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Protease-activated receptor-1 in human brain: localization and functional expression in astrocytes ☆

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Abstract

Protease-activated receptor-1 (PAR1) is a G-protein coupled receptor that is proteolytically activated by blood-derived serine proteases. Although PAR1 is best known for its role in coagulation and hemostasis, recent findings demonstrate that PAR1 activation has actions in the central nervous system (CNS) apart from its role in the vasculature. Rodent studies have demonstrated that PAR1 is expressed throughout the brain on neurons and astrocytes. PAR1 activation in vitro and in vivo appears to influence neurodegeneration and neuroprotection in animal models of stroke and brain injury. Because of increasing evidence that PAR1 has important and diverse roles in the CNS, we explored the protein localization and function of PAR1 in human brain. PAR1 is most intensely expressed in astrocytes of white and gray matter and moderately expressed in neurons. PAR1 and GFAP co-localization demonstrates that PAR1 is expressed on the cell body and on astrocytic endfeet that invest capillaries. PAR1 activation in the U178MG human glioblastoma cell line increased PI hydrolysis and intracellular Ca^{2+}, indicating that PAR1 is functional in human glial-derived tumor cells. Primary cultures of human astrocytes and human glioblastoma cells respond to PAR1 activation by increasing intracellular Ca^{2+}. Together, these results demonstrate that PAR1 is expressed in human brain and functional in glial tumors and cultures derived from it. Because serine proteases may enter brain tissue and activate PAR1 when the blood brain barrier (BBB) breaks down, pharmacological manipulation of PAR1 signaling may provide a potential therapeutic target for neuroprotection in human neurological disorders.

Keywords: Protease-activated receptor; PAR1; Thrombin; G-protein coupled receptor; Serine protease; Glioblastoma; Astrocyte

Introduction

Protease-activated receptor-1 (PAR1) is a member of a family of four unique G-protein coupled receptors, PAR1–4. PAR1 is well known for its actions in clotting and hemostasis (Coughlin, 2001; Macfarlane et al., 2001), but the role of PAR1 and its activators, serine proteases, in the central nervous system (CNS) has received increased attention in recent years. PAR1 is widely expressed throughout the rodent CNS in both neurons and glia and is upregulated following experimental ischemia in hippocampal slice cultures (Niclou et al., 1998; Rohatgi et al., 2003; Striggow et al., 2001; Weinstein et al., 1995). Protease-activated receptors have a novel mode of activation in which serine proteases cleave the extracellular N-terminal of the receptor and unmask a peptide sequence that acts as a tethered ligand to constitutively activate the receptor. Thrombin is the most potent activator of PAR1 (Vu et al., 1991). Other serine proteases including Factor Xa, the Factor VIIa/tissue factor complex, and streptokinase complexed with plasminogen can activate PAR1 (Camerer et al., 2000, 2002; McRedmond et al., 2000; Riewald et al., 2001), whereas plasmin and trypsin both activate and inactivate the receptor (Junge

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et al., 2003; Kawabata et al., 1999; Kuliopulos et al., 1999; Vu et al., 1991).

Experimental models of CNS disorders including ischemia, excitoxicity, seizure, and spinal cord injury suggest that serine proteases including tissue plasminogen activator (tPA), plasmin, and thrombin may have pathological effects in the CNS (Abe et al., 2003; Gingrich and Traynelis, 2000; Lee et al., 1997; Tsirka et al., 1995; Wang and Reiser, 2003; Wang et al., 1998). Whereas serine proteases are normally restricted to the vascular space due to the blood–brain barrier (BBB), some proteases including tPA and the plasmin precursor plasminogen are expressed in rodent and human brain, and may also enter the brain during BBB breakdown (Gingrich and Traynelis, 2000; Salles and Strickland, 2002; Teesalu et al., 2002). In addition, tissue plasminogen activator (tPA) is currently used as a thrombolytic therapy in a subset of stroke patients to facilitate reperfusion of ischemic tissue. Several substrates for the tPA/plasmin system in the brain have been identified (Nicole et al., 2001; Tsirka et al., 1997), including PAR1 (Junge et al., 2003).

In vivo, thrombin activation of PAR1 can induce apoptotic cell death in hippocampal cell cultures and motorneurons in the developing avian spinal cord (Donovan et al., 1997; Turgeon et al., 1998). In addition, activation of PAR1 may interfere with neuronal regeneration following cerebrovascular insult since PAR1 has been shown to cause neurite retraction, microglial proliferation, astrocyte proliferation and glial scar formation, which may prevent neurons from reestablishing connections (Nishino et al., 1993; Pindon et al., 2000; Sorensen et al., 2003; Suo et al., 2002, 2003). PAR1 activation also potentiates NMDA receptor responses (Gingrich et al., 2000), and activation of NMDA receptors has been implicated in pathological situations such as stroke and brain injury. In vivo studies indicate that the lack of PAR1 as well as pharmacological inhibition of PAR1 within the brain parenchyma is neuroprotective in rodent models of transient focal cerebral ischemia (Junge et al., 2003). Another recent report showed that PAR1 activation in the endothelium contributed to the neuroprotective phenotype resulting from injection of activated protein C (APC) in the same model of ischemia (Cheng et al., 2003). These studies demonstrate the complex and important role of PAR1 in pathological situations. Because of the accumulating evidence that serine proteases and their receptor PAR1 participate in neuropathological processes, we investigated the protein localization and function of PAR1 in human brain, astrocyte cultures, and astrocytic neoplasms.

Materials and methods

Immunoblot

Human cortical and hippocampal tissue (n = 5 human subjects) was retrieved by the Emory University autopsy service and immediately frozen. Post-mortem intervals ranged from 6 to 10 h. Brain tissue was collected only from subjects who had no history of neurologic disease and whose death was not neurologically related. Tissue was thawed and homogenized on ice in RIPA buffer [9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% Igepal CA-360 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS] containing 0.1 mg/ml PMSF (Sigma), 30 μl/ml aprotinin (Sigma), and 1 nM sodium orthovanadate (Sigma). Ten-microliter samples of homogenized protein were incubated with Laemmli SDS sample buffer (62.5 mM Tris–HCl, 25% glycerol, 2% SDS, 0.01% bromophenol blue) for 5 min at 100 °C. Samples were then loaded into 10% Tris–glycine precast gels (Invitrogen) and run at 200 mV for 1.5 h in running buffer (0.1% SDS, 125 mM Tris base, 1 M glycine). Gels were subsequently transferred onto polyvinylidine difluoride (PVDF) membranes in transfer buffer (10% methanol, 25 mM Tris, 192 mM glycine) for 2 h at 100 mV at 4 °C. Membranes were blocked in 2.5% milk in Tris-buffered saline (0.9%; TBS) for 30 min and then incubated in 4 μg/ml PAR1 primary antibody (monoclonal, WEDE15, Beckman Coulter) on a shaker at 4 °C overnight. Membranes were washed 3 × 10 min in TBS and incubated in goat anti-mouse secondary antibody (1:1000, Santa Cruz) in 2.5% TBS/milk on a shaker for 1 h at room temperature. Membranes were washed 3 × 10 min in TBS and secondary antibodies were detected by chemiluminescence (Amersham). When the primary or secondary antibody was omitted, the PAR1 antigen signal was not detected.

Immunohistochemistry

Post-mortem human brain was retrieved from the autopsy service of Emory University hospital within 4 h of death. The age range of the patients (n = 5, four females, one male) was between 60 and 74 years old. Frontal lobe, striatum, and temporal lobe including hippocampus were fixed in 3% paraformaldehyde for 48 h and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer. Brain tissue was frozen on dry ice and cut at 40 μm on a sliding microtome. Sections were placed in cryopreservative (30% sucrose, 30% ethylene glycol, 0.1 M phosphate buffer, pH 7.2) and stored at −20 °C. Sections were rinsed 6 × 10 min in cold TBS (pH 7.4) and incubated in endogenous peroxidase inhibitor (3% H_2O_2 in TBS) for 10 min. Sections were rinsed 5 × 10 min in TBS and blocked with 10% horse serum (Gibco) in TBS/0.1% Triton X-100 for 30 min at room temperature. Sections were incubated in the same solution in 2.5 μg/ml PAR1 primary antibody (monoclonal, WEDE15, Beckman Coulter) for 48 h on a shaker at 4 °C. Sections were rinsed 3 × 10 min in TBS and incubated in biotin-conjugated rat anti-mouse secondary antibody (1:200, Jackson Immunoresearch) for 1 h at room temperature. For single labeling studies, the avidin–biotin complex method was used to detect the antigen signal (ABC, Vector Laboratories) and 3,3′-diaminobenzidine tetrachloride was used to visualize the final product. When the primary or secondary antibody was omitted, no immunostaining was observed.

For double labeling studies, the tyramide signal amplification (TSA, Perkin Elmer) procedure with Cy3-conjugated tyramide was used to visualize PAR1; GFAP was visualized using a FITC-conjugated secondary antibody. To avoid cross-reactivity, labeling for PAR1 and GFAP was performed sequentially although the antibodies were raised in different hosts. Sections initially were incubated in GFAP primary antibody (rabbit polyclonal, 1:100; Sigma) followed by PAR1 primary antibody, as described above. Sections were then incubated in goat anti-rabbit FITC-conjugated secondary antibody for 1 h at room temperature. The avidin–biotin complex method followed by the TSA procedure was subsequently used to amplify the PAR1 signal, which was visualized with Cy3 conjugated to tyramide. When either the primary or secondary antibody was omitted, the PAR1 antigen signal was not detected.

Antibody preadsorption

Control experiments for both immunoblotting and immunohistochemistry were performed in which the primary antibody was preadsorbed against a peptide matching the epitope ($PAR1_{51-64}$, Emory University Microchemical Facility) against which the PAR1 antibody was made. The peptide $PAR1_{51-64}$ was anchored to sepharose and 500 μl of concentrated PAR1 primary antibody (1:40) was added to 500 μl of $PAR1_{51-64}$-sepharose in an Eppendorf tube and agitated for 1 h at room temperature. The tube was centrifuged at 100 × *g* for 3 min, and the supernatant was brought up to 1 ml resulting in a 1:80 dilution of the primary antibody (2.5 μg/ml). Brain sections were incubated in this solution and processed as described above. The primary antibody was similarly preadsorbed for immunoblotting.

Ca^{2+} imaging

Human glioblastoma cell line U178MG was maintained in standard Dulbecco's modified Eagle's medium culture medium (DMEM; Gibco) supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 units/ml). Resected glioma that was mechanically dissociated and primary human astrocytes (passage 7) were maintained in DMEM/fetal calf serum supplemented with penicillin/streptomycin for 12–72 h. Collection of tissue was in compliance with Emory University IRB review. U178MG glioblastoma cell line, acutely dissociated glioma cells, and primary astrocytes were plated on 12-mm glass coverslips that were coated with 0.1 mg/ml poly-D-lysine for Ca^{2+} imaging. For recording, cells were incubated with 5 μM Fura2-AM (Molecular Probes) for 30 min at room temperature (23°C). Cells were then placed on a microscope stage for imaging of intracellular Ca^{2+} concentration. External solution contained 150 mM NaCl, 10 mM HEPES, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM glucose, pH 7.3, and 325 mOsm. Intensity images of 510 nm wavelength were taken at 340 and 380 nm excitation wavelengths, and the two resulting images were taken for ratio calculations. Axon Imaging Workbench version 2.2.1 was used for acquisition of intensity images and conversion to ratios.

Analysis of resected glioblastoma maintained as a primary culture

Glioblastoma received by the Department of Pathology following surgical resection was formalin fixed and paraffin embedded for histologic processing. Standard 6-μm hematoxylin and eosin (H&E)-stained sections were reviewed histologically for diagnosis and for comparison to cells used in calcium mobilization experiments. Additional tissue was dissociated in trypsin and studied for calcium mobilization. Following calcium mobilization studies, cells on coverslips were fixed in cold paraformaldehyde and stained with a Diff-Quik Stain Set (Dade Behring, Newark, DE) or by immunocytochemistry for GFAP. Briefly, cells were washed in PBS for 5 min, permeabilized with proteinase K for 30 s, blocked sequentially with 0.3% H_20_2, avidin solution, and biotin solutions for 10 min each, and then incubated with anti-GFAP for 1 h (monoclonal, 1:100, Dako Co., Carpinteria, CA, USA). Antibodies were detected following incubation with a biotinylated secondary antibody (Dako) using the avidin-biotin-peroxidase complex (ABC) method (Dako). The antigen–antibody reaction was visualized using 3,3′-diaminobenzidine as the chromogen. Standard positive controls were used throughout, normal serum serving as the negative control. Cells were briefly counterstained with hematoxylin.

Measurement of phosphoinositide hydrolysis

Glioblastoma U178MG monolayers near confluence in 12-well plates were labeled overnight at 37°C with 4 μCi/ml *myo*-[2-^{3}H]inositol (20 Ci/mmol; American Radiolabeled Chemicals, Inc.) in DMEM supplemented with 10% fetal bovine serum. The following day, cells were incubated for 20 min at 37°C in DMEM buffered with 25 mM HEPES (pH 8) and containing 10 mM $LiCl_2$. Agonist (either thrombin or TFLLR at the indicated concentrations) in 0.1% BSA or 0.1% BSA alone was added to 1 ml of incubation media, and stimulation proceeded for the indicated times at 37°C. Following stimulation, media was aspirated and cells were lysed in 20 mM formic acid at 4°C for 30 min. Cell lysates were then collected into 1.5-ml microcentrifuge tubes (Eppendorf), and 214 μl 0.7 M NH_4OH was added to neutralize sample pH before column loading. Samples were loaded onto AG 1-X8 Dowex (Bio Rad) anion exchange columns that were then washed twice with 10 ml water, once with 8 ml 50 mM ammonium formate, and residual radiolabeled total inositol phosphates were eluted with 6 ml 1.2 M ammonium formate/0.1 M

formic acid. Inositol phosphate accumulation was then quantitated by liquid scintillation spectrophotometry.

Results and discussion

Verification of antibody specificity

The WEDE15 monoclonal antibody against human PAR1 has been extensively characterized and shown not to interact with human PAR2–4 (Molino et al., 1995; O'Brien et al., 2000). To confirm the WEDE15 PAR1 antibody specificity in our hands, preadsorption of the antibody with a control peptide corresponding to the antibody epitope was performed before use in immunoblots and immunohistochemistry. Preadsorbing WEDE15 with the control peptide completely blocked detection of the PAR1 antigen signal in brain tissue (Fig. 1A, right) compared with WEDE15 alone (Fig. 1A, left). Immunoblots demonstrated that WEDE15 detects a 66-kDa band in human cortical (Fig. 1B, left) and hippocampal (data not shown) brain membrane preparations, which corresponds with previous reports of the molecular weight of PAR1 (Molino et al., 1995; Woolkalis et al., 1995). Weak reactivity against lower-molecular-weight bands likely reflects PAR1 breakdown products that occurred during the delay before the tissue was obtained in autopsy. Preadsorption of WEDE15 with the control peptide also blocked detection of the PAR1 antigen signal in immunoblots (Fig. 1B, right). These results together suggest that WEDE15 specifically recognizes human PAR1.

PAR1 immunostaining in neurons in human brain regions

PAR1 protein expression was examined in human cortex, hippocampus, striatum, and cerebellum. Morphological examination of WEDE15-immunostained and hematoxylin-counterstained brain sections revealed that neuronal populations within the hippocampus, cortex, and striatum show modest to moderate immunostaining with intensities varying among neuronal subsets (data not shown). In contrast, neurons in the cerebellum, including Purkinje cells and granular cells, exhibit very little PAR1 immunostaining (data not shown). In the hippocampus, larger pyramidal neurons in the CA1–CA4 layers and the subiculum (Fig. 2A) show PAR1 immunoreactivity that is present in cell bodies and extends into major axonal and dendritic processes. Granule cells and interneurons in the dentate gyrus show very little PAR1 immunoreactivity. Similar to the hippocampus, larger pyramidal neurons in the cortex show moderate PAR1 immunoreactivity (Fig. 2B), with the immunostaining in layers 3 and 5 more prominent than that observed in the other cortical layers. In the caudate and putamen of the striatum, cell bodies morphologically identified as neurons show distinct PAR1 immunoreactivity. Differential expression of PAR1 by medium spiny and

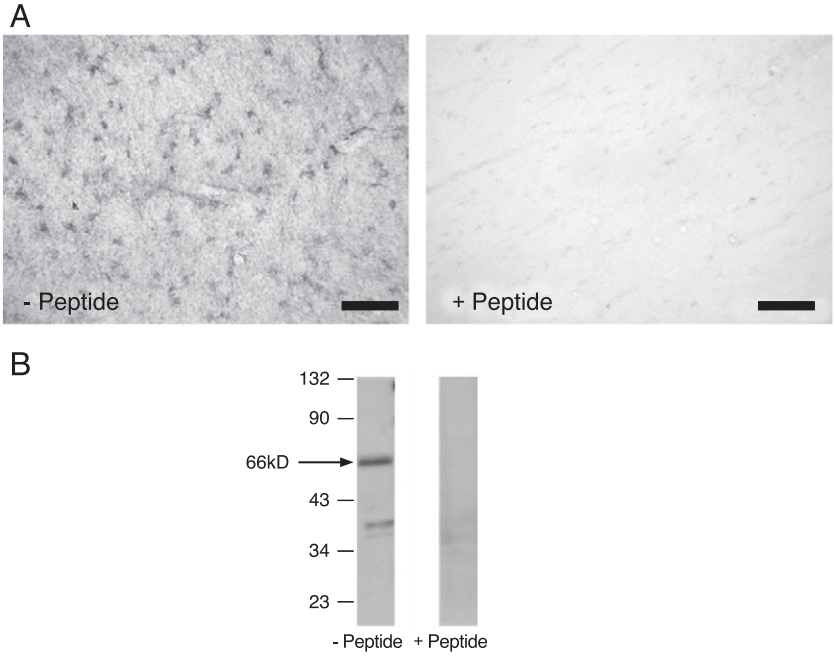

Fig. 1. PAR1 antibody preadsorption in brain tissue and immunoblots. (A) PAR1 immunostaining in human cortex with primary antibody (left) or primary antibody preadsorbed with the control peptide (right) showing lack of immunostaining when the primary antibody was preadsorbed with the control peptide (scale bar = 200 μm). (B) Immunoblot with PAR1 primary antibody (left) using human cortical brain membrane preparations. PAR1 antibody revealed a 66-kDa band, which is the previously reported molecular weight of PAR1. Primary antibody preadsorbed with the control peptide (right) showing lack of immunostaining when the primary antibody was preadsorbed with the control peptide.

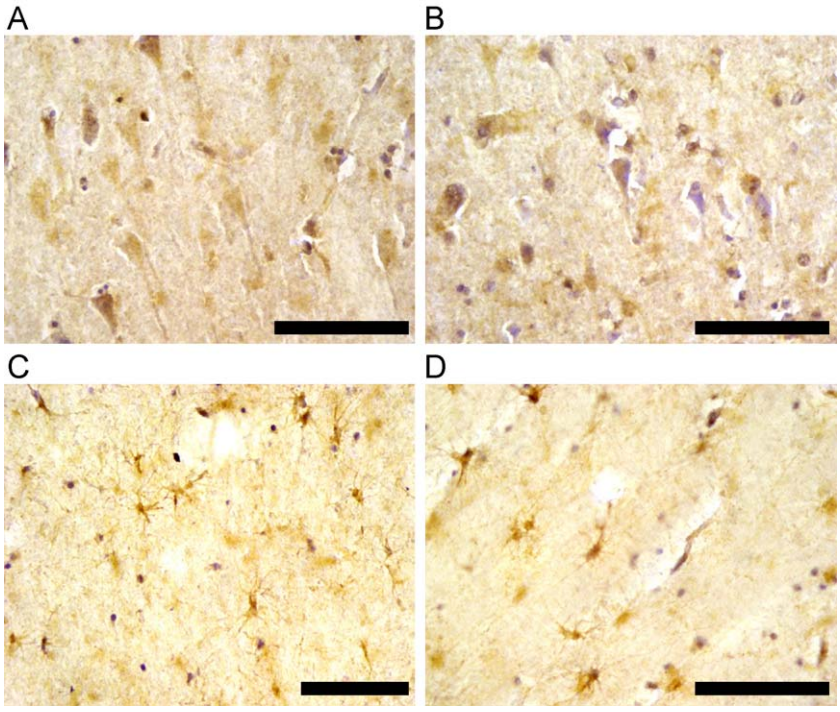

Fig. 2. High-power magnification of PAR1-immunostained and hematoxylin-counterstained human brain sections (scale bar = 100 μm). (A) Cell bodies and some processes of neurons in subiculum show moderate PAR1 immunoreactivity. (B) PAR1 immunostaining in cortical neuron cell bodies. (C) Astrocyte cell bodies and processes in cerebellar white matter show strong PAR1 immunoreactivity. (D) Astrocytes in temporal lobe white matter show similar PAR1 immunostaining. Oligodendrocyte cell bodies and processes do not show PAR1 immunostaining.

cholinergic neurons could not be observed. Overall, the neuronal immunostaining observed in the hippocampus, cortex, and striatum was moderate and slightly more intense than that observed in the surrounding neuropil. The neuropil, which is the delicate meshwork of neuronal and glial processes in gray matter structures, consistently showed mild immunoreactivity throughout all brain regions examined. Thus, PAR1 protein is moderately expressed in certain populations of neurons and shows diffuse, mild neuropil expression.

PAR1 immunostaining in astrocytes in human brain regions

The most intense PAR1 immunoreactivity was observed in astrocytes throughout all brain regions examined. Morphological examination of WEDE15-immunostained and hematoxylin-counterstained brain sections demonstrated that astrocytes in white and gray matter structures of the cerebellum (Fig. 2C), temporal lobe (Fig. 2D), frontal lobe (data not shown), and striatum (data not shown) showed PAR1 immunoreactivity. Ependymal cells lining the lateral ventricles (data not shown) as well as oligodendrocytes in white matter (Figs. 2C and D), which lack extensive processes, do not appear to express PAR1 protein. White matter showed less overall immunoreactivity than gray matter (Figs. 2A–D), most likely due to the lack of neuronal cell bodies and high density of neuronal processes present in gray matter. However, PAR1 staining of astrocytes showed marked contrast against the low background immunostaining seen in white matter tracts. Astrocytes in gray matter also exhibited strong PAR1 immunoreactivity, almost always with higher intensity than nearby neurons (Figs. 3 and 4).

Since PAR1 immunoreactivity is most prominently expressed in cells morphologically identified as astrocytes, we performed co-localization immunofluorescence studies with a glial fibrillary acid protein (GFAP) antibody and WEDE15 to unequivocally identify that the cells studied were astrocytes. GFAP-labeled cells (green) in white matter were also labeled with WEDE15 (red) indicating that PAR1 is expressed on astrocytes in white matter in the temporal lobe (Fig. 3A). This result was observed in white matter throughout the cortex, hippocampus, striatum, and cerebellum. WEDE15 and GFAP also co-localize on astrocytic cell bodies and processes in gray matter in all brain regions examined as well (Figs. 3B and C). Furthermore, astrocytes that extend endfeet to capillaries and blood vessels in the brain also show GFAP and PAR1 protein co-localization in the frontal lobe (Fig. 4A) and putamen of the striatum (Fig. 4B) as well as the hippocampus and cerebellum (data not shown). Capillaries also exhibit PAR1 and GFAP immunoreactivity (Figs. 4A and B). PAR1 protein expression on human endothelial cells in coronary arteries has previously been reported (Hamilton et al., 2002), and our results indicate that PAR1 protein is also expressed in the cerebral vascula-

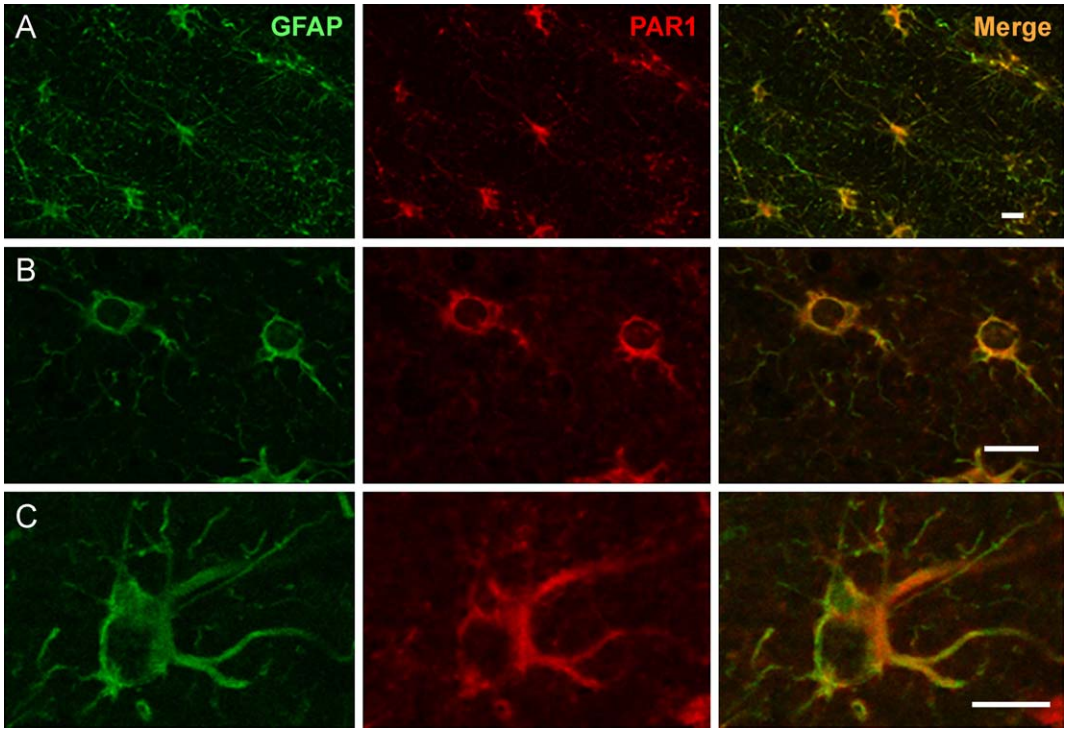

Fig. 3. Immunofluorescent images of PAR1 and GFAP co-localization. (A) Astrocytes cell bodies and processes in white matter of hippocampus show strong co-localization of GFAP (green, left) and PAR1 (red, middle). Merged image shown in right panel. (B and C) Gray matter astrocytes in frontal lobe also show strong PAR1 and GFAP co-localization at different magnification. Calibration bar is 10 μM.

ture. The GFAP immunoreactivity observed on the surface of capillaries is most likely astrocytic endfeet contacting capillaries. Thus, PAR1 protein is widely expressed in astrocytic cell bodies and processes throughout human brain.

PAR1 activation of Ca^{2+} mobilization in human glioblastoma cells and astrocytes

To examine whether PAR1 protein in human astrocytes is functional, we first utilized a glioma (U178MG) cell line

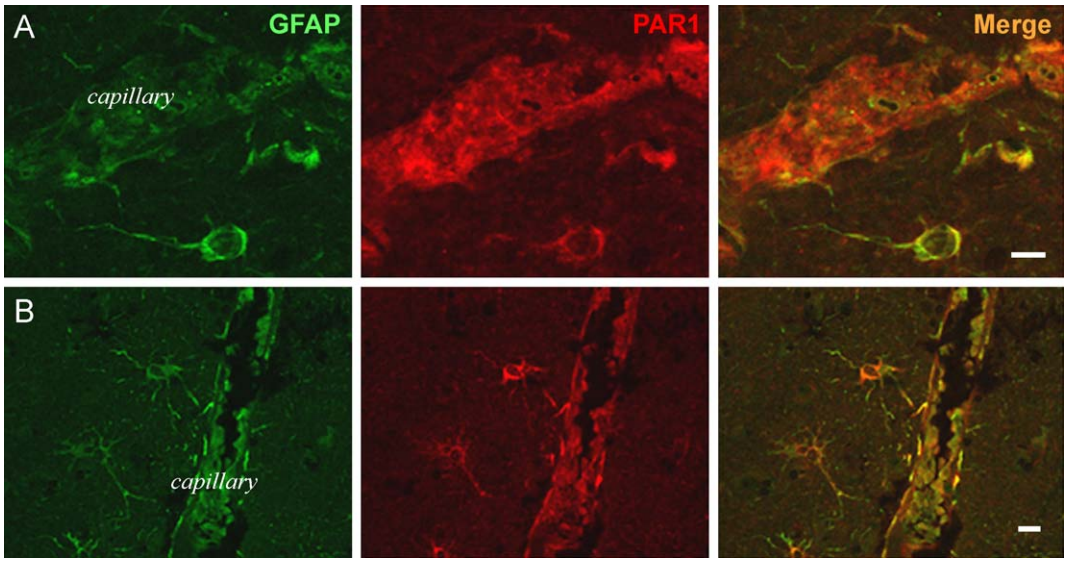

Fig. 4. PAR1 and GFAP co-localization in astrocyte cell bodies and processes contacting capillaries in gray matter of frontal lobe (A) and caudate (B). Photomicrographs show strong co-localization of GFAP (green, left) and PAR1 (red, middle); merged image shown in right panel. Capillaries also show PAR1 immunofluorescence, which is expected since human endothelial cells express PAR1. Capillaries show some GFAP immunofluorescence, which is most likely due to astrocytic end feet that invest capillaries. Calibration bar is 10 μM.

that was accessible for our experimental paradigm. To monitor functional expression, we examined the ability of PAR1 activation by both thrombin and the selective PAR1 activating peptide TFLLR-NH_2 (Hollenberg et al., 1997) to stimulate inositol phosphate signaling. We found that both thrombin and TFLLR-NH_2 evoked a concentration- and time-dependent increase in liberated inositol tris-phosphate, consistent with known coupling of PAR1 to Gαq signaling (Figs. 5A and B). We subsequently evaluated the effect of TFLLR-NH_2 using ratiometric Ca^{2+} imaging of Fura2-AM loaded U178MG cells. TFLLR-NH_2 (30 μM) induced an increase in Ca^{2+} concentration in 63% (n = 3 experiments) of U178MG cells (Fig. 5C). This increase was blocked when cells were perfused with the PAR1 antagonist BMS-200261 (1 μM; Fig. 5C; Bernatowicz et al., 1996). These results indicate that neoplastic cells that are derived from astrocytes express functional PAR1 that couples to an increase in intracellular Ca^{2+} concentrations. Our findings are consistent with similar conclusions in SNB19 and A172 cell lines (Kaufmann et al., 2000; Okamoto et al., 2001).

Whereas the U178MG glioblastoma cells are from human origin, they have been maintained in culture over many divisions. Therefore, we also tested whether primary cultures maintained 24–72 h after mechanical dissociation of acutely resected glioma had functional responses to the selective PAR1 activating peptide TFLLR-NH_2. Review of tissue sections from the resection specimen showed a high-grade infiltrative astrocytic neoplasm with necrosis and microvascular hyperplasia, fulfilling World Health Organization criteria for glioblastoma (Kleihues et al., 2002). Tumor tissue was composed of 90–95% neoplastic cells. Examination of cytologic preparations of dissociated glioblastoma cultures stained with Diff-Quik following calcium mobilizations studies demonstrated a highly atypical neo-

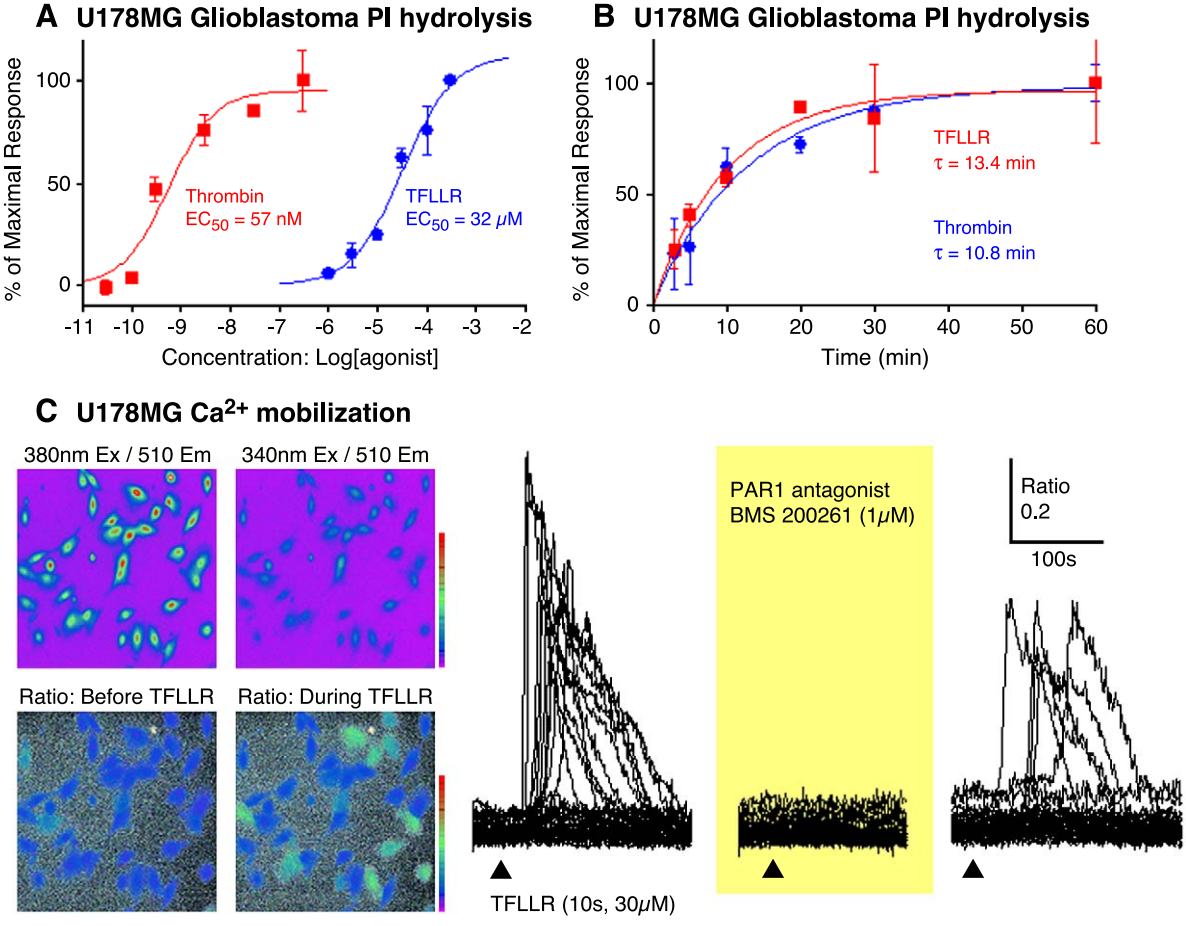

Fig. 5. Functional expression of PAR1 in U178MG glioblastoma cell line. (A) U178MG glioblastoma cells metabolically labeled with ^{3}H-*myo*-inositol were stimulated with varying concentrations of either TFLLR or thrombin for 30 min in the presence of 10 mM $LiCl_2$, and then lysed, subjected to anion-exchange chromatography, and recovered total [^{3}H]inositol phosphate accumulation was detected by liquid scintillation spectrometry. (B) U178MG glioblastoma cells metabolically labeled with ^{3}H-*myo*-inositol were stimulated with either 100 μM TFLLR or 3 nM thrombin for various times and accumulation of [^{3}H]inositol phosphates was measured. Data points are the mean ± SD and are representative of two separate experiments. (C, left panel) Pseudo-colored intensity image (380 or 340 excitation, 510 emission, above) and ratio images (below) of U178MG glioblastoma cells before and after TFLLR stimulation. (Right panels) Each trace represents changes in Fura2 intensity ratio in one cell. Filled triangles show the onset of 10-s TFLLR applications at 10-min intervals. Pre-application and co-application of 1 μM PAR1 antagonist, BMS-200261 (Bernatowicz et al., 1996), block the Ca^{2+} responses, which recovered in the subsequent TFLLR application.

plastic population with resemblance to H&E-stained tissue sections. Both the tumor tissue sections and dissociated tumor cells in culture showed cytoplasmic GFAP expression, confirming their astrocytic differentiation (Fig. 6A; Kleihues et al., 2002). In a subpopulation of these cells TFLLR-NH_2 stimulated an increase in Fura2 fluorescence ($n = 4$; Fig. 6B), which we interpret to suggest an increase in intracellular Ca^{2+} in primary cultured glioblastoma cells (Kaufmann et al., 1998). Finally, we obtained a primary culture of human astrocytes (passage 7). These cells showed a strong response to TFLLR-NH_2 ($n = 3$; Fig. 6C), consistent with results in glioblastoma cells as well as rodent

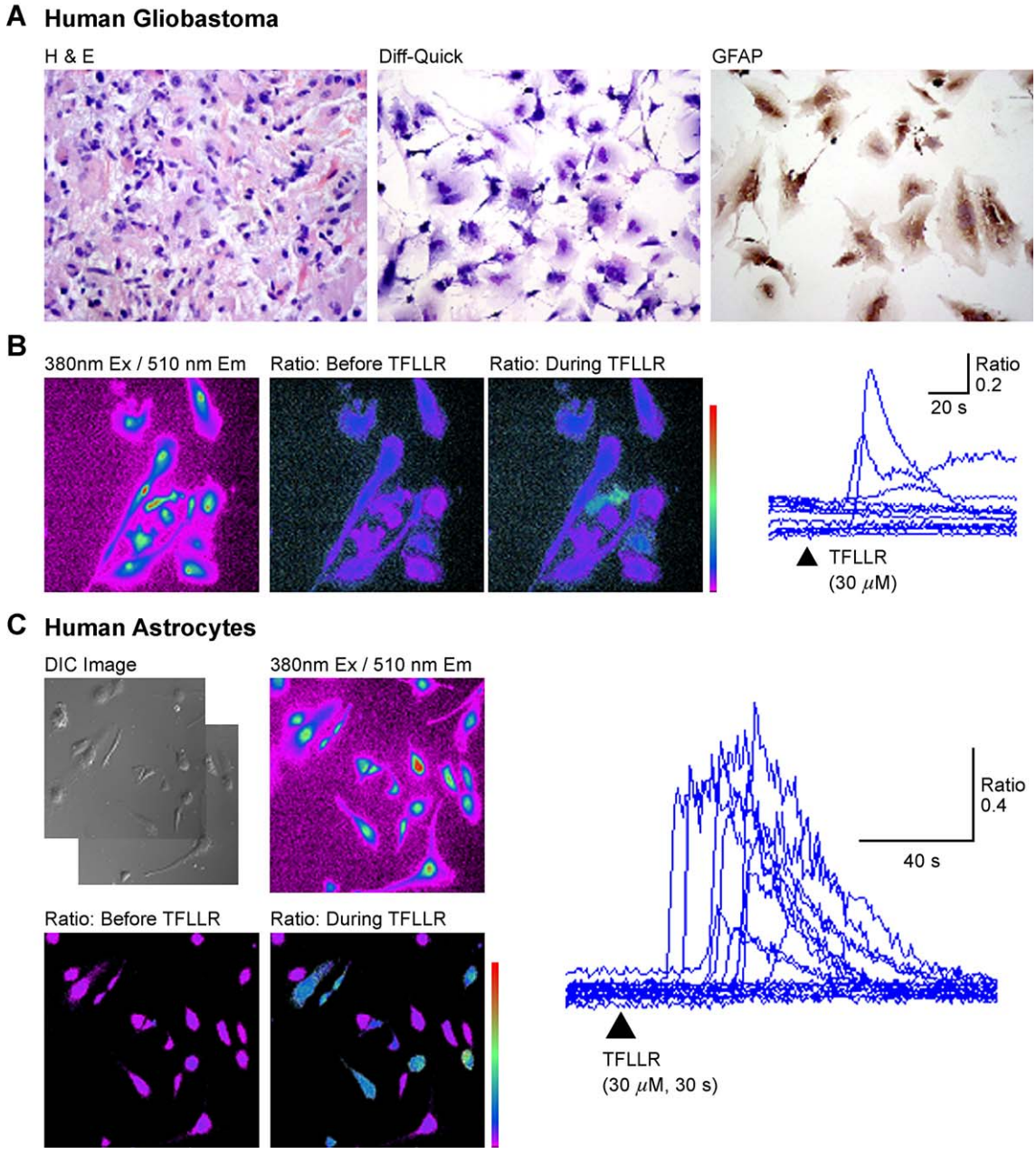

Fig. 6. Coupling of PAR1 to Ca^{2+} mobilization in acute primary culture of glioblastoma cells. (A) H&E-stained histologic sections (left panel) of acutely resected brain tumor showed a high-grade neoplasm with astrocytic differentiation (200×). Dissociated glioblastoma cells used for calcium mobilization studies were highly atypical, with enlarged, distorted nuclei, and irregular chromatin patterns (middle panel, Diff-Quik, 200×). Dissociated tumor cells showed cytoplasmic expression of GFAP, consistent with astrocytic differentiation (right panel, 200×). (B, left panel) Pseudo-colored intensity image (380 excitation, 510 emission) and ratio images before and during TFLLR-NH_2 application to dissociated glioblastoma cells. Each trace in the right panel represents changes in Fura2 intensity ratio in one cell. (C) Normarski and pseudo-color composite image (380 excitation, 510 emission) of cultured human astrocytes are shown in upper panels. Lower panels show TFLLR-NH_2-induced change in ratio of Fura2 fluorescence. Each trace in the right panel represents changes in Fura2 intensity ratio in one cell.

astrocyte cultures. These results suggest that human astrocytes express functional PAR1 in vitro and further support our interpretation that the immunostaining for PAR1 represents functional receptors.

Discussion

These results demonstrate that PAR1 is not only expressed in human vasculature, but also in multiple human brain regions. Our study reveals that PAR1 is most intensely expressed on astrocytes in white and gray matter of all brain regions examined, and is functional in cultured astrocytes and glioblastoma cells. PAR1 expression on astrocytes in humans has been suggested from numerous studies of PAR1 expression on rodent astrocytes in culture and in vivo (Boven et al., 2003; Sorensen et al., 2003; Wang et al., 2002a,b; Weinstein et al., 1995). In addition, PAR1 is moderately expressed in some neurons at a level detectable above the mild neuropil immunostaining. Several reports have suggested functional PAR1 expression in a subpopulation of rodent neurons in culture (Gingrich et al., 2000; Yang et al., 1997). PAR1 protein expression in adult human brain does not appear as widespread compared with in situ hybridization studies of PAR1 expression in prenatal and weanling rodents, in which PAR1 appears to be highly expressed in many cells (Niclou et al., 1998; Weinstein et al., 1995). PAR1 protein expression in adult rodents appears less widespread, which is similar to our observations in adult human brain (Striggow et al., 2001).

The presence of PAR1 in human brain supports the idea from experimental animal models and in vitro studies that the role for PAR1 in CNS disorders may be relevant for humans (Cheng et al., 2003; Junge et al., 2003). Furthermore, entry of serine proteases into brain tissue following cerebrovascular insult may activate PAR1-linked intracellular signaling cascades. It is well known that blood–brain barrier breakdown has harmful consequences in humans, and thus PAR1 activation may contribute to the associated pathological consequences. In addition, increased plasmin formation may occur during administration of recombinant tPA as a thrombolytic therapy for stroke, and plasmin may activate PAR1 in the brain. Therefore, tPA may have additional potentially harmful effects in brain tissue aside from its therapeutically beneficial effect of facilitating reperfusion. In summary, PAR1 may provide a new potential therapeutic target for preventing some of the pathological effects associated with entry of blood components into brain tissue.

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