Transforming Growth Factor-β Mediates Astrocyte-Specific Regulation of Brain Endothelial Anticoagulant Factors

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Background and Purpose—Astrocytes are potent regulators of brain capillary endothelial cell function. Recently, astrocytes were shown to regulate brain capillary endothelial expression of the fibrinolytic enzyme tissue plasminogen activator (tPA) and the anticoagulant thrombomodulin (TM). To study the mechanism of this process, we examined the hypothesis that astrocyte regulation of endothelial tPA and TM is mediated by transforming growth factor-β (TGF-β).

Methods—Brain capillary endothelial cells were grown in blood-brain barrier models. We examined astrocyte-endothelial cocultures, endothelial monocultures, and astrocyte-conditioned media (ACM) for the expression of TGF-β. We also incubated endothelial cells with ACM to determine the role of TGF-β. Following 24 hours of incubation, we assayed for tPA and TM mRNA, as well as tPA and TM activity.

Results—Astrocyte-endothelial cocultures and ACM exhibited significantly higher levels of active TGF-β than brain endothelial monocultures and endothelial cells grown in nonconditioned media, respectively. Brain endothelial cells incubated with ACM exhibited reduced tPA and TM mRNA and activity. Treatment with exogenous TGF-β produced dose-dependent reductions in tPA and TM. The effects of ACM on both tPA and TM were blocked by TGF-β neutralizing antibody.

Conclusions—These data indicate that TGF-β mediates astrocyte regulation of brain capillary endothelial expression of tPA and TM. (Stroke. 1999;30:1671-1677.)

Key Words: astrocytes ■ blood-brain barrier ■ endothelium ■ thrombomodulin ■ tissue plasminogen activator ■ transforming growth factors

Hemostasis is critically important in the pathogenesis of stroke. Acute stroke therapies and secondary stroke prevention strategies involve manipulation of coagulation pathways. Essential elements of coagulation pathways are endothelial dependent, including secretion of the fibrinolytic factor tissue plasminogen activator (tPA) and expression of the antithrombotic integral membrane protein thrombomodulin (TM).

tPA is a critical circulating fibrinolytic enzyme that proteolytically activates plasminogen to plasmin.1 Intravenous tPA improves neurological outcome in clinical stroke.2 Reduced expression of brain capillary tPA is associated with increased infarct size following transient middle cerebral artery occlusion in diabetic and nicotine stroke models.3,4 Brain capillary endothelial expression of tPA is limited,5-7 and understanding mechanisms underlying this limited expression has potential therapeutic value.

TM, an important antithrombotic protein, functions as a cofactor for the activation of circulating protein C.8,9 Both TM and activated protein C offer protection against a variety of thrombotic events. Treatment with purified or recombinant TM protects against thromboembolism in animal models.10-14 while TM neutralizing antibodies potentiate thrombin-induced thromboembolism.10 Treatment with activated protein C also provides protection against thromboembolic events.15,16 Resistance to the effects of activated protein C is closely linked to venous thrombosis in humans,17 including cerebral venous thrombosis.18 Low levels of circulating activated protein C are present in certain forms of ischemic stroke.19 Moreover, brain-specific protein C activation has been demonstrated in humans in vivo20; brain capillaries ex vivo also exhibit protein C activation.21 Brain TM expression is limited,22-26 and, like tPA, identification of mechanisms underlying this limited expression would be expected to have therapeutic value.

Astrocytes are regulators for a wide variety of brain capillary endothelial functions. Astrocytes are responsible for the induction of the blood-brain barrier (BBB), contributing to tight junction formation,27,28 increased electric resistance,29 and expression of highly selective transport systems delivering essential nutrients to the brain (eg, glucose transporter 1 [GLUT-1], γ-glutamyl transpeptidase).28,30-33 Astrocytes also

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modulate endothelial expression of the low-density lipoprotein receptor and Na-K-Cl cotransporter.

We have recently reported that astrocytes have an important role in the regulation of endothelial expression of critical hemostasis factors. We have demonstrated that astrocytes regulate endothelial expression of both tPA and TM in vitro. The mechanism of this action has been uncertain. Astrocytes can secrete a number of cytokines and growth factors known to modulate endothelial function, including transforming growth factor-β (TGF-β). TGF-β is known to regulate endothelial plasminogen activator activity and TM expression. We therefore hypothesized that astrocytic regulation of endothelial tPA and TM expression is mediated by TGF-β.

Materials and Methods

Cell Culture
We isolated bovine brain capillary endothelial cells by modification of techniques of Carson and Haudenschild, as previously described. Endothelial cells were maintained on gelatin-coated culture dishes in DMEM supplemented with 2.5% equine serum or 5.0% fetal calf serum (FCS). Endothelial cells were characterized by cobblestone-like morphology, uptake of acetylated low-density lipoprotein, and immunoreactivity for von Willebrand factor, as previously described. Experiments were performed on endothelial cells between passages 10 and 25.

Neonatal mouse astrocytes were isolated according to the methods of McCarthy and deVellis, as previously described, and were maintained in DMEM with 10% FCS. Astrocytes were characterized by >99% immunoreactivity for glial fibrillary acidic protein. Astrocytes used for these experiments were taken from primary cultures for establishment of astrocyte-endothelial cocultures. Experiments were performed with the use of primary culture astrocytes between 20 to 40 days postnatal. Mouse liver cells (CCL 9.1, ATCC, Rockville, Md) were maintained under conditions similar to those for astrocytes.

BBB Model
We prepared our BBB model as previously described. Endothelial cells elongated and formed capillary-like structures within 24 hours. Cocultures were established by the addition of astrocytes at 99% confluence. We have previously described the BBB model of techniques of Carson and Haudenschild, as previously described, and performed within institutional guidelines. Cells were maintained on DMEM with 10% FCS. Astrocytes were characterized by >99% immunoreactivity for glial fibrillary acidic protein. Astrocytes used for these experiments were taken from primary cultures for establishment of astrocyte-endothelial cocultures. Experiments were performed with the use of primary culture astrocytes between 20 to 40 days postnatal. Mouse liver cells (CCL 9.1, ATCC, Rockville, Md) were maintained under conditions similar to those for astrocytes.

Conditioned Media Experiments
We performed conditioned media experiments according to the methods of Maxwell et al. Briefly, astrocyte-conditioned media (ACM) and liver cell-conditioned media were prepared by first aspirating the growth media from these cultures, washing with PBS, and replacing media with 5% FCS-supplemented media. Forty-eight hours later the ACM or liver cell-conditioned media were collected, centrifuged to remove cellular debris, and stored at −80°C until use. Bovine brain endothelial cells were plated in 24-well tissue culture plates (4 × 10⁵ cells per well) in 1 mL 5% FCS-supplemented growth media. At confluence the cells were incubated for 24 hours with media containing 50% ACM (or liver cell-conditioned media) and 50% fresh growth media or fresh growth media alone in the presence or absence of neutralizing anti–TGF-β monoclonal antibody (20 μg, Genzyme Diagnostics). Anti–gp-120 monoclonal antibody (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from Dr Bruce Chesebro) was used as a control irrelevant antibody.

TGF-β Studies
Bovine brain endothelial cells were plated in 24-well tissue culture plates (4 × 10⁵ cells per well) as described above. At confluence, the cells were incubated with human recombinant TGF-β1 and TGF-β2 (Sigma) for 24 hours. Endothelial cell counts were performed after 24 hours of TGF-β incubation. In separate studies, total TGF-β activity was determined as a measure of proliferation inhibition with the use of the mitogen yeast cell line ATCC CCL-64, as previously described. Isoforms of TGF-β (TGF-β1 and TGF-β2) from conditioned media were measured by enzyme-linked immunosorbent assay (Promega). TGF-β concentrations were adjusted to endothelial cell count.

Polymerase Chain Reaction
Total RNA was isolated with the Glassmax DNA Spin Cartridge Isolation System (Gibco BRL). cDNA was synthesized from equal quantities of total RNA, as previously described, and the cDNA was stored at −20°C until use.

Polymerase chain reaction (PCR) primers for bovine tPA (forward primer, BTPA-1: 5′-AGGTTGACAAAGAAGTG-3′ and reverse primer, BTPA-2: 5′-GTGAGCCGGTGTCACCTCCTCTGGA-3′) and TM (forward primer, BTM-1: 5′-CTCUGCAACTACGCTG-CATCTGGGAGA3′ and reverse primer, BTM-2: 5′-GCACCACACCAGACGGCTTGAAT3′) were chosen from coding regions of the mRNA. β-Actin primers (Stratagene; forward primer, 5′-TGACGGGTTCACCAAACACTGTGCCCATCTA-3′; reverse primer, 5′-CTGAAAGCATTTGCGGATGAGGAGG-3′) were used to amplify β-actin mRNA as a housekeeping gene control. The PCR mixture contained 0.2 to 1.0 μg cDNA, 10 mMol/L Tris-HCl pH 8.3, 50 mMol/L KCl, 0.1 mMol/L dNTP, 1.0 mMol/L MgCl₂, 1.0 U Taq polymerase, and 0.5 μmol/L forward and reverse primers. Amplification was performed in a Geneamp PCR System 2400 (Perkin-Elmer Corp), as follows: initial denaturation at 94°C; each cycle consists of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 56°C; and 1 minute of extension at 72°C. All reverse transcription–PCR assays were performed within the linear range of the amplification curve. PCR products were visualized by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Negatives were prepared with a Polaroid camera (Polaroid Corp) and scanned by optical densitometry (Hoefer Instruments). Optical densitometric measurements were normalized to β-actin levels (ie, dividing by β-actin optical densitometric measurements or by percentage of β-actin relative to the mean).

Quantitative competitive PCR (QC-PCR) was used to quantify tPA and TM mRNA expression. QC-PCR analysis has been used extensively for the quantitation of mRNA and has been shown to be accurate. The PCR mixture contained all the amplification reagents (described above), a constant amount of target cDNA from each preparation, and serial dilutions of known concentrations of a competitor tPA or TM cDNA template. Competitor templates were generated according to the techniques of Tran et al. We have recently reported that astrocytes have an important role in the regulation of endothelial expression of critical hemostasis factors. We have demonstrated that astrocytes regulate endothelial expression of both tPA and TM in vitro. The mechanism of this action has been uncertain. Astrocytes can secrete a number of cytokines and growth factors known to modulate endothelial function, including transforming growth factor-β (TGF-β). TGF-β is known to regulate endothelial plasminogen activator activity and TM expression. We therefore hypothesized that astrocytic regulation of endothelial tPA and TM expression is mediated by TGF-β.

Plasminogen Activator Assay
We assayed cultured media from monoculture and coculture preparations for total plasminogen activator activity by amidolytic assay.

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Figure 1. QC-PCR analysis of tPA and TM mRNA. QC-PCR gels of tPA (A) and TM (B) are shown. A constant amount of unknown target cDNA was added to the PCR tubes containing serial dilutions of a competitor template. A, Lanes 1 through 6: 1.1×10^{-9}, 1.1×10^{-10}, 1.1×10^{-11}, 1.1×10^{-12}, 1.1×10^{-13}, 1.1×10^{-14} g. B, Lanes 1 through 6: 5.0×10^{-11}, 5.0×10^{-12}, 5.0×10^{-13}, 5.0×10^{-14}, 5.0×10^{-15}, 5.0×10^{-16} g. With decreasing amounts of competitor cDNA added, there is a decrease in competitor PCR products (bottom bands) and a concomitant increase in unknown target PCR products (top bands).

Protein C Assay

Endothelial TM activity was assayed by measuring the increase in protein C activation according to the methods of Tsiang et al. Cells were collected with a rubber policeman and resuspended in a 100-μL solution containing 50 mmol/L Tris-HCl, 2 mmol/L CaCl₂, 0.1 mol/L NaCl, 0.1% BSA, 0.1 μg bovine thrombin, 2 μg bovine protein C, pH 8.0. The solution was incubated at 37°C for 30 minutes. The reaction was terminated by adding anti–thrombin III (5 μg) and heparin (5 U), and the resulting mixture was centrifuged at 200,000 × g. Bovine thrombin, 2 μg bovine protein C, and bovine thrombin, 2 μg bovine protein C, pH 8.0. The solution was incubated at 37°C for 30 minutes. The reaction was terminated by adding anti–thrombin III (5 μg) and heparin (5 U), and the resulting mixture was centrifuged at 200,000 × g. Bovine protein C activation was considered significant for P<0.05.

Statistical Analysis

All data are expressed as mean±SD. Statistical comparisons between groups were performed with unpaired Student’s t tests and Pearson’s correlation coefficients. Differences were considered significant for P<0.05.

Results

We examined TGF-β levels in conditioned media in our BBB model. We previously reported downregulation of tPA and TM transcripts in astrocyte-endothelial cocultures in this model. Astrocyte-endothelial cocultures demonstrated a significant increase in active TGF-β (3038±323 pg/mL) compared with capillary-like structure monocultures (821±429 pg/mL; P<0.001). We analyzed our data for potential association between active TGF-β and mRNA levels. There was a significant inverse correlation between active TGF-β and mRNA (r=−0.73, P<0.04) and TM mRNA concentrations (r=−0.90, P<0.003).

To determine whether TGF-β can downregulate tPA and TM mRNA, we incubated brain capillary endothelial cells with human recombinant TGF-β1 and TGF-β2 (the 2 best-characterized isoforms). Treatment with TGF-β1 resulted in downregulation of endothelial tPA and TM mRNA in a dose-dependent manner (Figure 2). TGF-β2 treatment also produced dose-dependent downregulation of tPA and TM mRNA (Figure 2). Cell counts revealed no significant differences between TGF-β1- and TGF-β2-incubated and control endothelial cells (data not shown).

We next examined media conditioned by astrocytes for 48 hours (ACM) to determine whether astrocytes can produce TGF-β. ACM had significantly higher levels of active TGF-β1 (443±71 versus 90±36 pg/mL; P<0.03) and TGF-β2 (641±21 versus 86±21 pg/mL; P<0.002) than nonconditioned growth media. We then performed experiments using ACM to determine whether the astrocyte-induced mRNA downregulation is mediated by TGF-β. Bovine brain capillary endothelial cells were grown to confluence and incubated for 24 hours with ACM.

Figure 2. PCR analysis of tPA and TM mRNA expression. QC-PCR showed dose-dependent reductions in tPA (A, B) and TM (C, D) mRNA after incubation with human recombinant TGF-β1 and -β2. Data are presented as mean±SD. Data are from 1 experiment (performed in triplicate) and representative of 3 independent experiments (*P<0.05; **P<0.01; ***P<0.001).
of endothelial transcripts 24 hours after incubation with ACM revealed reductions in tPA (13.5±5.4 versus 34.3±3.2 pg/mL; P<0.006) (Figure 3) and TM mRNA (43.0±2.1 versus 68.8±4.8 pg/mL; P<0.002) (Figure 4) compared with endothelial cells incubated with nonconditioned media. Incubation with liver cell–conditioned media (as control) had no effects on endothelial tPA (32.3±9.9 pg/mL; P>0.6) or TM transcripts (70.2±3.0 pg/mL; P>0.7). The downregulation of tPA and TM mRNA was completely abolished with TGF-β neutralizing antibody (40.4±1.7 and 69.7±5.5 pg/mL, respectively); the control irrelevant antibody produced no effect (Figures 3 and 4).

To examine the functional consequences of mRNA down-regulation, we examined tPA activity in culture media and TM activity from cell lysates. There was a significant decrease in tPA activity in ACM incubated compared with control endothelial cells (15.3±1.8 versus 27.3±1.0 IU/mL; P<0.02). This tPA downregulation was attenuated by TGF-β neutralizing antibody (30.9±1.1 IU/mL); control antibody had no effect (17.7±1.3 IU/mL) (Figure 5). Concurrently, there was a significant reduction in TM activity in endothelial cells incubated with ACM compared with control (60±2% versus 100±1%; P<0.02) (Figure 6). This decrease was not observed in liver cell–conditioned media incubated cells (95±2% of control; P>0.1). The downregulation in TM activity was attenuated by TGF-β neutralizing antibody (97±4% of control; P>0.5) but not by control antibody (68±4% of control; P<0.05). These findings suggest that TGF-β mediates astrocyte regulation of tPA and TM expression at the mRNA level.

Discussion

The present study showed that TGF-β mediates astrocyte regulation of endothelial expression of anticoagulant factors. We demonstrated a significant increase in active TGF-β in astrocyte-endothelial cocultures compared with capillary-like structure monocultures. We showed a strong inverse correlation between active TGF-β concentrations and tPA and TM expression. We also demonstrated that ACM negatively regulates endothelial expression of tPA and TM transcripts. These effects were not demonstrable in endothelial cells...
incubated with conditioned media from control liver cells, thus indicating astrocyte-specific regulation. These changes in endothelial mRNA expression were blocked by neutralizing antibodies to TGF-β but not by isotype-matched control antibody. The reductions in endothelial cells transcripts were present with a concomitant reduction in tPA activity and TM activity. These functional changes were also blocked by TGF-β neutralizing antibodies. Our findings demonstrated that astrocytes specifically regulate 2 critical endothelial anticoagulant factors (tPA and TM) and that this regulation occurs via TGF-β.

Our data suggest that astrocytes contribute to TGF-β activation in astrocyte-endothelial cocultures. TGF-β is synthesized as a large precursor protein consisting of TGF-β and a latency-associated peptide. The latency-associated peptide is associated with TGF-β through noncovalent interactions and prevents TGF-β from binding to its receptor. TGF-β can be activated in vitro by a variety of exogenous treatments, including acidification, alkalization, heat, or protease treatment. The cellular mechanisms for TGF-β activation are not well understood; however, increases in active TGF-β have also been reported following heterotypic cocultures of endothelial cells with pericytes. We have shown that both latent and active TGF-β are present in conditioned media from astrocyte monocultures. These data suggest that astrocytes not only secrete TGF-β but can also regulate its activation.

We found that astrocytes express both TGF-β1 and TGF-β2 isoforms. Astrocytes have been reported to express TGF-β1, TGF-β2, and TGF-β3 mRNA but only secrete TGF-β1 and TGF-β2 in vitro. TGF-β1 and TGF-β2 are isoforms found most frequently in the central nervous system. TGF-β can be produced by a number of cells in the central nervous system, including astrocytes and endothelial cells. Both astrocyte-derived and endothelial-derived TGF-β were secreted into the growth media in our coculture preparations. Our assays for TGF-β cannot assess the relative contributions of TGF-β by each of these cell types.

Analysis of TGF-β demonstrated higher concentrations of TGF-β in media from astrocyte-endothelial cocultures than from ACM. This difference may be attributed to differing culture conditions. (1) Astrocyte-endothelial cocultures represent an angiogenic model in which endothelial cells develop into capillary-like structures; TGF-β plays an important role in the angiogenic process. (2) Coculture media were collected from 7 day astrocyte-endothelial cocultures, whereas ACM were conditioned by astrocytes for only 48 hours. With longer exposure to astrocytes, one would expect to see greater levels of TGF-β secreted into the culture media. (3) The enhanced expression of TGF-β in astrocyte-endothelial cocultures may result from autocrine or paracrine regulation. TGF-β secreted by astrocytes and/or endothelial cells may upregulate TGF-β expression and activation by astrocytes. (4) Finally, astrocyte-endothelial cocultures and endothelial monocultures were grown in 2.5% equine serum, whereas astrocyte and endothelial monolayers were grown in 5.0% fetal calf serum. We cannot exclude the role of serum in the expression of TGF-β.

TGF-β has been shown to induce growth inhibition on a number of cell types in vitro, including endothelial cells. It is possible that tPA and TM downregulation could reflect TGF-β-induced growth inhibition. However, this explanation is unlikely because our experiments were performed on confluent endothelial cells, and cell counts were similar for TGF-β-incubated and control endothelial cells.

The extent of tPA downregulation by ACM was comparable to those previously reported by direct astrocyte-endothelial cocultures. These findings further corroborate the role of an astrocyte-derived soluble factor, namely TGF-β, in astrocyte-mediated tPA regulation. In contrast to our tPA findings, the extent of TM downregulation was not as extensive as previously reported in direct astrocyte-endothelial cocultures. The differences in TM regulation may reflect differences in our experimental paradigm: (1) In our coculture model, astrocytes were added to subconfluent endothelial cells, whereas ACM were incubated with confluent endothelial monolayers. Proliferating endothelial cells have been reported to be more responsive to astrocytes than confluent endothelial monolayers. (2) Astrocytes were cocultured with endothelial cells for 7 days, while endothelial monolayers were exposed to ACM for 24 hours. TGF-β-mediated TM downregulation may occur in a time-dependent manner. (3) TGF-β mediates TM downregulation in a dose-dependent manner. In our coculture model, astrocytes are in direct contact with endothelial cells; local concentrations of TGF-β at this astrocyte-endothelial interface may be significantly higher than TGF-β concentrations found in coculture media or ACM, eliciting greater biological effects. (4) TM regulation in cocultures may occur through multiple mechanisms; TGF-β may constitute only 1 component of the astrocyte–endothelial cell interactions. Cellular contact between the 2 cell types may induce further changes in endothelial function. Astrocytic cellular membrane has been reported to modulate selective endothelial function in vitro. Moreover, cellular contact in our astrocyte-endothelial cocultures may function as a positive feedback mechanism by enhancing production and/or activation of TGF-β.

The regulatory roles of astrocytes on endothelial cells through direct cell contact are well defined. Astrocyte-induced effects can also occur via ACM. Our findings suggest that astrocyte regulation of endothelial hemostasis function is mediated in part by astrocyte-derived TGF-β. Astrocytes can elaborate other growth factors (eg, fibroblast growth factor, pleiotrophin, vascular endothelial growth factor) known to affect endothelial function. Our data cannot exclude the roles of these soluble factors in the regulation of endothelial function. Moreover, we cannot rule out the potential contributions of direct cell contact on regulation of endothelial hemostasis.

Our findings provide further support for the existence of a unique hemostatic regulatory apparatus of the brain. This regulation is astrocyte dependent, occurs in the microcirculation, and is mediated by TGF-β. While it is important to be cautious in extrapolating our in vitro findings to in vivo phenomena, the negative regulation of tPA and TM suggests a prothrombotic milieu within the brain microvasculature. This prothrombotic milieu may provide evolutionary advan-
tage, protecting the brain from neonatal intracranial hemorrhage. However, with age and development of stroke risk factors, this prothrombic milieu may become counterproductive and predispose to infarction. Modulation of astrocyte-dependent endothelial hemostasis regulation may lead to new strategies for the treatment and prevention of ischemic stroke.

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References


Endogenous endothelial anticoagulant factors, including tPA and TM, are known to be neuroprotective against a variety of thrombotic events that occur in acute stroke.1,2 The expression of these endogenous anticoagulant proteins in the brain is rather limited and is known to be tightly regulated. The identification of the mechanisms of the regulation of the expression of these factors is of therapeutic significance.

Using several well-established in vitro culture systems involving primary astrocytes and endothelial cells, concomitant with up-to-date molecular techniques, Tran and colleagues have presented strong evidence demonstrating that TGF-β, produced by astrocytes, appears to be the culprit responsible for the downregulation of the endothelial iPA and tPA mRNA levels. The study is carefully done, the results are convincing, and the findings are novel. The following issues have evolved from this study: (1) The mechanisms causing the activation of astrocytic release of TGF-β during acute stroke and the subsequent regulation of TGF-β on tPA and TM transcription are unknown at present. The current findings do provide an impetus for further studies in this area. (2) The astrocytic response to cerebral ischemia is very complex and can be neuroprotective despite the production of TGF-β. In addition to being a major cellular component forming the intact BBB with endothelial cells, astrocytes are known to provide the necessary microenviron-
ment for neurons to survive under pathological conditions such as ischemia, oxidative injury, and glutamate toxicity.\textsuperscript{3,4} Thus, future molecular or pharmacological approaches can be directed to amplify the good side (i.e., neuroprotection) and to minimize the bad side (i.e., production of TGF-\(\beta\)) of the astrocytic response in acute stroke therapy.

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