreactivity was observed (data not shown).

DISCUSSION

The importance of regulating the activity of serine proteases in the CNS is evident in numerous pathophysiological conditions, where the balance between serine proteases and serpins may have an impact on outcome. Thus, identifying factors regulating serpin expression is important in order to gain further insight into the mechanisms that modulate proteolytic activities within the human brain. Our study provides an extensive characterization of serpin expression in cultured human astrocytes in response to injury-related factors.

Limited oxygen supply (i.e. hypoxia) has been implicated in the pathology of many disorders. Thus, the effect of hypoxia on PAI-1 gene regulation has been extensively studied in various cell types. Collectively, data presented so far show that this regulation is highly complex and involves different transcription factors and gene regulatory elements, which may vary depending on the cell type and species of origin (Dimova and Kietzmann 2008) (Nagamine 2008). We found that exposure of astrocytes to hypoxia induced an increased PAI-1 expression. Although the response evoked in the present study was quite modest, our results show that hypoxia may contribute to increased PAI-1 levels associated with cerebral ischemia (Dietzmann et al. 2000).

The brain response to an ischemic insult or injury is associated with an acute and prolonged inflammatory process, characterized by activation of glial cells and increased production of both pro- and anti-inflammatory cytokines (Stoll et al. 1998) (Tuttolomondo et al. 2008) (Planas et al. 2006). Pro-inflammatory cytokines are known to mediate neurodegeneration and exacerbate cell death following brain injury. However, in some situations they can also have beneficial actions. For example, they may stimulate the expression of serpins within the CNS, thus serving a protective role against excessive serine protease activity. We here demonstrate that the major pro-inflammatory cytokines IL-1β, IL-6 and TNF-α regulate the expression of both PAI-1 and PN-1 in astrocytes. Shorter exposures to these cytokines increased the expression levels of PAI-1, which is in line with previous studies on astrocytes of human origin (Kasza et al. 2002) (Yoshida et al. 2006). Interestingly, prolonged treatment with IL-1β or TNF-α resulted in a down-regulation. A similar effect of IL-1β on astrocytic PAI-1 expression has previously been described by Kasza *et al* (Kasza et al. 2002), although the mechanism behind this inhibitory effect is yet to be determined. Anti-inflammatory cytokines, such as IL-10, are associated with protective effects following brain injury (Strle et al. 2001). In the present study, we demonstrate for the first time that IL-10 is a potent stimulus for PAI-1 expression in cultured human astrocytes.

As regards PN-1, our observation of a stimulatory effect of pro-inflammatory cytokines on its expression in astrocytes is in agreement with a previous report on neuroblastoma cells (Vaughan and Cunningham 1993). In addition, we demonstrate a pronounced induction of astrocytic PN-1 gene expression in response to IL-10. The exact role for PN-1 in the brain is still unclear, but the general idea is that the main function of this serpin is to block the activity of thrombin. *In vitro* studies have demonstrated that thrombin mediates apoptotic cell death in neurons and astrocytes (Donovan et al. 1997), and that it contributes to ischemic damage in neural tissue (de Castro Ribeiro et al. 2006). Furthermore, inhibition of thrombin is protective in animal models of cerebral ischemia (Ohyama et al. 2001). These data indicate that excessive thrombin activity may have deleterious effects in the brain. It can be speculated that the release of inflammatory cytokines, at sites of injury, may stimulate the expression of both PAI-1 and PN-1 in surrounding astrocytes, thus functioning as a protective mechanism against the neurotoxic effects exerted by serine proteases during pathological conditions in the CNS.

TGF-β is considered as one of the most important regulators of PAI-1 gene expression (Nagamine 2008). In various cell types, including astrocytes, TGF-β strongly up-regulates

PAI-1 expression (Docagne et al. 1999) (Nagamine 2008). In the present study, treatment with TGF-β induced an early up-regulation of PAI-1 in human astrocytes, similar to that described in human retinal glial cells (Schacke et al. 2002). However, the induction of PAI-1 expression by TGF-β was modest compared to that previously found in cultured astrocytes of non-human origin (Docagne et al. 1999). In addition to TGF-β, we found that treatment with TGF-α induced a pronounced up-regulation of both PAI-1 and PN-1 expression levels. A similar PAI-1 response to TGF-α has previously been demonstrated in mouse astrocytes (Gabriel et al. 2003). Numerous studies have shown that the levels of TGF-α and TGF-β are increased in the brain following ischemia-induced brain injury (Krupinski et al. 1996) (Ali et al. 2001). Evidence largely point to beneficial effects of this up-regulation, as these cytokines have the potential to reduce neuronal cell death *in vitro* (Prehn et al. 1993) (Gabriel et al. 2003) and infarct volume in experimental models of cerebral ischemia (Prehn et al. 1993) (Justicia et al. 2001). The mechanisms behind these effects are not entirely clear. However, recent studies conducted by Vivien and co-workers may suggest that astrocyte-derived PAI-1 may function to mediate the neuroprotective activities exerted by TGF-α and TGF-β (Gabriel et al. 2003) (Docagne et al. 1999). Thus, induction of astrocytic serpin expression by these cytokines may represent an important protective mechanism against excessive serine protease activity within the CNS.

In the present study, we confirmed the presence of PAI-1 in astrocytes in human brain tissue, where the most prominent immunoreactivity was observed in reactive astrocytes within areas of human temporal cortex. In agreement with this, astrocytic expression of PAI-1 in the human CNS has primarily been related to infectious disease or ischemic lesions (Dietzmann et al. 2000), conditions also associated with astrocyte activation and reactive gliosis. However, in contrast to the study by Choi and coworkers (Choi et al. 1990), we were not able to demonstrate a convincing immunoreactivity for PN-1. Thus, further studies on PN-1 expression in human brain tissue are warranted. In addition to the present finding on PAI-1, we have previously demonstrated immunohistochemical localization of t-PA in reactive astrocytes in human brain tissue derived from a patient operated for intractable epilepsy (Hultman et al. 2008). It is of interest to note that the expression of t-PA is rapidly induced after seizures (Qian et al. 1993). Thus, the presence of t-PA and its inhibitors in reactive astrocytes in gliotic areas might suggest an involvement of these factors in the pathological processes associated with or resulting from epileptic seizures.

There are some limitations to this explorative study worth noting, the major one being that it is performed on isolated cells *in vitro*. As the astrocytes in their normal *in vivo* setting are involved in a complex interplay between different cell-types, e.g. neurons, microvascular endothelial cells, microglia, and pericytes, their response to injury is likely to be affected by these surrounding cells. Extrapolations to the *in vivo* situation should therefore be made with caution. However, we have chosen to work with human cells with the anticipation that the results would be more relevant to the clinical situation.

In conclusion, the findings presented here show that human astrocytes express PAI-1 and PN-1, and demonstrate that the expression of these serpins is regulated in a dynamic manner by injury-related factors in cultured astrocytes. Induction of serpins in theses cells may be part of a defence mechanism to protect brain tissue from excessive serine protease activity following an acute insult. Full elucidation of the factors regulating the expression of serpins and the specific roles of serine proteases in the human brain may provide a basis for novel neuroprotective strategies.

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Table I. Expression of PAI-1 mRNA in human astrocytes following treatment with cytokines.

	Fold increase in PAI-1 mRNA in human astrocytes, mean (SEM)					
	0 h	**3 h**	**6 h**	**14 h**	**20 h**	ANOVA
IL-6	1.0	1.76 (0.05) ‡	1.84 (0.05) ‡	1.35 (0.06) ‡	1.28 (0.04) †	< 0.001
IL-1β	1.0	1.23 (0.08) *	1.15 (0.04)	1.02 (0.03)	0.57 (0.08) ‡	< 0.001
TNF-α	1.0	2.08 (0.11) ‡	1.26 (0.04) *	0.70 (0.03) †	0.82 (0.04) *	< 0.001
TGF-β	1.0	1.98 (0.11) ‡	1.77 (0.07) ‡	1.33 (0.04) †	1.10 (0.10)	< 0.001

Confluent astrocyte cultures were treated with interleukin-6 (IL-6, 10 ng/ml), interleukin-1β (IL-1β, 10 ng/ml), tumor necrosis factor-α (TNF-α, 5 ng/ml) or TGF-β (1 ng/ml) for the indicated time periods. Total cellular RNA was extracted, and mRNA was converted into cDNA. Target mRNA (cDNA) was quantified by real-time RT-PCR and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Results are expressed as fold increase compared to control cultures. Each data point represents the average of three treatment series, performed on two separate occasions (n = 6). Response to treatment was evaluated by one-way ANOVA and post-hoc analysis by Tukey's test; *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ vs 0 h.

Table II. Expression of PN-1 mRNA in human astrocytes following treatment with cytokines.

	Fold increase in PN-1 mRNA in human astrocytes, mean (SEM)					
	0 h	**3 h**	**6 h**	**14 h**	**20 h**	ANOVA
IL-6	1.0	1.13 (0.06)	1.25 (0.06) †	1.28 (0.04) †	1.25 (0.04) †	< 0.001
IL-1β	1.0	1.30 (0.05) *	1.40 (0.07) ‡	1.28 (0.05) *	1.32 (0.09) †	< 0.001
TNF-α	1.0	1.14 (0.02)	1.23 (0.07) *	1.10 (0.05)	1.09 (0.08)	ns
TGF-β	1.0	1.47 (0.10) †	1.51 (0.11) ‡	1.45 (0.07) †	1.11 (0.06)	< 0.001

Confluent astrocyte cultures were treated with interleukin-6 (IL-6, 10 ng/ml), interleukin-1β (IL-1β, 10 ng/ml), tumor necrosis factor-α (TNF-α, 5 ng/ml) or TGF-β (1 ng/ml) for the indicated time periods. Total cellular RNA was extracted, and mRNA was converted into cDNA. Target mRNA (cDNA) was quantified by real-time RT-PCR and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Results are expressed as fold increase compared to control cultures. Each data point represents the average of three treatment series, performed on two separate occasions (n = 6). Response to treatment was evaluated by one-way ANOVA and post-hoc analysis by Tukey's test; *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ vs 0 h. ns = not significant.

Table III (Appendix). PAI-1 protein secretion in human astrocytes following treatment with cytokines or hypoxia.

	Fold increase in PAI-1 protein in human astrocytes, mean (SEM)					
	0 h	**3 h**	**6 h**	**14 h**	**20 h**	ANOVA
IL-6	1.0	1.06 (0.03)	1.43 (0.06) ‡	1.31 (0.06) †	1.20 (0.04) *	< 0.001
IL-1β	1.0	1.25 (0.05) *	1.11 (0.03)	1.22 (0.04) *	0.84 (0.03) *	< 0.001
TNF-α	1.0	1.19 (0.04)	1.65 (0.08) ‡	1.21 (0.04) *	0.87 (0.05)	< 0.001
IL-10	1.0	1.23 (0.06) *	1.53 (0.12) ‡	1.30 (0.11) †	1.21 (0.06) *	< 0.001
TGF-α	1.0	1.52 (0.09) ‡	1.49 (0.06) ‡	1.09 (0.05)	1.03 (0.07)	< 0.001
TGF-β	1.0	1.47 (0.07) ‡	1.76 (0.09) ‡	1.08 (0.05)	1.02 (0.07)	< 0.001
Hypoxia	1.0	1.37 (0.07) ‡	1.66 (0.15) ‡	1.97 (0.11) ‡	1.82 (0.13) ‡	< 0.001

Confluent astrocyte cultures were treated with interleukin-6 (IL-6, 10 ng/ml), interleukin-1β (IL-1β, 10 ng/ml), tumor necrosis factor-α (TNF-α, 5 ng/ml), interleukin-10 (IL-10, 10 ng/ml), transforming growth factor-α (TGF-α, 1 ng/ml) or TGF-β (1 ng/ml) or hypoxia (1% O_2) for the indicated time periods. Cell culture media was collected and PAI-1 protein secretion was determined by ELISA. Results are expressed as fold increase compared to control cultures. Each data point represents the average of three treatment series, performed on two separate occasions (n = 6). Response to treatment was evaluated by one-way ANOVA and post-hoc analysis by Tukey's test; *P < 0.05, †P < 0.01, ‡P < 0.001 vs 0 h.

Figure I

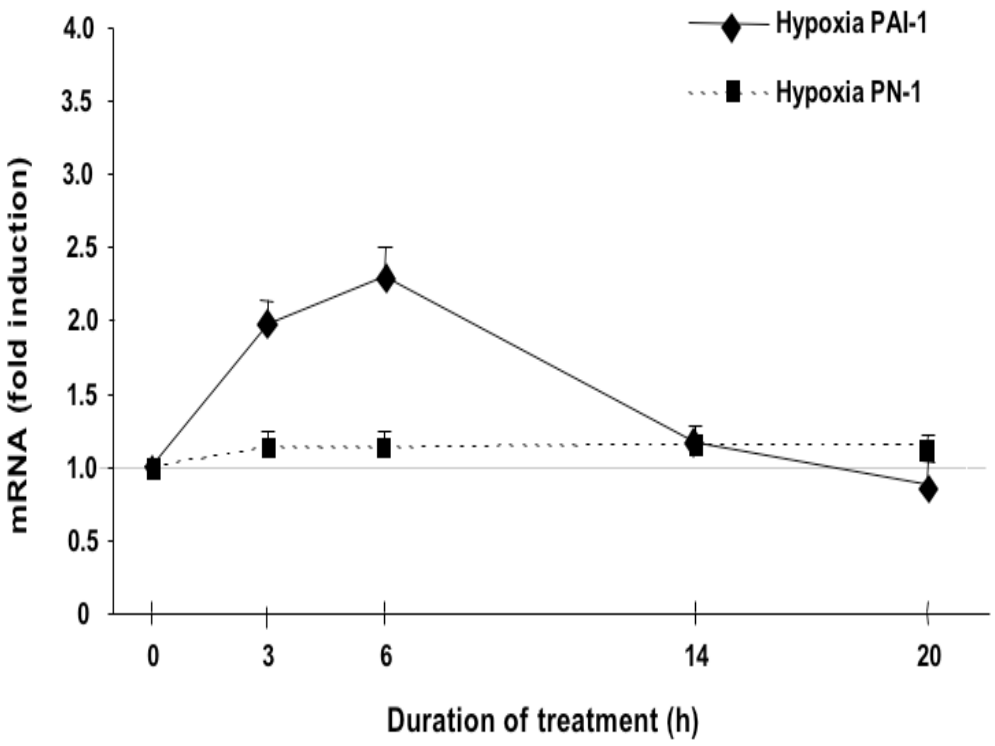

Figure I. PAI-1 mRNA levels are increased during hypoxia in astrocytes.
Confluent human astrocyte cultures were exposed to hypoxia (1% O_2) for the indicated time periods. Control cultures were kept under standard cell culture conditions. Total cellular RNA was extracted and mRNA was converted into cDNA. The levels of PAI-1 mRNA (cDNA) were quantified by real-time RT-PCR and normalized relative to 18S mRNA. Results are expressed as fold induction compared to control cultures and presented as mean and SEM. Three treatment series were performed on two separate occasions (n = 6). Response to treatment was evaluated by one-way ANOVA. Post-hoc analysis by Tukey's test; †$P < 0.01$, ‡$P < 0.001$ vs 0 h are shown.

Figure II

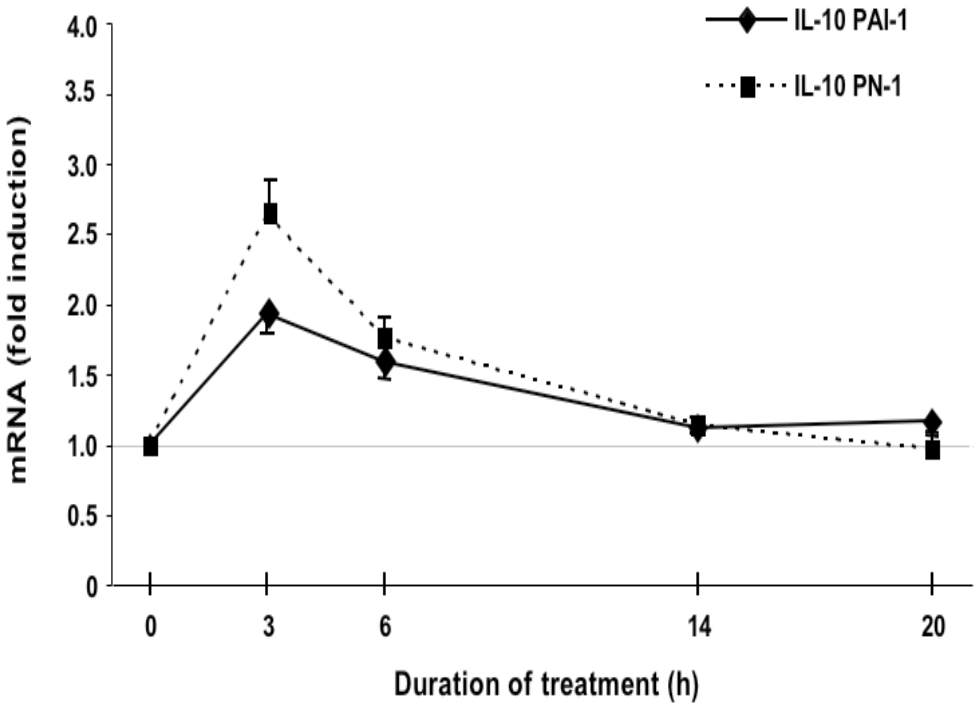

Figure II. IL-10 treatment induces an early up-regulation of PAI-1 expression in astrocytes.

Confluent human astrocyte cultures were treated with interleukin-10 (IL-10, 10 ng/ml) for the indicated time periods. Total cellular RNA was extracted and mRNA was converted into cDNA. The levels of PAI-1 mRNA (cDNA) were quantified by real-time RT-PCR and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Results are expressed as fold induction compared to control cultures and presented as mean and SEM. Three treatment series were performed on two separate occasions (n = 6). Response to treatment was evaluated by one-way ANOVA. Post-hoc analysis by Tukey's test; †$P < 0.01$, ‡$P < 0.001$ vs 0 h are shown.

Figure III

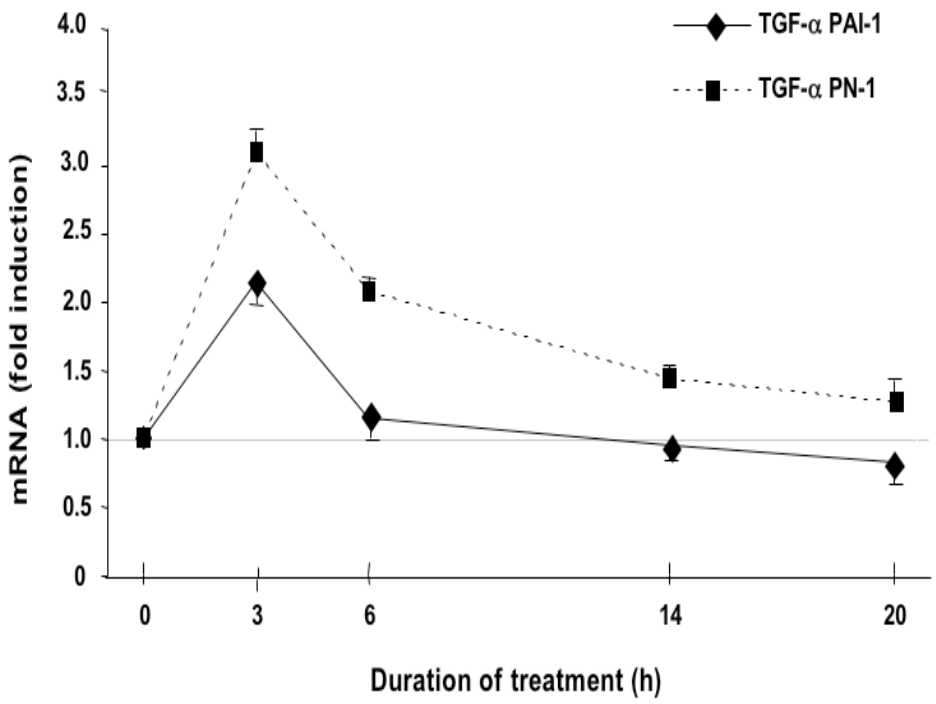

Figure III. TGF-α treatment induces an early up-regulation of PAI-1 expression in astrocytes.

The experiments were performed as described in Fig. II, with the modification that human astrocyte cultures were treated with transforming growth factor-α (TGF-α, 1 ng/ml). Results are expressed as fold induction compared to control cultures and presented as mean and SEM. Three treatment series were performed on two separate occasions (n = 6). Response to treatment was evaluated by one-way ANOVA. Post-hoc analysis by Tukey's test; †$P < 0.01$, ‡$P < 0.001$ vs 0 h are shown

Figure IV

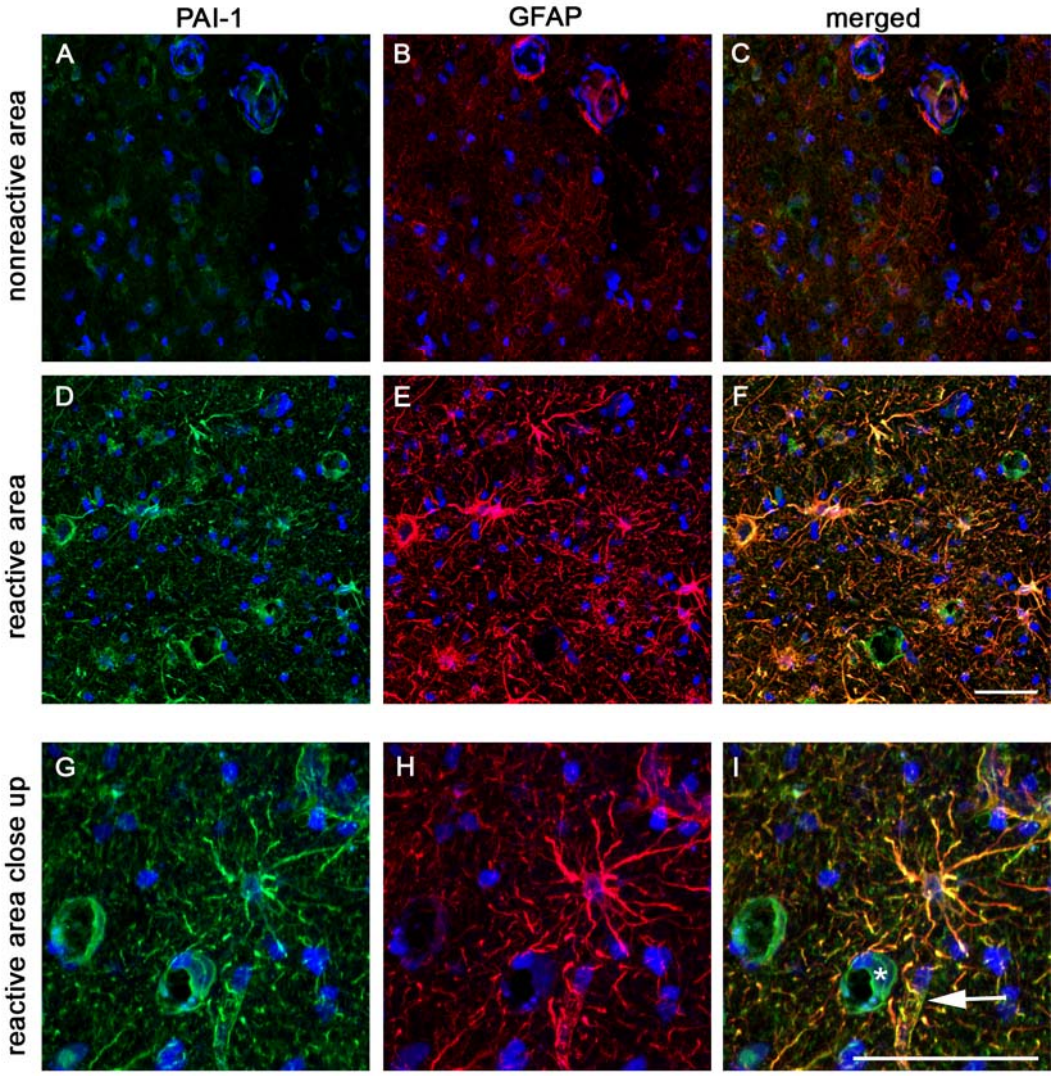

Figure IV. PAI-1 is present in reactive astrocytes *in vivo*.
Intense immunoreactivity for PAI-1 was demonstrated in astrocytes and in cerebral endothelial cells within gliotic areas of human temporal cortex (A-C: non-reactive area, D-F: reactive area, G-I: close-up view of reactive area). I: Double labeling with antibodies against PAI-1 and GFAP show that these two proteins are co-expressed in astrocytes. Arrow shows astrocytic processes extending to the vascular wall. Asterisk denotes PAI-1 immunoreactivity of cerebral endothelial cells. (PAI-1, green; GFAP, red; nuclei, blue). Scale bar 50 µm.

Figure V

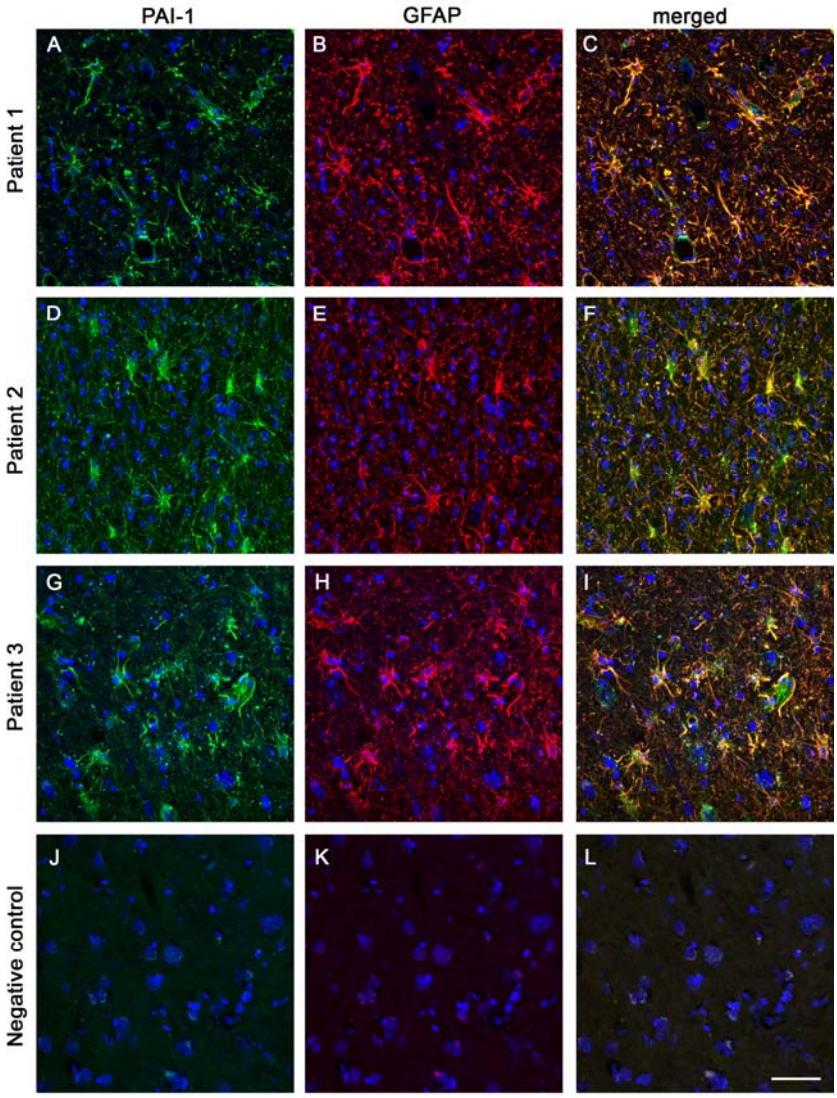

Figure V. Similar staining patterns of PAI-1 in human brain biopsy samples derived from three different individuals.
A-C: Patient 1, D-F: Patient 2, G-I: Patient 3 and J-L: negative control (only secondary antibodies). (PAI-1, green; GFAP, red; nuclei, blue). Scale bar 50 µm.