Potentiating effect of endothelial cells on astrocytic PAI-1 gene expression in an in vitro model of the blood-brain barrier

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Personal manuscript version of the accepted article (not publishers version).
Published in Neuroscience. 2010;166:408-415, www.elsevier.com, (IF 3.22)

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ABSTRACT

There is accumulating evidence of the importance of cellular communication between the cells that compose the blood-brain barrier (BBB). Astrocytes are known to affect the expression of tissue-type plasminogen activator (t-PA) and its inhibitor plasminogen activator inhibitor type-1 (PAI-1) in endothelial cells. We investigated the influence of endothelial cells on astrocytic gene expression of PAI-1, protease nexin-1 (PN-1) and t-PA using an in vitro model of the BBB. Primary rat astrocyte-enriched cultures were cocultured with primary adult rat brain microvascular endothelial cells on opposite sides of a transwell membrane. After coculturing for 9-11 days, the cultures were treated with lipopolysaccharide (LPS) for 8 h or 24 h. The levels of PAI-1, PN-1 and t-PA mRNA in untreated and treated monocultures and cocultures were analyzed by Real-Time RT-PCR. Cocultivation of astrocytes and endothelial cells increased astrocytic PAI-1 mRNA expression, and this response was further amplified by LPS treatment. The levels of PN-1 and t-PA mRNA expression in astrocytes were unaffected by cocultivation and/or LPS treatment. Analysis of endothelial PAI-1 and t-PA gene expression revealed increased PAI-1 mRNA levels in cocultured cells, whereas t-PA mRNA levels remained unchanged. These results demonstrate that the cocultivation of astrocytes and endothelial cells induces a pronounced increase in astrocytic PAI-1 gene expression, and that this effect is amplified by LPS treatment. These findings imply an important role for intercellular crosstalk in modulating PAI-1 gene expression within the BBB, under both physiologic and pathophysiologic conditions.

Key words: Endothelial-astrocyte cocultures; protease-nexin-1; tissue-type plasminogen activator; plasminogen activator inhibitor-1.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a serine protease that converts plasminogen into the active enzyme plasmin, which in turn degrades the fibrin of the forming thrombus. Thus, t-PA functions as the main activator of the fibrinolytic process in the intravascular compartment (Emeis, 1992). The vascular endothelium is the principal contributor of circulating t-PA. Plasminogen activator inhibitor type-1 (PAI-1) is the major inhibitor of t-PA in the blood.

The blood brain barrier (BBB) is composed of brain capillary endothelial cells, which are in close association with perivascular astrocytes and pericytes (Abbott et al., 2006). These endothelial cells possess specific functional properties, such as intercellular tight junctions and specific membrane transporter systems (Persidsky et al., 2006). Astrocytes appear to play an important role in the development and maintenance of the specialized BBB phenotype of the brain endothelium (Bauer and Bauer, 2000). The mechanisms underlying this process are unclear, although direct contact between the different cell types (Tontsch and Bauer, 1991) and astrocyte-derived soluble factors (Haseloff et al., 2005) may be required. On the other hand, factors secreted by brain endothelial cells can induce maturation and differentiation of astrocytes (Estrada et al., 1990). Thus, the reciprocal influences of different cell types appear to be of importance in the development and maintenance of the BBB.

In experimental *in vitro* models of the BBB, astrocytes show a protective effect against BBB disruption induced by ischemia (Kondo et al., 1996), and lipopolysaccharide (LPS)-mediated injury (Descamps et al., 2003). Astrocytes also play an important role in the regulation of endothelial-dependent fibrinolysis. In cocultures of astrocytes and brain endothelial cells, increased endothelial expression of PAI-1 and reduced expression of t-PA have been demonstrated (Tran et al., 1998). Similar patterns of PAI-1 and t-PA expression have been observed for endothelial cells cocultured with pericytes (Kim et al., 2006). Moreover, pericyte-induced enhancement of endothelial PAI-1 expression was potentiated by LPS treatment (Kim et al., 2006).

Apart from its role in the circulation, t-PA exerts important functions in the central nervous system (CNS). Under physiologic conditions, t-PA is involved in mechanisms of synaptic remodeling (De Stefano et al., 2007) and neuronal plasticity (Calabresi et al., 2000). In contrast, during pathologic conditions, excessive microglial activation and neuronal depolarization result in the accumulation of t-PA in the extracellular space, which can potentiate neurodegeneration (Tsirka et al., 1997) and induce breakdown of the BBB (Yepes et al., 2003). The damaging effects of serine proteases in CNS pathology can be reduced by serine protease inhibitors (serpins), such as PAI-1 and protease nexin-1 (PN-1), both of which are expressed by astrocytes (Gravanis and Tsirka, 2005).

Previous studies have focused on the regulatory role of astrocytes in endothelial-dependent fibrinolysis. In the present study, we investigated whether endothelial cells influence the gene expression of PAI-1, PN-1 and t-PA in astrocytes, in an *in vitro* model of the BBB. We show that cocultivation of astrocytes and endothelial cells induces a significant increase in astrocytic PAI-1 gene expression and that this effect is potentiated by LPS treatment.

EXPERIMENTAL PROCEDURES

All the experiments conformed to the guidelines on the ethical use of animals and were approved by the Ethics Committee at the University of Gothenburg (Nos. 294-2004; 349-2004; 232-2007; 255-2007). All efforts were made to minimize the number of animals used and any potential suffering.

Cell culturing

Astroglial primary cultures

Primary astroglial cultures were prepared from newborn rat cerebral cortices (Charles River, Sulzfeldt, Germany) and cultured as described previously (Hansson et al., 1984), with some modifications. Briefly, pups were dipped into 70% ethanol and decapitated. The cerebral cortex was dissected and gently passed through an 80 µm nylon mesh into Eagle´s minimum essential medium MEM with Earle´s salts (Invitrogen, Paisley, UK) that was supplemented with 7.5 mM glucose, 2 × amino acids, 4 × vitamins, 52.4 mM $NaHCO_3$, 2 mM L-glutamine, 20% fetal calf serum, 250,000 IU/l penicillin (Invitrogen), and 0.5% streptomycin (Invitrogen). The pH was adjusted to 7.3 using $NaHCO_3$. The cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C and pH 7.3, and the medium was changed three times per week.

Microvascular endothelial primary cultures

Brain capillary fragments were isolated, and endothelial cells were cultured according to the method of Hansson et al, (2008), using the modification of Abbott and co-workers (Abbott et al., 1992). Three male Sprague-Dawley rats (Charles River), weighing 225-250 g, provided sufficient capillary fragments for plating 48 inserts, which had a diameter of 12 mm and a pore size of 0.4 mm (Transwell-clear, tissue-culture-treated polyester membrane, Corning Costar, Cambridge, MA, USA). The inserts were pre-coated with a mixture of 0.33 mg/ml collagen IV from human placenta and 20 µg/ml fibronectin from bovine plasma. The cerebral cortices were dissected and the meninges and choroid plexus were peeled off. The tissue was chopped and spun at 600 × g, 4°C, for 5 min in a bovine serum albumin (BSA) buffer (calcium- and magnesium-free Hanks´ balanced saline solution, buffered with 10 mM Hepes (Research Organics Inc., Cleveland, OH, USA), pH 7.3, containing 1% penicillin-streptomycin (PEST, Invitrogen) and 0.5% BSA (30% solution, Serologicals Corporation, Norcross, GA, USA)). The preparation involved a two-step enzymatic dissociation to degrade the extracellular matrix. The cells were first treated with 10 ml of 0.1% collagenase II solution (containing 50 µg/ml gentamycin and 2 mM L-glutamine) at 37°C for 2 h on a thermomixer with gentle shaking every 10 min. The suspension was then centrifuged spun at 600 x g, 4°C, for 15 min. The supernatant was removed and a 20% BSA buffer was added. The solution was mixed and centrifuged at 1000 x g for 20 min at 4°C, to separate the capillary fragments from myelin, neurons, astrocytes and other single-cell contaminations. The top layer was removed to eliminate traces of myelin. The capillary pellet was resuspended in 5 ml of 0.1% collagenase/dispase solution (Roche Diagnostic GmbH, Penzberg, Germany), in Dulbecco´s modified Eagle´s medium (DMEM), low glucose (1000 mg/l) containing 50 µg/ml gentamycin and 2 mM L-glutamine, incubated at 37°C for 1.5 h on a thermomixer with gentle shaking every 10 min. The suspension was centrifuged at 600 x g for 5 min at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml DMEM solution and layered onto a prepared Percoll gradient. After centrifugation for 20 min at 1000 x g, 4°C, the white-grayish band of endothelial cell clusters located above the red

blood cells was aspirated and added to 10 ml of DMEM solution. The tube was spun at 600 × g for 5 min at 4°C, and the pellet was resuspended in DMEM. This procedure was repeated twice. After the last centrifugation step the supernatant was aspirated and the pellet was mixed with 2.5 ml of culture medium and plated into inserts on top of the astrocytic cultures in multiwell plates. The culture medium was based on DMEM that was supplemented with 20% donor horse serum (ICN Biomedicals GmbH, Meckenheim, Germany), 2 mM L-glutamine, 50 µg/ml gentamycin and 1 ng/ml bFGF (Roche). The cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C and pH 7.3, and the medium was changed three times per week.

Astrocytes cocultured with adult rat brain microvascular primary cultures

Six-day-old astroglial cultures were cocultured with newly prepared microvascular endothelial cell cultures. The endothelial cells were grown on transwell inserts that were placed above the astrocyte cultures. The bottom of each insert consisted of a permeable polyester membrane. The cells from the two different cultures were never in contact.

Extensive morphologic and immunocytochemical characterization of astrocytes and brain microvascular endothelial cells during coculturing has previously been described by our group (Hansson et al., 2008).

Experimental design

At the time of the experiments, astrocytes and endothelial cells had been cocultured for 9-11 days, at which point the astrocytes were 15-17 days old in total. To determine cell type-specific gene expression, gene expression was investigated at the mRNA level. Our main aim was to compare the mRNA levels in monocultured compared with cocultured astrocytes. In all the experiments, the gene expression levels in endothelial cells were also determined for comparative purposes.

In the first experimental series, mRNA expression in monocultured and cocultured cells was determined (Fig. 1 A). In the second experimental series (Fig. 1 B), monocultures and cocultures were treated with LPS (1 ng/m, from *Escherichia coli*, Sigma-Aldrich, St Louis, MO, USA) in unsupplemented, serum-free MEM, for 8 h or 24 h. In the cocultures, LPS was administered to the two cell types simultaneously. The dosage and timing of LPS treatment were based on previous *in vitro* studies (Kim et al., 2006) (Lee et al., 2008). In the control cultures (i.e., untreated monocultured or cocultured cells), the media were replaced with unsupplemented MEM. In the third experimental series (Fig. 1 C), the insert that contained the endothelial cells was removed and placed in a new empty well, and the media of the pre-cocultured astrocyte and endothelial cultures were replaced with fresh serum-free medium that contained LPS (1 ng/ml), and incubated for 8 h or 24 h. All experiments were performed in three different wells on two separate occasions (n=6). At the end of each experiment, the culture medium was removed and the cells were lysed by the addition of lysis buffer (Buffer RLT; Qiagen, Hilden, Germany), and the lysates were stored at -70°C until the preparation of total RNA.

Measurements of mRNA expression

Total cellular RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) and mRNA was transcribed to cDNA using a 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), 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. For the amplification of t-PA, PAI-1, PN-1 and control gene GAPDH, pre-designed primers and probes were used (Applied Biosystems Assay-on-demand Rn01482584_m1, Rn01481341_m1, Rn01400470_m1 and Rn01462662_g1, respectively). Relative quantification of gene expression was computed as a treatment-to-control expression ratio using the comparative C_T method. Each sample was analyzed in triplicate for both the target and control gene.

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. Differences in the mRNA levels between groups were evaluated by the unpaired Student's *t*-test. The SPSS software for Macintosh (SPSS Inc., Chicago, IL, USA) was used for all statistical calculations.

RESULTS

Coculturing of endothelial cells and astrocytes induces PAI-1 gene expression in astrocytes

When astrocytes were cocultured with endothelial cells, the PAI-1 mRNA levels in astrocytes were significantly increased by approximately 5.6-fold, as compared with the levels in monocultured astrocytes (Fig. 2). No significant alterations in the levels of PN-1 and t-PA mRNA expression were observed in cocultured astrocytes, as compared with the astrocyte monocultures (Table 1).

PAI-1 induction by LPS is potentiated in cocultured astrocytes

The effects of LPS treatment on the astrocyte levels of PAI-1, PN-1 and t-PA transcripts were investigated. Significant increases in PAI-1 mRNA levels were observed after 8 h and 24 h of LPS treatment in both the monocultured and cocultured astrocytes, as compared with their respective control culture ($P<0.001$) (Fig. 3). Induction of PAI-1 gene expression by LPS was significantly higher in the cocultures than in the monocultures of astrocytes ($P<0.001$) (Fig. 3). In contrast, LPS did not induce any significant alterations in PN-1 or t-PA gene expression in either monocultured or cocultured astrocytes (data not shown).

To investigate whether the potentiating effect of LPS treatment on astrocytic PAI-1 gene expression is a persistent effect of endothelial-dependent conditioning of astrocytes or an effect of direct intercellular crosstalk, an additional set of experiments was performed in which the endothelial cells were removed from the cocultures prior to treatment with LPS. Pre-cocultured LPS-treated astrocytes did not display a significantly different level of PAI-1 mRNA induction, as compared with cocultured LPS-treated astrocytes (Fig. 4).

Effects of coculturing and LPS treatment on endothelial PAI-1, PN-1 and t-PA gene expression

For comparative purposes, the influences of astrocytes on endothelial PAI-1, PN-1 and t-PA mRNA expression were also studied. For the cocultured endothelial cells, the PAI-1 mRNA levels were increased approximately 6.5-fold as compared with the monocultured endothelial cells (Fig. 5). Treatment with LPS induced significant increases in PAI-1 mRNA levels after 8 h and 24 h of the moncultured and cocultured endothelial cells, as compared with the levels in the respective control cultures (Fig. 6). For the cocultured endothelial cells, this response was significantly greater than that of the monocultured endothelial cells ($P<0.001$) (Fig. 6). The t-PA mRNA levels were not significantly different between the cocultured cells and the monocultured endothelial cells (Table 1).

In contrast to astrocytes, when the pre-cocultured endothelial cells were incubated with LPS, no significant increase in PAI-1 mRNA expression was observed (Fig. 7). The levels of endothelial PN-1 mRNA was at the threshold of detection for the assay, and it was not affected by either coculturing with astrocytes or by treatment with LPS.

DISCUSSION

We investigated the modulation by brain microvascular endothelial cells of PAI-1, PN-1 and t-PA gene expression in astrocytes using an *in vitro* model of the BBB. Our results demonstrate that cocultivation of these two cell types induces a pronounced increase in astrocytic PAI-1 gene expression, and that this effect is potentiated by LPS treatment. Therefore, in addition to the well-known influence of astrocytes on endothelial PAI-1 and t-PA expression, we show that there is an intercellular crosstalk in the opposite direction.

Few studies have addressed the regulatory effects of endothelial cells on astrocytes. It has been demonstrated that coculturing of astrocytes and endothelial cells induces the maturation and differentiation of astrocytes (Estrada et al., 1990). Recent data published by our group suggest that astrocytes exhibit a more developed intracellular signal transduction system when cocultured with endothelial cells, as evidenced by the increased astrocytic intracellular Ca^{2+} responses evoked by inflammatory or pain-associated stimuli (Hansson et al., 2008) (Delbro et al., 2009). Furthermore, cocultivation leads to a mutual up-regulation of antioxidant enzymes in astrocytes and endothelial cells (Schroeter et al., 1999).

The present study demonstrates, for the first time, endothelial cell-dependent up-regulation of PAI-1 gene expression in astrocytes. The mechanism behind this effect remains to be established. However, since the astrocytes and endothelial cells were separated by a permeable transwell membrane and never in direct contact, it seems likely that intercellular crosstalk, mediated by a soluble factor or factors, is required for astrocytic PAI-1 up-regulation during endothelial coculturing. Interestingly, we observed that the potentiating effect of LPS on PAI-1 gene expression in cocultured astrocytes persisted after the endothelial cells were removed, which suggests a persistent endothelial-dependent conditioning effect on astrocytes. This may be a result of astrocytic maturation and/or increased expression of soluble factors that are subsequently secreted by astrocytes, i.e. cytokines and/or growth factors. Further studies are warranted to fully elucidate the mechanisms behind these findings. However, astrocytes express several cytokines that are potential mediators of PAI-1 gene expression, such as transforming growth factor-β (TGF-β) (Treichel et al., 1998), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1β) (Faber-Elman et al., 1995). Moreover, autocrine regulation of gene expression by cytokines has been described for astrocytes (Morganti-Kossmann et al., 1992). Therefore, it is tempting to speculate that these factors are involved in an autocrine signaling loop that is triggered by endothelial cocultivation and that is further potentiated by LPS treatment.

LPS is an endotoxin that is commonly used to induce inflammatory responses in experimental settings, and it is known to induce PAI-1 gene expression (Emeis and Kooistra, 1986) (Lee et al., 2008). It has been shown that stimulation with LPS produces a strong immunologic activation of astrocytes *in vitro*, through its action on Toll-like receptor 4 (TLR4), resulting in the over-expression of inflammatory mediators, such as pro-inflammatory cytokines, in these cells (Kielian, 2006). Interestingly, recent data published by our group demonstrate that LPS-induced release of IL-1β is more pronounced in astrocytes that are cocultured with endothelial cells, as compared with monocultured astrocytes (Delbro et al., 2009). Given the acute inflammatory response present in cerebral ischemia (Emsley et al., 2008), induction of astrocytic PAI-1 gene expression by pro-inflammatory mediators may provide an important endogenous defense mechanism against the neurotoxic effects of t-PA in the brain tissue during these conditions.

Several CNS disorders, such as ischemic stroke and multiple sclerosis, are associated with breakdown of the BBB (Weiss et al., 2009). In experimental studies of cerebral ischemia, t-PA has been suggested as a critical factor in the potentiation of BBB disruption (Yepes et al., 2003). Although the mechanisms behind this effect are not fully understood, previous

studies indicate that this, through the interaction between t-PA and the LDL-receptor-related protein (LRP), may involve activation of platelet-derived growth factor CC (PDGF-CC) (Su et al., 2008). t-PA may also promote BBB breakdown by up-regulating brain matrix metalloproteinases (MMPs), in particular MMP-9 (Wang et al., 2003), thereby facilitating degradation of the vascular basement membrane. Under these pathophysiologic conditions, astrocyte-derived PAI-1 might also play an important protective role against t-PA-mediated opening of the BBB.

Knowledge regarding the regulation of PN-1 gene expression in brain-derived cells is scarce. In the present study, we confirm the expression of PN-1 in cultured astrocytes, and demonstrate that neither endothelial coculturing nor treatment with LPS affects the levels of PN-1 expression. Immunohistologic analyses of adult human brain tissue have demonstrated positive staining for PN-1 in perivascular astrocytes, particularly in and around blood vessels (Choi et al., 1990). Although the role of PN-1 remains unclear, its localization in brain tissue suggests that the primary function of PN-1 is to protect against extravasated thrombin following injury or alteration of the BBB (Choi et al., 1990).

As expected (Tran et al., 1998), we observed a pronounced increase in PAI-1 gene expression in cocultured endothelial cells. In addition, we found that LPS treatment further amplified the level of PAI-1 expression in cocultured endothelial cells. This response was completely abolished in pre-cocultured LPS-treated endothelial cells, thus indicating that the presence of an astrocyte-derived soluble factor or factors is required for the potentiating effect of LPS on endothelial PAI-1 expression. TGF-β has previously been suggested as a likely mediator of astrocyte-dependent up-regulation of endothelial PAI-1 expression, since conditioned media from astrocyte-endothelial cocultures exhibit increased levels of active TGF-β (Kim et al., 2003). Furthermore, a neutralizing anti-TGF-β antibody has been shown to attenuate astrocyte-mediated up-regulation of endothelial PAI-1 expression (Kim et al., 2003).

In contrast to PAI-1, we did not observe a significant effect of coculturing on endothelial t-PA mRNA expression. Previous studies have demonstrated pronounced reductions in the mRNA and protein levels of t-PA in endothelial cells cocultured with astrocytes (Tran et al., 1998). In the present study, cells were cocultured for 9-11 days, whereas previous studies used culturing times that ranged from 2 to 7 days. Therefore, we performed an additional set of experiments in which mRNA levels were analyzed after 7 days of coculturing. In agreement with previous studies, we found that t-PA mRNA levels were significantly reduced in endothelial cells exposed to coculturing for a shorter time period, i.e. 45% reduction compared with monocultured endothelial cells ($n=6$, $P<0.001$). Thus, these apparent discrepancies may be attributed differences in the periods of coculturing.

There are some notable limitations to the present study. This *in vitro* model of the BBB does not fully mimic the *in vivo* situation, in which astrocytes and endothelial cells are involved in a close interplay with other cell types (i.e., pericytes and neurons). Moreover, we have used a coculture model in which the astrocytes and endothelial cells were physically separated by a permeable membrane, which allowed the diffusion of soluble factors between the two compartments. Since this model does not allow identification of the cellular source of a specific secreted protein present in the conditioned media, gene expression was investigated at the mRNA level.

Our data show that the cocultivation of astrocytes and brain microvascular endothelial cells induces a pronounced increase in astrocytic PAI-1 gene expression, and that this effect is amplified by treatment with endotoxin. These findings are consistent with an important role for intercellular crosstalk in modulating PAI-1 gene expression within the BBB, under both physiologic and pathophysiologic conditions.

ACKNOWLEDGEMENTS

The present study was supported by the Swedish Research Council (K2008-65X-14605-06-3) (to CJ) and the Swedish Research Council (33X-06812) (to EH), the Swedish Heart-Lung Foundation (20070404), grants from the Swedish State under the LUA/ALF agreement (ALFGBG-11206) and the Yngve Land Foundation. We would also like to acknowledge the assistance of The SWEGENE Göteborg Genomics Core Facility platform, which is funded by a grant from the Knut and Alice Wallenberg Foundation.

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FIGURE LEGENDS

Fig. 1. Schematic illustration of the experimental series. 1) Comparison of the cell type-specific gene expression profiles of astrocyte-endothelial cell cocultures and monocultures of astrocytes and endothelial cells (Panel A). 2) Comparison of the cell type-specific gene expression profiles of astrocyte-endothelial cocultures and monocultures of astrocytes and endothelial cells in response to LPS (Panel B). 3) Comparison of the cell-type-specific gene expression profiles of pre-cocultured astrocytes and endothelial cells in response to LPS.

Fig. 2. PAI-1 gene expression is induced in astrocytes cocultured with endothelial cells. Total cellular RNA was extracted and mRNA was reverse-transcribed into cDNA. The levels of PAI-1 mRNA (cDNA) was quantified by Real-Time RT-PCR and normalized to the levels of GAPDH mRNA. Results are expressed as mean ± SEM fold-induction compared with monocultured astrocytes. Three treatment series were performed on two separate occasions (n=6). Statistical analysis was performed using the unpaired Student's *t*-test: *** $P< 0.001$.

Fig. 3. PAI-1 gene expression in cocultured astrocytes is potentiated by LPS treatment. Monocultured or cocultured astrocytes were treated with LPS for 8 h or 24 h. In control cultures (i.e. untreated monocultured and cocultured cells), the medium was replaced with unsupplemented medium. Total cellular RNA was extracted and mRNA was reverse-transcribed into cDNA. The levels of PAI-1 mRNA (cDNA) were quantified by Real-Time RT-PCR and normalized relative to the levels of GAPDH mRNA. Results shown are mean ± SEM fold-induction compared with the respective control culture. Three treatment series were performed on two separate occasions (n=6). Statistical analysis was performed using the unpaired Student's *t*-test: *** $P< 0.001$.

Fig. 4. Potentiating effect of LPS on PAI-I gene expression persists in pre-cocultured astrocytes. The experiments were performed as described in Figure 3, with the exception that the insert that contained the endothelial cells was removed and the pre-cocultured astrocytes were then treated with LPS. Results shown are mean ± SEM fold-induction compared with non-separated LPS-treated cocultured astrocytes. Three treatment series were performed on two separate occasions (n=6). Statistical analysis was performed using the unpaired Student's *t*-test: ns = non-significant.

Fig. 5. PAI-1 gene expression is induced in cocultured endothelial cells. The level of PAI-1 mRNA was quantified by Real-Time PCR as described in Figure 1. Results shown are mean ± SEM fold-induction compared with monocultured endothelial cells. Three treatment series were performed on two separate occasions (n=6). Statistical analysis was performed using the unpaired Student's *t*-test: *** $P< 0.001$.

Fig. 6. PAI-1 gene expression in cocultured endothelial cells is potentiated by LPS treatment. Monoculutred or cocultured endothelial cells were treated with LPS for 8 h or 24 h. In control cultures (i.e. untreated monocultured and cocultured cells), the medium was replaced with unsupplemented medium. Total cellular RNA was extracted, and mRNA was reverse-transcribed into cDNA. The levels of PAI-1 mRNA (cDNA) was quantified by Real-Time RT-PCR and normalized relative to the levels of GAPDH mRNA. Results shown are mean ± SEM fold-induction compared with the respective control culture. Three treatment series were performed on two separate occasions (n=6). Statistical analysis was performed using the unpaired Student's *t*-test: *** $P< 0.001$.

Fig. 7. PAI-I induction in response to LPS is abolished in pre-cocultured endothelial cells. The experiments were performed as described in Figure 6, with the exception that the insert that contained the endothelial cells was removed, and placed in a new empty well, and then treated with LPS. All results are expressed as fold-induction (mean ± SEM) compared with non-separated LPS-treated cocultured endothelial cells. Three treatment series were performed on two separate occasions (n=6). Statistical analysis was performed using the unpaired Student's *t*-test: *** $P < 0.001$.

Table 1. PN-1 and t-PA mRNA expression in cocultured astrocytes and endothelial cells compared to monocultures.

Table 1.

Fold increase in mRNA expression in cocultured compared to monocultured cells			
		mean (SEM)	**unpaired Student's *t*-test**
Cocultured astrocytes	**PN-1**	1.10 (0.12)	ns
	t-PA	1.08 (0.09)	ns
Cocultured endothelial cells	**PN-1**	---	---
	t-PA	1.15 (0.12)	ns

Total cellular RNA was extracted and mRNA was converted into cDNA. The levels of PAI-1 mRNA (cDNA) was quantified by real-time PCR and normalized relative to glyceraldehyde 3-phosphatedehydrogenase (GAPDH) mRNA. Results are expressed as fold induction compared to mono-cultured cells and presented as mean and SEM. Three treatment series were performed on two separate occasions (n=6). Response to treatment was evaluated by unpaired Student's t-test. ns = not significant.

Figure 1

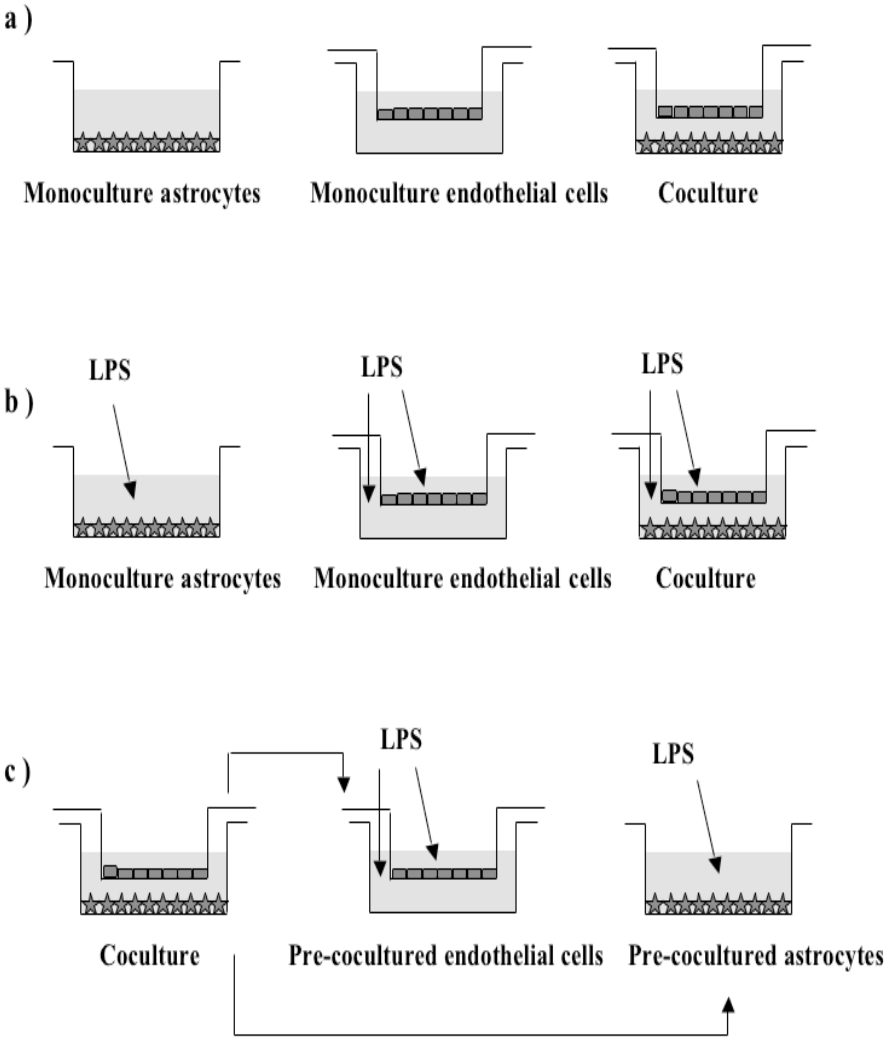

a)
Monoculture astrocytes
Monoculture endothelial cells
Coculture
b)
LPS
LPS
LPS
Monoculture astrocytes
Monoculture endothelial cells
Coculture
c)
LPS
LPS
Coculture
Pre-cocultured endothelial cells
Pre-cocultured astrocytes

Figure 2

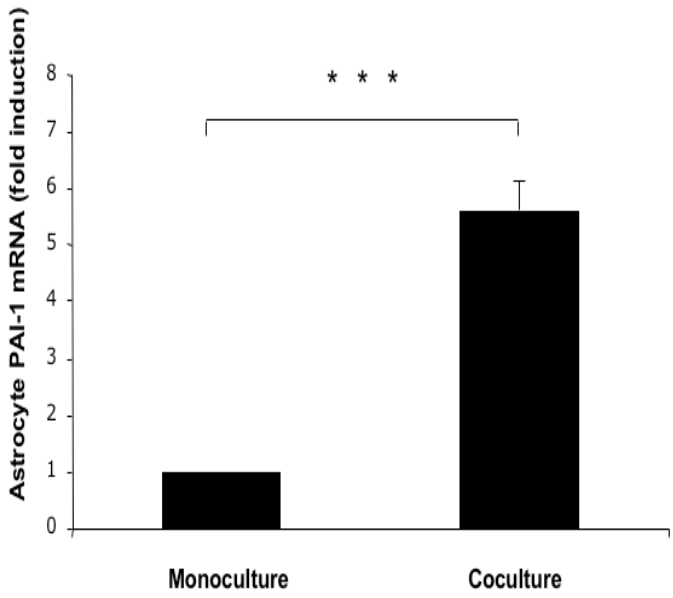

Astrocyte PAI-1 mRNA (fold induction)
0
1
2
3
4
5
6
7
8
* * *
Monoculture
Coculture

Figure 3

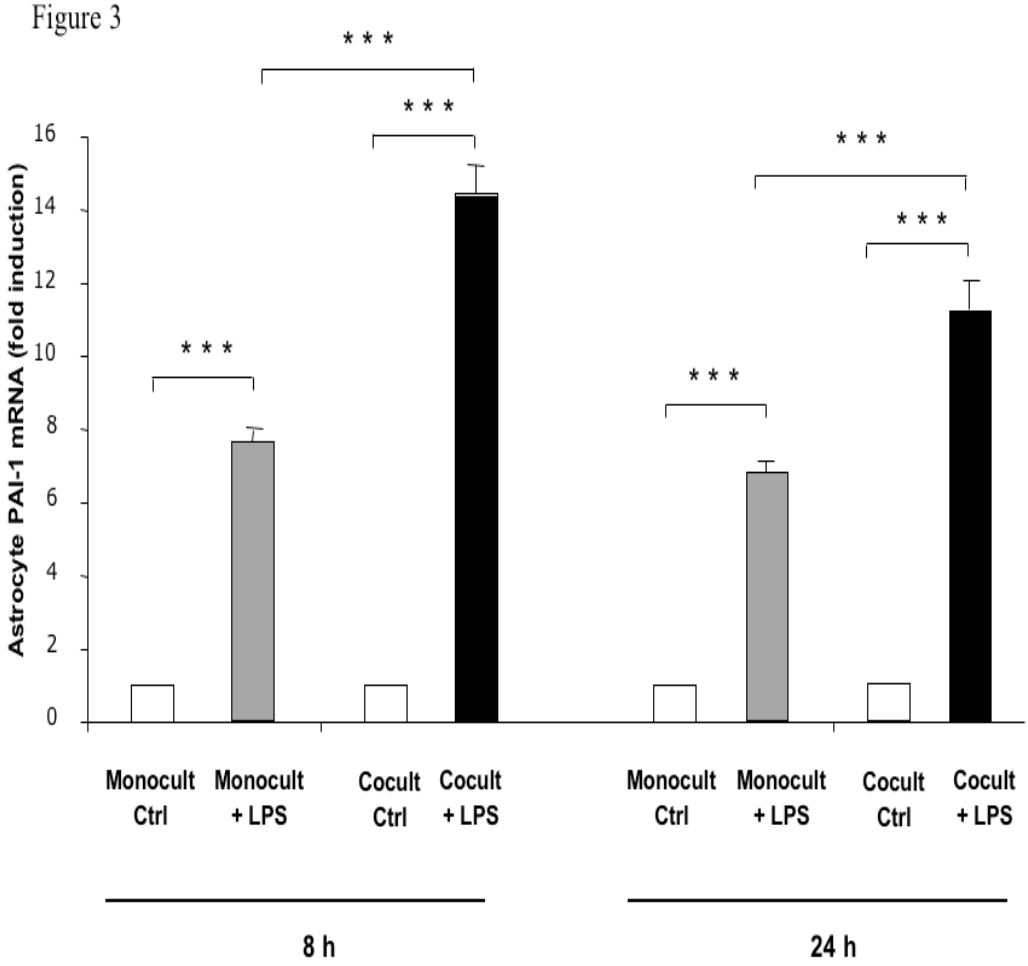

Astrocyte PAI-1 mRNA (fold induction)
0
2
4
6
8
10
12
14
16
* * *
* * *
* * *
* * *
Monocult Ctrl
Monocult + LPS
Cocult Ctrl
Cocult + LPS
Monocult Ctrl
Monocult + LPS
Cocult Ctrl
Cocult + LPS
8 h
24 h

Figure 4

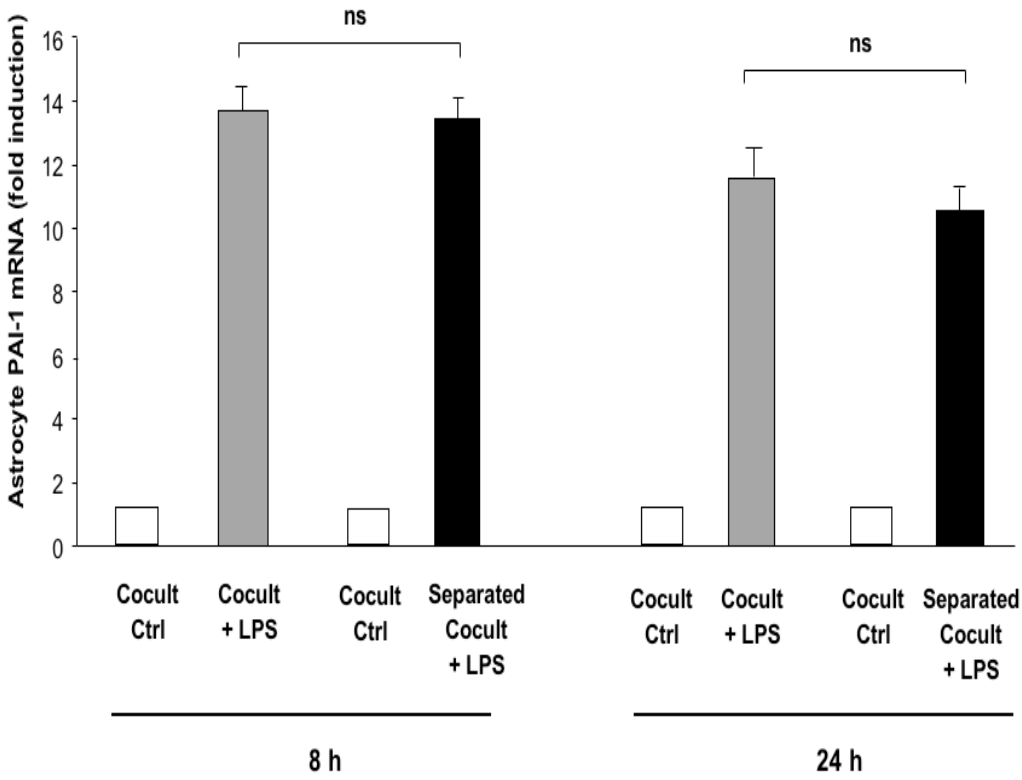

ns
ns
Astrocyte PAI-1 mRNA (fold induction)
16
14
12
10
8
6
4
2
0
Cocult Ctrl
Cocult + LPS
Cocult Ctrl
Separated Cocult + LPS
Cocult Ctrl
Cocult + LPS
Cocult Ctrl
Separated Cocult + LPS
8 h
24 h

Figure 5

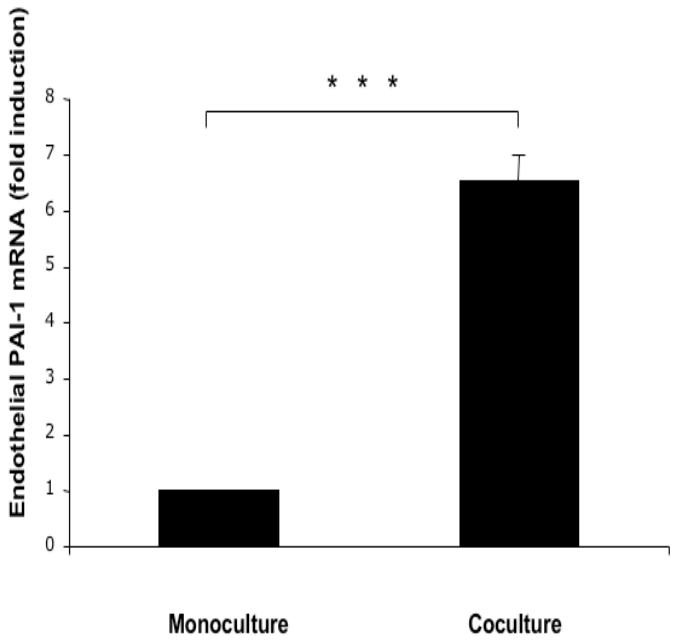

Endothelial PAI-1 mRNA (fold induction)
* * *
8
7
6
5
4
3
2
1
0
Monoculture
Coculture

Figure 6

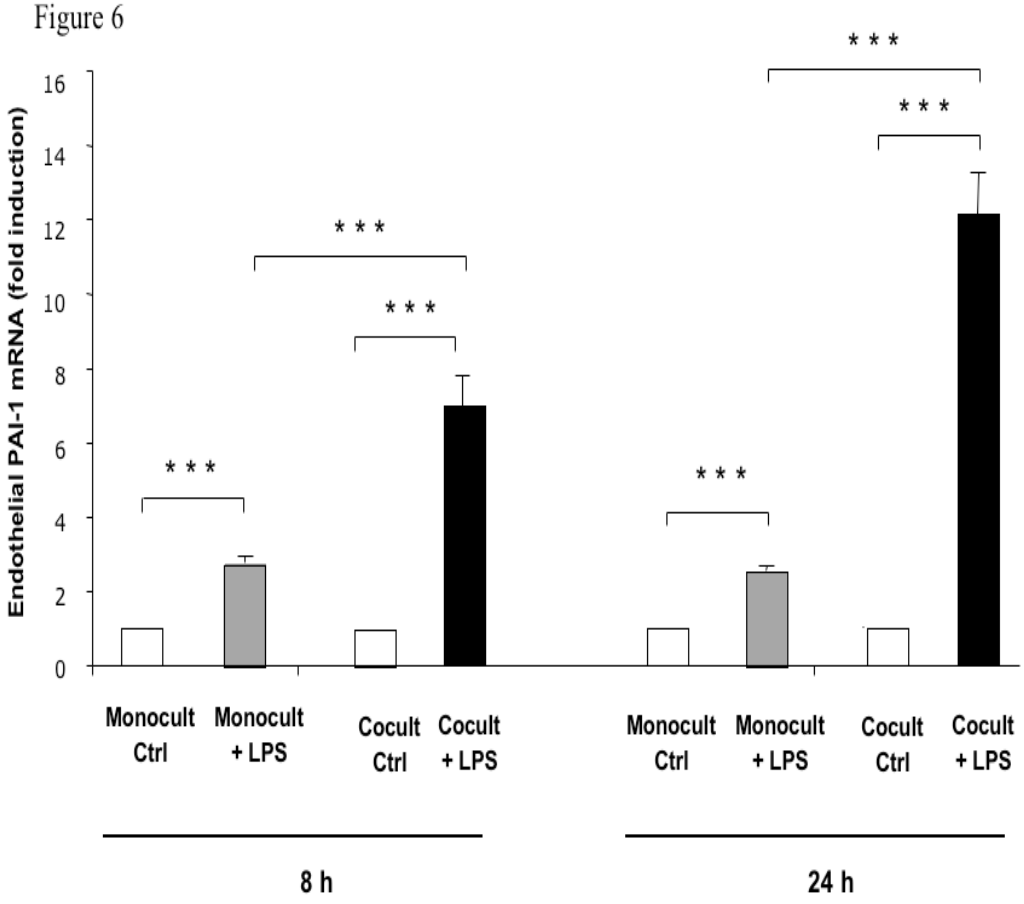
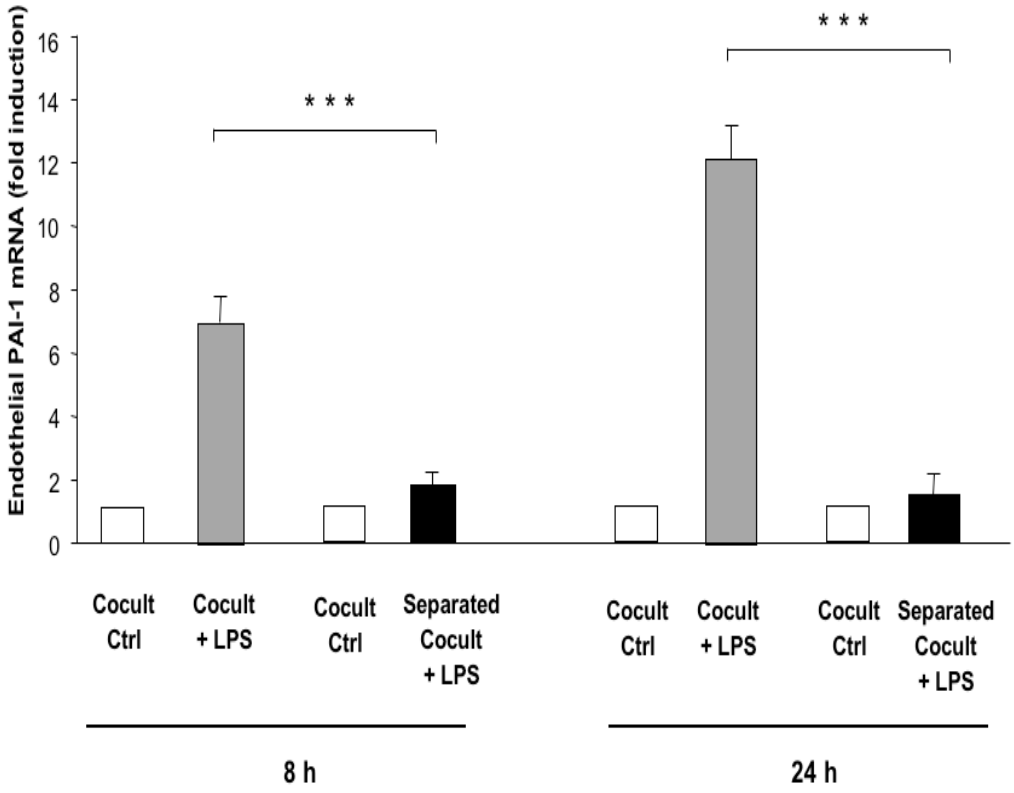

Endothelial PAI-1 mRNA (fold induction)
16
14
12
10
8
6
4
2
0
* * *
* * *
* * *
* * *
* * *
Monocult Ctrl
Monocult + LPS
Cocult Ctrl
Cocult + LPS
Monocult Ctrl
Monocult + LPS
Cocult Ctrl
Cocult + LPS
8 h
24 h

Figure 7


Endothelial PAI-1 mRNA (fold induction)
16
14
12
10
8
6
4
2
0
* * *
* * *
Cocult Ctrl
Cocult + LPS
Cocult Ctrl
Separated Cocult + LPS
Cocult Ctrl
Cocult + LPS
Cocult Ctrl
Separated Cocult + LPS
8 h
24 h