



Protease-activated receptor-2 (PAR2) mediates VEGF production through the ERK1/2 pathway in human glioblastoma cell lines

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ARTICLE INFO

Article history:

Received 16 March 2012
Available online 4 April 2012

Keywords:

Glioblastoma
Protease-activated receptor
Mitogen-activated protein kinase
Vascular endothelial growth factor
Angiogenesis

ABSTRACT

Glioblastoma (GBM) is a highly aggressive cancer type characterized by intense neovascularization. Several lines of evidence indicate that blood clotting enzymes play an important role in the tumor micro-environment, mainly through the activation of protease-activated receptors (PAR). In particular, PAR1 and PAR2 isoforms may activate signal transduction pathways that promote a number of pro-tumoral responses. However, little is known concerning the role of PAR1/PAR2 in GBM progression. In this study, we investigated the expression and function of PAR1 and PAR2 in the human GBM cell lines A172 and U87-MG. We also evaluated the effect of agonist peptides for PAR1 (PAR1-AP) and PAR2 (PAR2-AP) on signaling pathways and the expression of vascular endothelial growth factor (VEGF). Immunoblotting assays showed that A172 and U87-MG constitutively express PAR1 and PAR2. Treatment of GBM cells with PAR1-AP or PAR2-AP enhanced Akt (protein kinase B) and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in a time-dependent manner. LY29042 and PD98059, inhibitors of the phosphatidylinositol 3-kinase (PI3 K) and mitogen-activated protein kinase (MAPK) pathways, decreased PAR-mediated activation of Akt and ERK1/2, respectively. In addition, we observed that PAR2, but not PAR1, activation increased VEGF secretion in U87-MG and A172 cells. Notably, only PD98059 reduced PAR2-mediated VEGF production by GBM cells. Our results suggest that PAR2 modulates VEGF production through the MAPK/ERK1/2 pathway, and not the PI3 K/Akt pathway, in human GBM cell lines. Therefore, the PAR2/MAPK signaling axis might be regarded as a relevant target for adjuvant treatment of GBM with a possible impact on tumor angiogenesis.

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1. Introduction

There is a close link between the activation of blood coagulation and cancer [1]. Blood clotting proteins are thought to play an important role in the tumor microenvironment by facilitating tumor growth, invasion and metastasis [2,3]. Certain proteases may affect cellular functions by activating G protein-coupled receptors known as protease-activated receptors (PARs) [4]. PARs comprise a family of receptors (PAR1, PAR2, PAR3 and PAR4) that are uniquely activated by the proteolytic cleavage of their extracellular portion. This cleavage unmasks a new N-terminus, which serves as a tethered ligand that binds to the second extracellular domain of the protein, resulting in a variety of cellular responses [5]. PAR1 can be cleaved and activated by thrombin and factor Xa (FXa) as well as by plasmin, activated protein C and MMP1. PAR2 can be activated by FVIIa, FXa, tryptase and trypsin, but not

thrombin. Experimentally, PARs can also be activated by synthetic peptides that mimic the neo-amino terminus of the cleaved receptor [5]. Such agonist peptides are useful for the study of PARs without using proteases that also have biological effects unrelated to PAR activation. Once activated, PARs can elicit a variety of cellular responses through multiple signaling cascade pathways [6].

PAR1 has been shown to be overexpressed in various human cancer types including breast [7], melanoma [2], colon [8], prostate [9], ovarian [10], esophagus [11], aggressive leukemias [12] and others. Moreover, studies using cultured cells have demonstrated a strong correlation between PAR1 expression and aggressive tumor behavior [2,7,13]. PAR1 has been associated with several pro-tumoral responses, including primary growth, invasion, metastasis and angiogenesis [2,7,13].

As seen with PAR1, PAR2 is overexpressed in a number of tumor types including pancreatic and breast cancers [14,15]. PAR2 activation has been correlated with invasion, primary tumor growth and angiogenesis [16,17]. Notably, a gene expression study of the human breast tumor cell line MDA-MB-231 showed that many of the PAR2-regulated genes are also regulated by a PAR1 agonist peptide. This suggests extensive redundancy between PAR2- and PAR1-mediated signaling in tumor cells [18]. Accordingly, PAR1

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and PAR2 activation have been reported to be coupled to redundant signaling pathways, including Ca^{2+} mobilization and activation of the mitogen-activated protein kinase (MAPK) pathway [16,19,20].

Malignant gliomas are the most common primary brain tumors in adults. Glioblastoma [GBM; World Health Organization (WHO) grade IV] is the highest grade astrocytoma and is characterized by rapid cell proliferation and a marked propensity to invade and damage surrounding tissues [21]. In addition, intense angiogenesis is a distinguishing pathological hallmark of GBM relative to lower-grade gliomas. More recently, tumoral intravascular thrombosis was reported as an additional distinction between GBM and lower grade astrocytomas [22]. In fact, the prothrombotic properties of GBM cell lines have been correlated with the expression of the clotting initiator protein, tissue factor (TF) and surface exposure of the procoagulant lipid phosphatidylserine (PS) [23,24]. As a result, the GBM microenvironment is prone to the generation of PAR-activating enzymes.

In this study we investigated the expression of PAR1 and PAR2 in the human GBM cell lines U87-MG and A172. In addition, we analyzed the effect of specific agonist peptides of PAR1 and PAR2 on signaling pathways and VEGF production. Our results show that PAR1 and PAR2 agonists promote significant cell activation through ERK1/2 and Akt phosphorylation. However, PAR2, but not PAR1, activation increases VEGF production in both cell lines mediated by MAPK pathways. Altogether, PAR2 may play a relevant role in GBM angiogenesis and could be a possible target for adjuvant therapy of this aggressive cancer type.

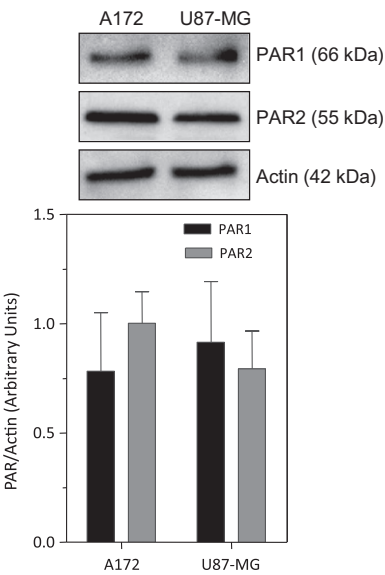


Fig. 1. A172 and U87-MG cells constitutively express PAR1 and PAR2. Tumor cells were serum-starved overnight. Then, the total levels of PAR1, PAR2 and actin were determined by immunoblotting as described in the materials and methods section. Proteins were quantified by densitometry using Scion Image. The data are shown as the mean \pm SD of three independent experiments.

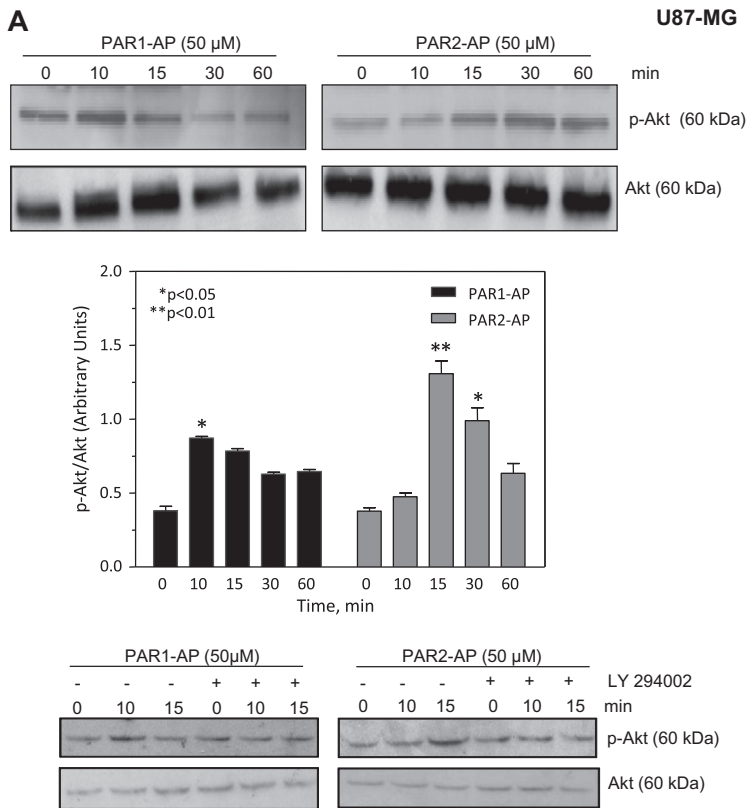


Fig. 2. PAR1 and PAR2 induce Akt activation in GBM cell lines. (A) U87-MG and (B) A172 cells were serum-starved for 30 min and then treated with PAR1-AP (50 μM) or PAR2-AP (50 μM) for different times. In some experiments, the cell lines were serum-starved for 30 min in the presence of LY294002 (25 μM) and then treated with PAR1-AP (50 μM) or PAR2-AP (50 μM) for 0, 10 or 15 min at 37 $^{\circ}\text{C}$ in a 5% CO_2 atmosphere. The levels of total and phosphorylated Akt in the cell lysates were determined by immunoblotting as described in the materials and methods section and quantified by densitometry using Scion Image. The data are shown as the mean \pm SD of three independent experiments. ** $P < 0.01$, * $P < 0.05$ compared with time zero in both treatments.

2. Material and methods

2.1. Reagents

ATAP2 anti-PAR1, SAM-11 anti-PAR2, anti-actin, anti-phospho-ERK 1/2, anti-phospho-Akt 1/2/3, polyclonal anti-ERK 1/2 and polyclonal anti-Akt-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated with biotin and peroxidase-conjugated streptavidin were obtained from Zymed (Invitrogen). PAR1 agonist peptide (PAR1-AP, TFLLR-NH2) and PAR2 agonist peptide (PAR2-AP, SLIGKL-NH2) were synthesized by Biosynthesis Inc. (Lewisville, TX). 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) was obtained from Sigma (St. Louis, MO). 2-(2-diamino)-3-methoxyphenyl-4H-1-benzopyran-4-one (PD98059) was obtained from Calbiochem (San Diego, CA).

2.2. Cell culture

The human glioblastoma cells lines A172 and U87-MG were maintained in Dulbeccó's Modified Eagle Medium (DMEM-F12, GibcoBRL) supplemented with 10% FBS (Cultilab, BR), 60 mg/L penicillin, 100 mg/L streptomycin and 1.2 g/L sodium bicarbonate in culture flasks, in a 5% CO₂-air mixture at 37 °C. Subconfluent cultures were washed twice with PBS, and cells were detached with Hank's solution containing 10 mM HEPES and 0.2 mM EDTA. Cells were seeded at 2×10^5 cells/well in 12-well plates for ELISA or at 5×10^5 cells/well in 6-well plates for western blotting assays.

2.3. Western blot analysis of signal transduction pathways induced by stimulation with the agonist peptides PAR1-AP or PAR2-AP

A172 and U87-MG cells were seeded at 5×10^5 cells/well in 6-well plates. For the quantitative analysis of PAR1 and PAR2, cells

were washed with phosphate-buffered saline and lysed in cold buffer containing a phosphatase inhibitor cocktail. The relative levels of PAR1 and PAR2 were expressed as a ratio to actin. The levels of the downstream products of two major signal transduction pathways in PAR activation were examined. These were phospho-Akt in the PI3 K/Akt pathway and phospho-ERK 1/2 in the MAPK/ERK pathway. Briefly, cells were kept in DMEM-F12 in the absence of FBS for 30 min and then treated with PAR1-AP or PAR2-AP (50 μ M) for 10, 15, 30 or 60 min. In some cases, cells were incubated with LY294002 or PD98059 for 1 h prior to the addition of PAR1-AP or PAR2-AP. The relative levels of phospho-Akt and -ERK1/2 were expressed as a ratio to Akt and ERK1/2, respectively. After 10 and 15 min incubation periods, respectively, the cells were harvested for western blot analysis. Cell lysates (20 μ l) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10–12%). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) and further blocked with Tris-buffered saline (TBS) containing 5% BSA and 0.1% Tween 20 for 1 h at room temperature; the membranes were then probed with primary antibodies overnight at 4 °C. The membranes were washed three times with TBS/Tween before the addition of secondary antibody for 1 h at room temperature. The membranes were further washed and probed with peroxidase-conjugated streptavidin for 1 h at room temperature. Immunodetection was carried out by a chemiluminescent method using the Western Lightning ECL kit (Amersham Pharmacia Biotech). The blots were quantified by Scion Image software.

2.4. Immunoassay for the quantitation of VEGF

Cells were serum-starved for 30 min prior to stimulation with PAR1-AP or PAR2-AP for 24 h at 37 °C. VEGF protein secretion into the cell supernatants was measured using a human VEGF ELISA kit from Pepro Tech Inc. (Rocky Hill, NJ) according to the manufacturer's instructions. In some cases, cells were incubated with

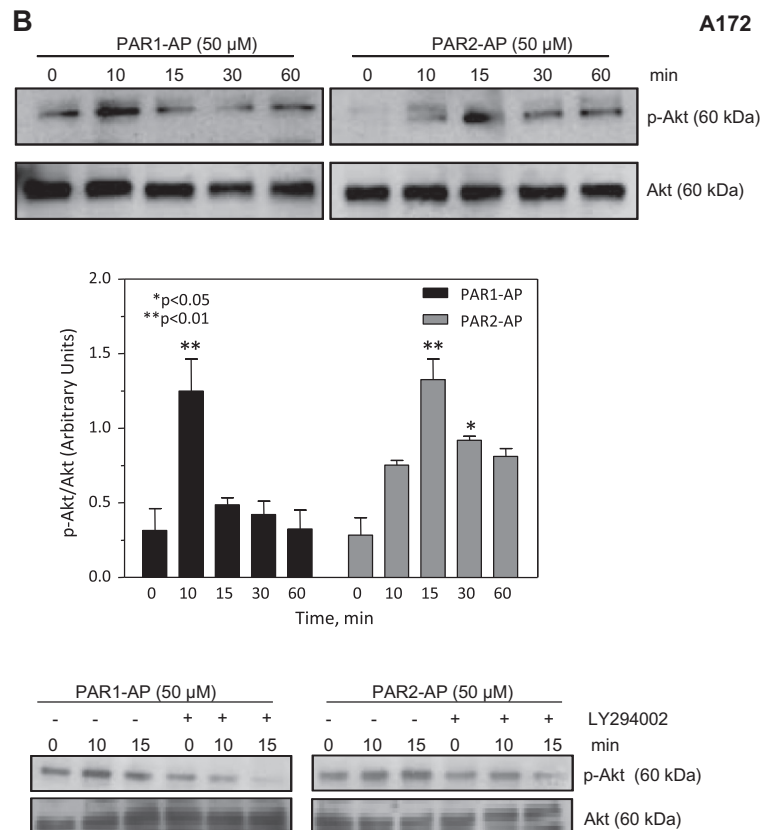


Fig. 2 (continued)

LY294002 (25 μ M) or PD98059 (20 μ M) for 1 h prior to stimulation with agonist peptides.

2.5. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). Two-way analysis of variance (ANOVA) complemented by the Bonferroni post hoc test was used for pairwise comparisons between test groups. Differences were considered significant when $P < 0.05$.

3. Results

3.1. A172 and U87-MG constitutively express PAR1 and PAR2

Several studies have demonstrated that PAR1 and PAR2 are constitutively expressed by a variety of tumor cell types. Thus, we investigated the expression of PAR1 and PAR2 in the human GBM cell lines A172 and U87-MG. Western blotting assays (Fig. 1) show that both cell lines constitutively express PAR1 and PAR2. In addition, the expression levels of these receptors were similar in both cell lines.

3.2. PAR1 and PAR2 induce Akt activation in GBM cells

The PI3 K/Akt signaling pathway has been suggested to be a major determinant for poor prognosis in high-grade gliomas [25]. Therefore, we evaluated the effect of PAR activation on Akt signaling in GBM cell lines. Fig. 2 shows that PAR1-AP and PAR2-AP both induced an increase in Akt phosphorylation in the U87-MG (Fig. 2A) and A172 (Fig. 2B) cell lines, demonstrating that both PAR1 and PAR2 mediate the activation of the PI3 K signaling pathway. In order to confirm this hypothesis, we used LY294002, a known PI3 K inhibitor. Fig. 2 shows that LY294002 efficiently inhibited PAR1-AP- and PAR2-AP-induced Akt phosphorylation in both cell lines.

3.3. PAR1 and PAR2 mediate ERK1/2 activation in GBM cell lines

PAR1 and PAR2 have been implicated in a number of tumor cell responses to a variety of extracellular proteases in the tumor micro-environment. We further investigated whether PAR1 and PAR2 activate ERK 1/2 in GBM cell lines by exposing U87-MG and A172 cells to PAR1 or PAR2 agonist peptides (PAR1-AP and PAR2-AP, respectively). Both agonists triggered ERK1/2 phosphorylation in the

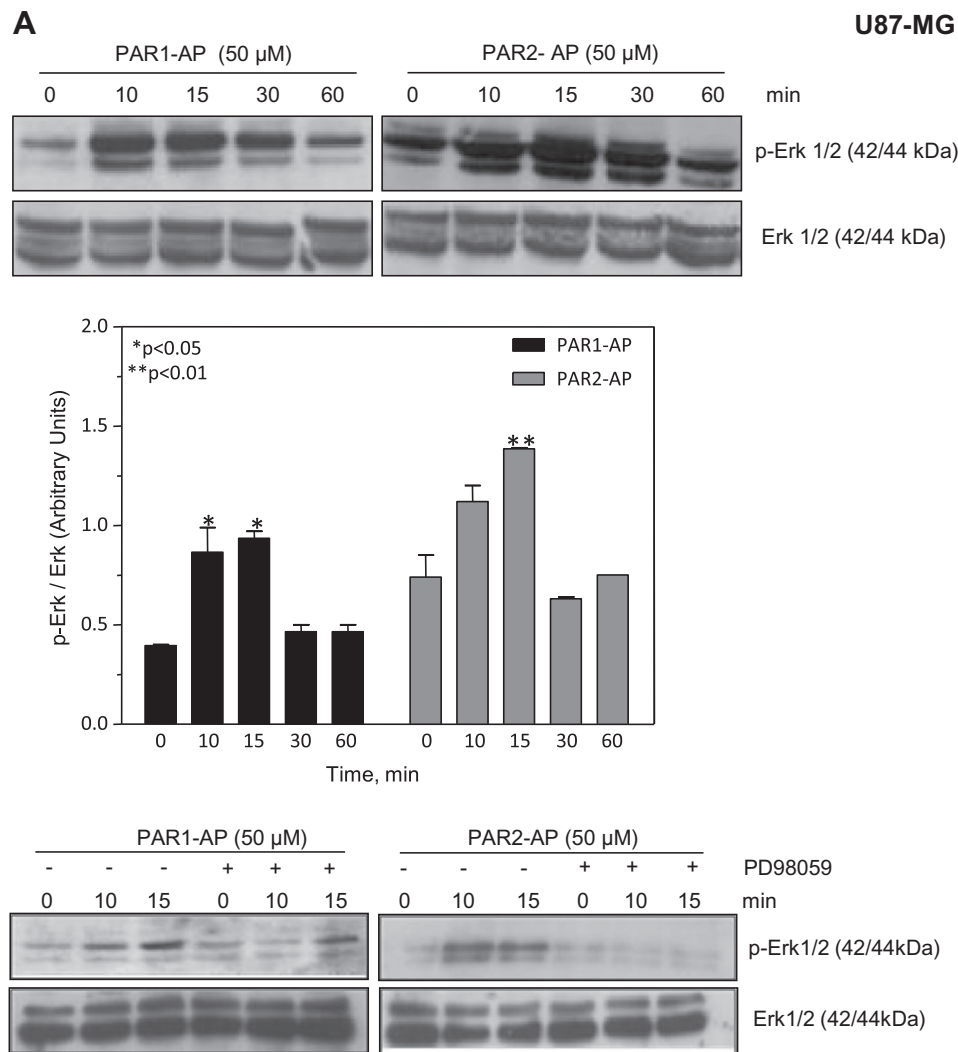


Fig. 3. PAR1 and PAR2 mediate ERK 1/2 activation in GBM cell lines. (A) U87-MG and (B) A172 cells were serum-starved for 30 min and then treated with PAR1-AP (50 μ M) or PAR2-AP (50 μ M) for different lengths of time. In another set of experiments, cell lines were serum-starved for 30 min in the presence of PD98059 (20 μ M) and then treated with PAR1-AP (50 μ M) or PAR2-AP (50 μ M) for 0, 10 or 15 min at 37 $^{\circ}$ C in a 5% CO₂ atmosphere. The levels of total and phosphorylated ERK 1/2 in the cell lysates were determined by immunoblotting as described in the materials and methods section and quantified by densitometry using Scion Image. The data are shown as the mean \pm SD of three independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared with time zero in both treatments.

U87-MG (Fig. 3A) and A172 (Fig. 3B) cell lines in a time-dependent fashion. To confirm the inhibitor effects (PD98059) on ERK1/2 phosphorylation induced by PAR1 and PAR2 activation, the cells were treated with the inhibitor. Fig. 3 shows that, as expected, both PAR1-AP- and PAR2-AP-induced ERK 1/2 phosphorylation was inhibited upon treatment with PD98059, a known MAPK pathway inhibitor, in both cell lines. Remarkably, the effect of PD98059 on ERK 1/2 phosphorylation was greater in cells treated with PAR2-AP. These results demonstrate that both PAR1 and PAR2 activation induce ERK 1/2 activation, thus promoting signaling through MAPK pathways.

3.4. PAR2 activation induces VEGF expression in GBM cell lines

GBM is one of the most highly vascularized malignant tumors and there is strong evidence that VEGF plays a key role in this process [26]. Furthermore, PAR1 and PAR2 have been implicated in VEGF production by tumor cells [27]. To determine the effect of PAR1 and PAR2 activation on VEGF production by GBM cell lines, U87-MG and A172 cells were treated with agonist peptides, and VEGF was quantified in the cell-conditioned medium. As demonstrated in Fig. 4, PAR2, but not PAR1, activation increased VEGF production in U87-MG (1.8-fold increase) and A172 (0.7-fold increase) cells. We also evaluated whether the PI3 K pathway was

involved in PAR2-induced VEGF production by GBM cells. VEGF production by A172 cells was not affected by treatment with LY294002 (Fig. 4B). However, treatment with LY294002 produced a significant increase in VEGF production in U87-MG cells stimulated by either PAR1-AP or PAR2-AP (Fig. 4A). We also evaluated whether the MAPK pathway was involved in PAR2-induced VEGF production by GBM cells. PAR2-induced VEGF protein secretion was significantly decreased by the pretreatment of U87-MG (Fig. 4A) and A172 (Fig. 4B) cells with PD98059. Taken together, these data strongly suggest that the ERK1/2 signaling pathway is involved in PAR2-induced VEGF production.

4. Discussion

Protease-activated receptors (PARs) are G protein-coupled receptors that signal in response to a variety of extracellular proteases. These receptors play crucial roles in hemostasis and thrombosis, as well as inflammation and vascular development [4]. Several lines of evidence have implicated PARs in tumor progression. In particular, PAR1 and PAR2 are overexpressed in several cancer types and have been associated with tumor growth, invasion, metastasis and other pro-tumoral responses. In the present study, we evaluated the expression and function of PAR1 and PAR2 in the human GBM cell lines U87-MG and A172. Our data

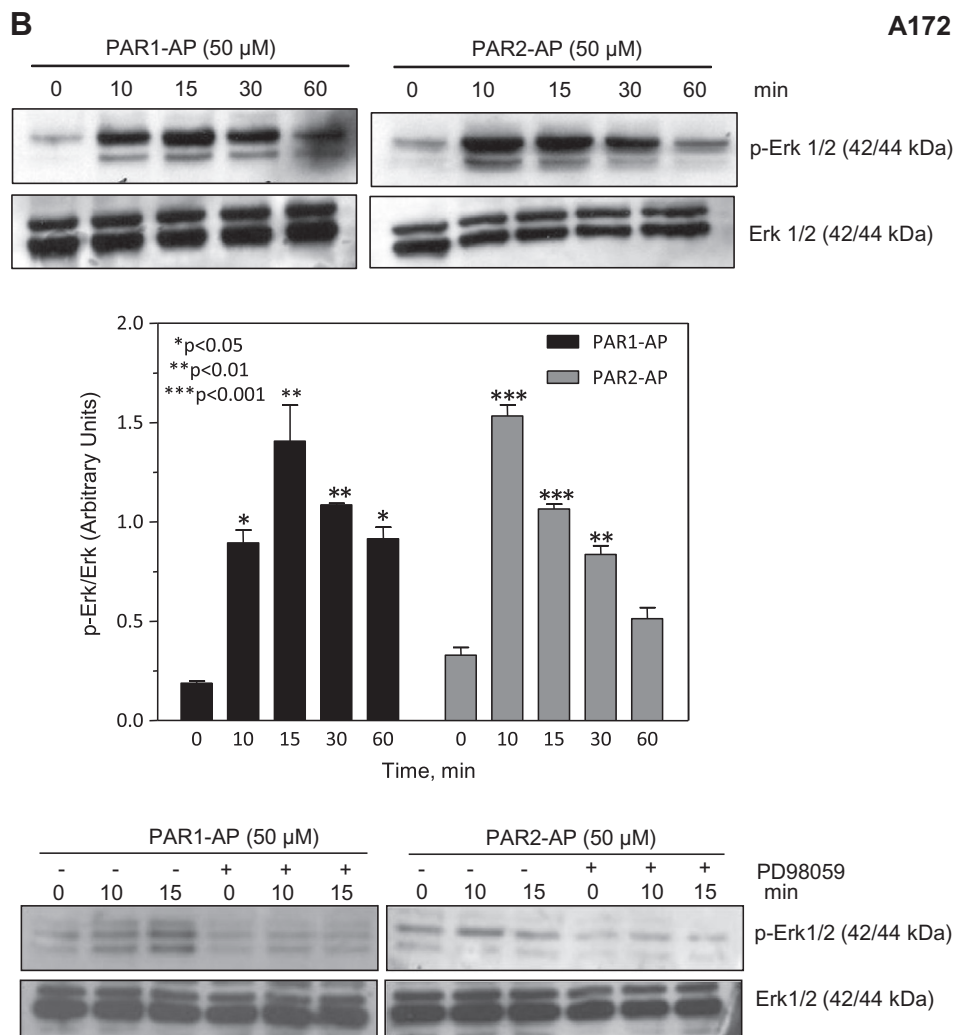


Fig. 3 (continued)

show that both cell lines constitutively express these receptors. Activation of either PAR1 or PAR2 activated signaling pathways involving Akt and ERK 1/2. Notably, PAR2, but not PAR1, increased VEGF production through the MAPK/ERK 1/2 pathways.

Intense angiogenesis is a distinguishing pathological hallmark of GBM relative to lower-grade gliomas. GBM is among the most highly vascularized malignant tumors and there is strong evidence that VEGF plays a key role in this process [26]. Studies using genetically modified mice have consistently demonstrated that PAR2 deficiency delays tumor development and the angiogenic process in a mouse breast cancer model [28]. Furthermore, the exogenous TF/FVIIa complex inhibitor, Ixolaris, reduces *in vivo* tumor angiogenesis in a human GBM model [24], possibly through the suppression of PAR2 signaling. Those observations support a proangiogenic role for PAR2 and coincide with our observation that PAR2 activation increases VEGF production by GBM cells *in vitro*.

Recent studies have shown that PAR2 mediates proliferation, migration and invasion of malignant glioma cells through the ERK signaling pathway [29]. Notably, PAR2 activation in glioma cells seems to be driven essentially by FVIIa/TF complex-mediated cleavage because silencing of TF interrupts the activation of the ERK pathway in tumor cells. The involvement of the ERK 1/2 pathway in VEGF production has been demonstrated in fibroblasts, consistent with a hypoxia-independent route for VEGF production [30]. Furthermore, activation of ERK 1/2 has been directly associated with PAR2 signaling that increases VEGF production by human breast cancer cells [27]. Our data show that PAR2 activation drives VEGF production through the MAPK/ERK 1/2 pathway. This conclusion was demonstrated by the inhibition of tumor cell VEGF production by treatment with PD98059 prior to stimulation with PAR2 agonist peptide.

The occurrence of intra-tumoral thrombosis in GBM has been shown to be a distinct feature of GBM in relation to lower-grade

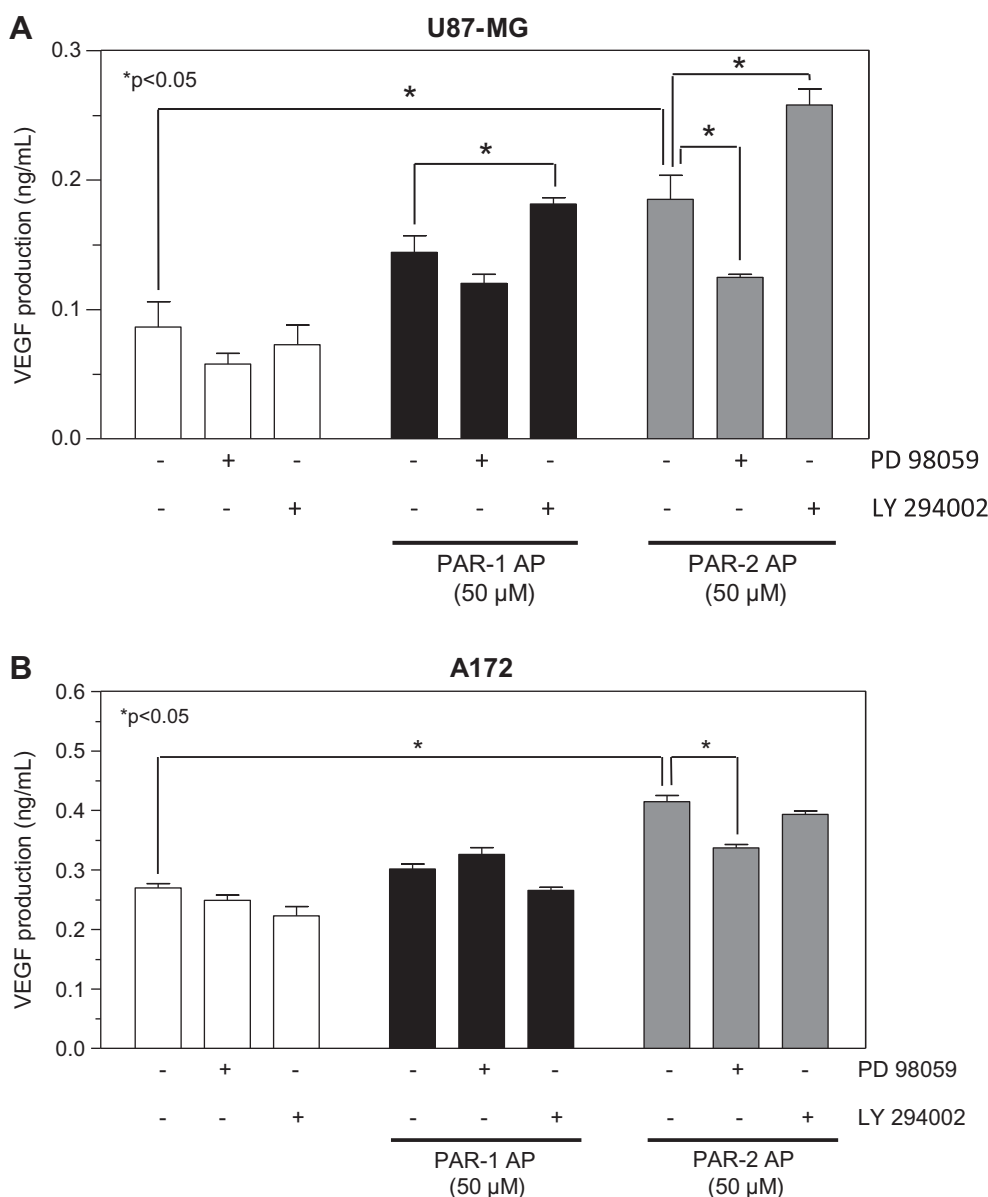


Fig. 4. PAR2 activation induces VEGF expression in GBM cell lines. (A) U87-MG and (B) A172 cell lines were treated with PAR1-AP (50 μ M) or PAR2-AP (50 μ M) for 24 h, in serum-free condition, at 37 $^{\circ}$ C in a 5% CO₂ atmosphere. In some cases, cells were incubated with LY294002 (25 μ M) or PD98059 (20 μ M) for 6 h prior to treatment with agonist peptides. VEGF secretion into the cell supernatants was quantified as described in the materials and methods section. The data are shown as the mean \pm SD of three independent experiments. * P < 0.05 compared with the treatment group.

gliomas [22]. It has been proposed that intra-tumoral thrombosis contributes to the establishment of hypoxic areas that in turn stimulate the production of pro-tumoral factors such as VEGF and IL-8 [31]. Interestingly, hypoxia upregulates TF [32] and PAR1 [33] expression in tumor cells, thus contributing to the remodeling of the tumor microenvironment. It remains to be determined whether hypoxia can modulate PAR2 expression in GBM, which could possibly synergize to promote the development of tumor vessels.

The aberrant activation of the PI3K/Akt signaling pathway has been described in high-grade gliomas and has been correlated with poor prognosis in GBM [25,34]. A number of studies have correlated Akt activation with the treatment of tumor cells with coagulation proteases [35,36]. In addition, suppression of PAR1 signaling suppresses Akt signaling in breast cancer cells [37]. In this study, we observed that both the PAR1 and PAR2 activating peptides induce Akt activation in GBM cell lines. It remains to be determined whether PAR1 and/or PAR2 modulate cell survival in GBM models.

Considering that the GBM microenvironment is prone to generating PAR-activating enzymes, it is possible to speculate that a blockade of coagulation activation may affect GBM progression [38]. We recently demonstrated that pharmacological inhibition of the TF/FVIIa complex decreases primary tumor growth in a mouse xenograft model using U87-MG cells [24]. Moreover, Hua and colleagues showed that the specific thrombin inhibitor argatroban can reduce the *in vivo* growth of rat glioblastoma, although only a modest survival improvement was shown [39]. Taken together, our results suggest that PAR1 and PAR2 may contribute to GBM progression. Furthermore, the PAR2/MAPK signaling axis might be regarded as a relevant target for adjuvant treatment of GBM with a possible impact on tumor angiogenesis.

Financial support

This research was supported by the Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro Carlos Chagas Filho (FAPERJ), and Fundação do Câncer.

Acknowledgments

We thank Dr. I.M.B. Francischetti (National Institutes of Health, USA) for careful revision of the manuscript and Thaís de Medeiros Gameiro for her technical assistance.

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