Sphingosine-1-Phosphate and Interleukin-1 Independently Regulate Plasminogen Activator Inhibitor-1 and Urokinase-Type Plasminogen Activator Receptor Expression in Glioblastoma Cells: Implications for Invasiveness

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Abstract
Glioblastoma multiforme is an invasive primary brain tumor, which evades the current standard treatments. The invasion of glioblastoma cells into healthy brain tissue partly depends on the proteolytic and nonproteolytic activities of the plasminogen activator system proteins, including the urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI-1), and a receptor for uPA (uPAR). Here we show that sphingosine-1-phosphate (S1P) and the inflammatory mediator interleukin-1 (IL-1) increase the mRNA and protein expression of PAI-1 and uPAR and enhance the invasion of U373 glioblastoma cells. Although IL-1 enhanced the expression of sphingosine kinase 1 (SphK1), the enzyme that produces S1P, down-regulation of SphK1 had no effect on the IL-1–induced uPAR or PAI-1 mRNA expression, suggesting that these actions of IL-1 are independent of S1P production. Indeed, the S1P-induced mRNA expression of uPAR and PAI-1 was blocked by the S1P2 receptor antagonist JTE013 and by the down-regulation of S1P2 using siRNA. Accordingly, the inhibition of mitogen-activated protein kinase/extracellular signal–regulated kinase 1/2 and Rhokinase, two downstream signaling cascades activated by S1P2, blocked the activation of PAI-1 and uPAR mRNA expression by S1P. More importantly, the attachment of glioblastoma cells was inhibited by the addition of exogenous PAI-1 or siRNA to uPAR, whereas the invasion of glioblastoma cells induced by S1P or IL-1 correlated with their ability to enhance the expression of PAI-1 and uPAR. Collectively, these results indicate that S1P and IL-1 activate distinct pathways leading to the mRNA and protein expression of PAI-1 and uPAR, which are important for glioblastoma invasiveness. (Mol Cancer Res 2008;6(9):1469–77)

Introduction
Glioblastoma multiforme is one of the most common and most malignant tumors of the central nervous system (1, 2). Due to the invasive phenotype and diffuse penetration of glioblastoma cells into normal regions of the brain, standard treatments such as surgery and radiotherapy are ineffective (3). It is for these reasons that patients diagnosed with glioblastoma multiforme survive an average of 10 to 12 months (4). The invasion of glioblastoma cells requires the degradation of the extracellular matrix, which depends on the activation/inhibition of proteases and their inhibitors, respectively. These processes include two main proteolytic systems: the plasminogen activator system, which controls the activation of the protease plasmin from inactive plasminogen, and the matrix metalloproteinases and their inhibitors (5-8).

In the brain, microglia produce inactive plasminogen, whereas astrocytes and glioma cells produce and secrete the components of the plasminogen activator system. The plasminogen activator system includes the plasminogen activators [urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator], their inhibitors [plasminogen activator inhibitor (PAI)-1, PAI-2, PAI-3, and protease nexin 1], and a receptor for uPA (uPAR; ref. 5). The binding of uPA to uPAR leads to the localization of proteolytic activity to the cell surface, the enhancement of plasmin production, and the activation of several signaling pathways via uPAR (9, 10). Significantly, the expression of both uPA and uPAR has been correlated with the invasiveness and migration of several cancer cell lines (11). Moreover, the knockdown of uPAR expression in gliomas, using RNA interference, leads to a significant decrease in cell invasion in both Matrigel and spheroid invasion assays (12). Furthermore, transfecting glioblastoma cells with antisense uPA disrupted actin cytoskeleton formation, reduced the amount of cell-bound uPA, and decreased cell migration (13). Surprisingly, high levels of PAI-1, which inhibit uPA, have been associated with highly invasive glioblastomas (14). Similarly, breast cancer patients with high levels of PAI-1 have a poor prognosis for survival (15). Together, these observations support the recent findings.
that PAI-1 binds to the uPA/uPAR/integrin complex, which promotes the internalization of this complex and subsequent cell detachment and metastasis (16, 17).

The expression of the components of the plasminogen activator system is regulated by growth factors and cytokines, such as epidermal growth factor (EGF) and interleukin (IL)-1, respectively (18, 19). Importantly, increased glioblastoma invasiveness and decreased patient survival correlate with PAI-1 and EGF receptor overexpression (14, 18). Moreover, inhibition of EGF receptor tyrosine kinase suppresses the invasion of glioblastoma cells and decreases uPAR protein levels (20). Recently, we have described a novel signaling pathway of EGF-mediated up-regulation of PAI-1 expression in glioblastoma cells, which requires the sequential activation of c-Src, protein kinase C, and sphingosine kinase 1 (SphK1; ref. 21). SphK1 produces the potent lipid mediator sphingosine-1-phosphate (S1P) by phosphorylating sphingosine, and its expression correlates with the poor survival of patients with glioblastoma multiforme (22). S1P has been shown to be mitogenic for several glioma cell lines and to enhance their motility and invasiveness (23). S1P acts through five G-protein–coupled receptors (S1P1–S1P5) to activate multiple signaling pathways; however, it may also have intracellular actions through mechanisms that are still not understood.

IL-1 is a proinflammatory cytokine released by inflammatory cells in the brain due to inflammation caused by an injury or a growing tumor. In addition, glioblastomas have recently been shown to secrete substantial amounts of IL-1 (24), which incites the secretion of other cytokines, such as IL-6 and IL-8, as well as promotes glioblastoma proliferation (19). IL-1 has also been shown to stimulate the expression of several matrix metalloproteinases, tissue-type plasminogen activator, and uPA (19), yet nothing is known of its role in invasion of glioblastomas. Because IL-1 has been shown to activate SphK1 in other cell types (25), we investigated the effects of IL-1 and S1P on the regulation of the components of the plasminogen activator system and invasion in glioblastoma cells. We show that S1P requires S1P2 to activate PAI-1 and uPAR mRNA expression, and although IL-1 markedly up-regulated the mRNA expression of SphK1, it acts independently of S1P. Moreover, S1P and IL-1 cooperatively increase the invasion of U373 glioblastoma cells as well as primary nonestablished GBM6 glioblastoma cells. Our data highlight the role of S1P and IL-1 in the regulation of PAI-1 and uPAR mRNA and protein expression, which are critical for glioblastoma invasiveness.

Results

S1P and IL-1 Regulate the Expression of the Plasminogen Activator System in Glioblastoma Cells

Previous studies have suggested that S1P might regulate invasion of glioblastoma cells (22, 23), yet the mechanism involved remained elusive. To this end, U373 glioblastoma cells were stimulated with both S1P and the neuroinflammatory cytokine IL-1 because glioblastomas have recently been shown to secrete substantial amounts of IL-1 (24), which also activates PAI-1 (26). Both S1P and IL-1 efficiently stimulated the mRNA expression of PAI-1 (both transcripts generated by the alternative cleavage and polyadenylation; ref. 27) and uPAR, whereas the mRNA expression of uPA was unaffected (Fig. 1A and B). Accordingly, protein levels of PAI-1 secreted into the

**FIGURE 1.** S1P and IL-1 up-regulate the expression of uPAR and PAI-1 in glioblastoma cells. U373 cells were treated with 10 ng/mL IL-1 or 5 μmol/L S1P for 18 h (A–C), stimulated with 5 μmol/L S1P for the indicated times (D, left), or stimulated with the indicated amounts of S1P or 10 ng/mL IL-1 (D, right). A, B, and D, RNA was isolated and subjected to analysis either by Northern blotting or quantitative PCR using TaqMan technology as described in Materials and Methods. A and D, Bottom, 28S RNA stained with ethidium bromide on the membrane as a loading control. The quantitative PCR data were normalized to GAPDH mRNA and expressed as a ratio to mRNA levels in untreated cells. C, Media were collected and cell lysates prepared and analyzed by Western blotting as described in Materials and Methods. Tubulin was used as a loading control.
media and uPAR present in the cells were up-regulated by IL-1 and S1P (Fig. 1C). The effect of exogenous S1P on PAI-1 and uPAR mRNA expression was time and dose dependent, with activation observed as early as 1 hour after stimulation (Fig. 1D). The strongest activation of PAI-1 and uPAR mRNA expression was observed at 5 μmol/L S1P, but the effect was significant at a concentration as low as 10 nmol/L. These results indicate that S1P affects the expression of the plasminogen activator system components in glioblastoma cells, and its effects are further enhanced by IL-1.

S1P Activates PAI-1 and uPAR mRNA Expression via Multiple Signaling Pathways

The additive activation of both PAI-1 and uPAR mRNA expression by the combination of S1P and IL-1 (Fig. 1B) suggests that they may regulate the mRNA expression of these genes by distinct pathways. Both S1P and IL-1 have been shown to activate various signaling pathways in many cell types, leading to the reprogramming of gene expression profiles (24, 25). In glioblastoma cells, both S1P and IL-1 induced rapid phosphorylation of extracellular signal–regulated kinase (ERK)-1/2; however, the phosphorylation of c-jun NH2-terminal kinase and the efficient degradation of IkBα, which is important for the activation of nuclear factor κB, were restricted to IL-1 (Fig. 2A). We used several pharmacologic inhibitors to identify the signaling pathway(s) regulating PAI-1 and uPAR mRNA expression in response to S1P in U373 cells. The inhibition of phosphatidylinositol 3-kinase and p38 (using LY294002 and SB202190, respectively) diminished the intrinsic mRNA expression of PAI-1 but did not affect the relative fold activation by S1P (Fig. 2B). In contrast, the inhibition of mitogen-activated protein kinase/ERK kinase 1/2 and Rho-kinase (using U0126 and Y27632, respectively) blocked PAI-1 and uPAR up-regulation by S1P. In addition, the S1P-mediated uPAR activation was also inhibited by LY294002. These results suggest that diverse signaling molecules, including mitogen-activated protein kinase/ERK kinase/ERK and Rho-kinase, regulate the mRNA expression of PAI-1 and uPAR in response to S1P. Because both mitogen-activated protein kinase/ERK kinase/ERK and Rho-kinase can activate activator protein 1 (AP-1) and both PAI-1 and uPAR expression are regulated by AP-1 in response to various stimuli (28-30), we tested whether this potent transcription factor may be implicated in the regulation of PAI-1 and uPAR mRNA expression in response to S1P and IL-1. We used U373-TAM67 cells inducibly overexpressing dominant-negative c-jun (TAM67), which quenches the expression of AP-1–dependent genes (31). The intrinsic mRNA expression of PAI-1, its fold activation by both S1P and IL-1, and the marked increase by their combination were diminished, thus indicating that AP-1 is involved in the regulation of PAI-1 mRNA expression (Fig. 2D). In contrast, the mRNA expression

FIGURE 2. Signaling pathways involved in S1P-stimulated expression of PAI-1 and uPAR. A. U373 cells were stimulated with 10 ng/mL IL-1 or 5 μmol/L S1P for the indicated times. The cell lysates were prepared and analyzed by Western blotting with anti–phospho-ERK (p-ERK), anti–phospho-c-jun NH2-terminal kinase (p-JNK), anti-IκBα, and anti-ERK. Blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer. B and C. U373 cells were pretreated with 1 μmol/L U0126, 10 μmol/L LY294002, 1 μmol/L SP600125, 10 μmol/L SB202190, 5 μmol/L Go6983, or 5 μmol/L Y27632 for 1 h and subsequently stimulated with 5 μmol/L S1P for 18 h. RNA was isolated and the expression of PAI-1 (B) and uPAR (C) was analyzed by quantitative PCR. The data were normalized to GAPDH mRNA and expressed as a ratio to mRNA levels in untreated cells. Asterisks indicate statistically significant inhibition. D. U373-TAM67 cells were preincubated with 1 μg/mL tetracycline for 24 h and then stimulated with 10 ng/mL IL-1 or 5 μmol/L S1P for 18 h; RNA was isolated and analyzed as in B and C.
of uPAR was not affected by c-jun(TAM67) overexpression (Fig. 2D). These results argue that whereas genes encoding PAI-1 and uPAR are regulated by signals initiated by S1P and IL-1, the precise mechanisms of their regulation are distinct.

**IL-1–Activated PAI-1 and uPAR mRNA Expression Is SphK1 Independent**

Because IL-1 rapidly activates (32) and up-regulates the expression of SphK1 in several other cell types (33), we examined the effect of IL-1 on SphK1 mRNA expression in glioblastoma cells. The mRNA expression of SphK1 was significantly up-regulated by IL-1 in U373 cells (Fig. 3A). Thus, the IL-1–induced PAI-1 and uPAR mRNA expression could potentially be due to elevated levels of S1P produced in response to IL-1. Because in many other cell types, S1P produced by agonist-stimulated SphK1 can activate cell surface S1P receptors in an autocrine/paracrine manner (34), this possibility was examined by down-regulating the expression of SphK1 with specific siRNA in U373 cells. However, IL-1–induced mRNA expression of PAI-1 and uPAR was not affected by the down-regulation of SphK1 (Fig. 3B and C), thus indicating that this activation is SphK1 independent.

**S1P Activates PAI-1 and uPAR mRNA Expression via the S1P2 Receptor**

Given that most of the effects of S1P are mediated by binding to specific cell surface receptors (23, 35-37), of which S1P1, S1P2, and S1P3 are expressed by U373 cells (23), it was of interest to identify which of the S1P receptors mediates the activation of PAI-1 and uPAR mRNA expression. To this end, we used both pharmacologic and molecular approaches. First, antagonists VPC23019 and JTE-013 were used to block the S1P1 and S1P2 receptors, respectively. Inhibition of S1P2 abrogated the activation of PAI-1 and uPAR mRNA expression by S1P, whereas the inhibition of S1P1 was ineffective (Fig. 4A and B). Moreover, inhibition of either S1P1 or S1P2 did not have any effect on the IL-1–activated mRNA expression of PAI-1 and uPAR (Fig. 4C and D), thus suggesting that the activation by IL-1 does not require these two receptors. Second, the mRNA expression of S1P2 was down-regulated by >80% using S1P2 specific siRNA (Fig. 5A). In agreement with the pharmacologic inhibition of S1P2, the down-regulation of S1P2 expression abolished the activation of PAI-1 and uPAR mRNA expression by S1P, without affecting the response to IL-1 (Fig. 5B and C). These results indicate that S1P specifically activates PAI-1 and uPAR mRNA expression via the S1P2 receptor.

**PAI-1 and uPAR Are Critical for the IL-1– and S1P-Induced Invasion of Glioblastoma Cells**

Subsequently, we examined the roles of PAI-1, uPAR, S1P, and IL-1 in the invasion of glioblastoma cells. The invasion of U373 cells into Matrigel was significantly increased in response to both S1P and IL-1 (Fig. 6B). More importantly, the S1P- and IL-1–induced invasion was abrogated in U373 cells when the expression of either PAI-1 or uPAR was down-regulated (Fig. 6A and B). Thus, both IL-1 and S1P increase the invasion of glioblastoma cells via the activation of PAI-1 and uPAR expression. Because PAI-1 binds to and induces the internalization of the PAI-1/uPA/uPAR/integrin complex, which causes cell detachment in breast cancer cells (16, 17), we evaluated whether PAI-1 can interfere with the attachment of U373 cells. Indeed, the down-regulation of uPAR expression or the addition of 1 μmol/L PAI-1 significantly inhibited the attachment of U373 cells to vitronectin-coated dishes (Fig. 6C). These results
suggest that uPAR is important for the attachment of glioblastoma cells, whereas PAI-1 may regulate detachment.

**PAI-1 and uPAR mRNA Expression Is Regulated by S1P and IL-1 in Primary Nonestablished Glioblastomas**

Glioblastoma cell lines, commonly used in *in vitro* studies, are not invasive in *in vivo* animal models (38, 39). Therefore, we examined the effects of S1P and IL-1 on primary nonestablished glioblastoma cells (GBM6 and GBM12), which were shown to produce invasive, diffuse tumors in severe combined immunodeficient mice (38). RNA expression of PAI-1 and uPAR was increased by S1P and IL-1 in GBM6 cells (Fig. 7A and B). However, whereas S1P increased PAI-1 in GBM12 cells, IL-1 increased uPAR without affecting PAI-1 (Fig. 7A and B). In contrast to U373 cells, the expression of uPA mRNA was also up-regulated by S1P and IL-1 in both of these nonestablished glioblastoma cells (Fig. 7C). Moreover, the invasion of GBM6 cells into Matrigel was significantly increased in response to both S1P and IL-1 (Fig. 7D), whereas GBM12 cells were not invasive (Fig. 7D), which may be due to the extremely low levels of uPAR in these cells. Thus, both S1P and IL-1 play important roles in regulating the expression of the plasminogen activator system components and the invasion of primary glioblastoma cells.

**Discussion**

The invasive phenotype of glioblastoma cells is the major obstacle in the successful treatment of patients diagnosed with glioblastoma multiforme. Whereas the precise mechanisms leading to the diffuse penetration of individual cells into normal regions of the brain are not understood, it is known that their invasion is regulated by growth factors, cytokines, and other signaling molecules, including bioactive lipids. Among cytokines and growth factors, IL-1 and EGF have attracted most of the attention. EGF is produced in the brain and readily crosses the blood-brain barrier (40), whereas its receptor is frequently amplified (41, 42), overexpressed (41, 42), or mutated (41, 43) in glioblastomas. Furthermore, the amplification and overexpression of EGF receptor is associated with high-grade progression (44), and patients expressing high levels of both EGF receptor and PAI-1 have a shorter prognosis for survival (14). Similarly to EGF, IL-1 is readily found in the brain and can be produced by activated microglia and astrocytes surrounding the necrotic center of a glioblastoma tumor. In addition, significant amounts of IL-1 are produced by glioblastomas (24). IL-1, as a potent neuroinflammatory cytokine, activates the expression of many genes in astrocytes and glioblastoma cells, including the genes encoding PAI-1 and SphK1. Similarly, we have recently shown that EGF stimulates PAI-1 expression in glioblastomas via the activation of SphK1 (21), the enzyme that produces S1P.

In this article, we show that exogenous S1P activates PAI-1 and uPAR expression in both U373 cells (Fig. 1) and primary invasive glioblastoma cells (Fig. 7). In addition, S1P enhances uPA mRNA expression in primary glioblastoma cells. However, the precise cellular localizations of PAI-1, uPA, and uPAR are likely critical because the attachment at the leading edge of a migrating cell and the concurrent detachment at its trailing edge are imperative for efficient migration and invasion. More importantly, the enhanced expression of the plasminogen activator system components may provide both the attachment and the detachment depending on the ratio of PAI-1, uPA, and uPAR at specific locations on the cell membrane. Indeed, uPAR is mainly found at focal adhesion areas, rafts, and caveolae at the leading edge of migrating cells (45, 46). uPAR is bound to catalytically active uPA, leading to the degradation of the extracellular matrix and providing attachment via the uPA/uPAR/integrin complex. In contrast, the enhanced expression of PAI-1 on the trailing edge of migrating cells may induce the internalization of the uPA/uPAR/integrin complex and result in cell detachment.

Our data indicate that the S1P-induced expression of PAI-1 and uPAR is mediated by S1P2, as shown using pharmacologic inhibitors and specific siRNA. S1P2 inhibits glioma cell...
migration through Rho activation and the Rho-kinase signaling pathway (47), which is probably mediated by PTEN activation (48). However, a recent study shows that S1P2 inhibits glioma cell migration through Rho signaling pathways independent of PTEN (49). In agreement with our results, S1P2 was recently reported to enhance, rather than suppress, invasion, most likely by increasing cell adhesion (50). In addition, we show that S1P2 may also control the generation of plasmin, which is critical for the degradation of the extracellular matrix, by regulating the expression of PAI-1 and uPAR. The S1P2-mediated up-regulation of PAI-1 and uPAR mRNA required functional Rho-kinase and mitogen-activated protein kinase/ERK kinase 1. The increased mRNA expression of uPAR induced by both S1P and IL-1 was AP-1 independent as shown in U373-TAM67 cells. In contrast, the intrinsic mRNA expression of PAI-1 and its stimulation by both S1P and IL-1 were decreased in U373-TAM67 cells, suggesting that this gene is regulated, at least in part, by AP-1.

Moreover, we showed that IL-1 acts independently of S1P to stimulate PAI-1 and uPAR expression in U373 cells and primary glioblastoma cells. This may be relevant in vivo because IL-1 is secreted by the majority of glioblastoma cells (24). IL-1 can also regulate the levels of S1P in vivo because it strongly up-regulates the mRNA expression of SphK1 (Fig. 3A). However, the IL-1–mediated increase of PAI-1 and uPAR mRNA expression is independent of SphK1, and thus independent of S1P formation. These data are further supported by the observation that the knockdown of S1P2 expression does not affect the IL-1–induced mRNA expression of PAI-1 and uPAR. An additional role for IL-1 may lie in its ability to stimulate the expression of SphK1, thus maintaining a sustained pool of S1P, which is known to be critical for glioblastoma cell growth and survival (22). More importantly, we show that in vitro invasion of both U373 cells and primary GBM6 cells was increased in response to both S1P and IL-1. Furthermore, the down-regulation of both PAI-1 and uPAR expression abrogated S1P- and IL-1–induced invasion. Therefore, we propose that IL-1 and S1P independently stimulate the invasion of glioblastoma cells via up-regulation of PAI-1 and uPAR expression. These observations have important implications for development of future therapeutic agents to limit the invasion of glioblastoma cells into surrounding healthy tissue, thus allowing for the effective removal of the tumor.

Materials and Methods

Cell Culture

Human glioblastoma U373-MG cells were obtained from the American Type Culture Collection, whereas primary human nonestablished glioblastoma GBM6 and GBM12 cells were kindly provided by Dr. C. David James (University of California, San Francisco, CA). The U373-TAM67 cells expressing the dominant-negative c-jun(TAM67) were described previously (31). Cells were maintained in DMEM supplemented with 10% FCS, antibiotics, sodium pyruvate, and nonessential amino acids. For the experiments, cells (5 x 10⁵ per well) were cultured in six-well culture plates in the presence of 1% serum.

Cytokines and Cell Stimulation

Cells were stimulated with 10 ng/mL IL-1 (a gift from Immunex Corp.) or the indicated amounts of S1P (Biomol Research Laboratories), as described previously (21). For the inhibitor studies, cells were pretreated with 1 μmol/L SP600125, 10 μmol/L SB202190 (Sigma-Aldrich), 1 μmol/L JTE-013 (Tocris), 0.3 μmol/L VPC23019 (Avanti Polar Lipids), 5 μmol/L Y27632, 5 μmol/L Gö6983 (EMD Biosciences, Inc.), 10 μmol/L LY294002, or 1 μmol/L U0126 (Cell Signaling Technology, Inc.) 1 h before stimulation.

Northern Blot Analysis

Total RNA was prepared by phenol extraction exactly as described previously (51). Five-microgram samples of RNA were subjected to formaldehyde gel electrophoresis using standard procedures (52) and transferred onto Hybond-XL membranes (GE Healthcare Bio-Sciences Corp.) according to
FIGURE 6. PAI-1 and uPAR are indispensable for S1P- and IL-1–enhanced invasion of U373 cells. A. Duplicate cultures of U373 cells were transfected with control, uPAR, or PAI-1 siRNA for 48 h, as indicated. RNA was isolated from one of the duplicates, and the expression of uPAR and PAI-1 was analyzed by quantitative PCR. The data were normalized to GAPDH mRNA and expressed as a ratio to mRNA levels in untreated cells. B. Cells from the second duplicate were stimulated with 10 ng/mL IL-1 or 100 nmol/L S1P for 12 h, as indicated, and allowed to migrate through Matrigel-coated polycarbonate filters for 7 h. The invasion was measured as described in Materials and Methods. Columns, mean from three independent experiments; bars, SD. C. Left, U373 cells were incubated with 1 µmol/L PAI-1 or 1 µmol/L bovine serum albumin (BSA) for 10 min, plated onto vitronectin-coated dishes, and allowed to adhere for 10 min. Subsequently, the medium containing the nonattached cells was removed and the number of cells present in the medium was counted using a hemocytometer. Right, expression of PAI-1 and uPAR was down-regulated as described in A and the attachment was analyzed as described above. Columns, mean from two independent experiments done in triplicate; bars, SD.

Quantitative PCR
uPAR, uPA, PAI-1, SphK1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were measured using TaqMan technology (Applied Biosystems) according to the supplier’s instructions. Briefly, 1 µg of total RNA was reverse transcribed using the high-capacity cDNA archive kit. Subsequently, the cDNA was diluted 10-fold (uPAR, uPA, PAI-1, and SphK1) or 100-fold (GAPDH). For real-time PCR, premixed primer-probe sets and TaqMan Universal PCR Master Mix were purchased from Applied Biosystems, and the cDNA was amplified using an ABI 7900HT cycler. The data are means ± SD from three independent experiments done in triplicate, unless indicated otherwise in the figure legends.

Western Blotting and Antibodies
Cells (5 x 10^6) were lysed in 275 µL of 10 mmol/L Tris (pH 7.4), 150 mmol/L sodium chloride, 1 mmol/L EDTA, 0.5% NP40, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche). Media were collected and concentrated 6-fold using Microcon 3,000 MWCO filters (Millipore) according to the manufacturer’s instructions. The protein amounts were quantified using the bicinchoninic acid assay (Sigma-Aldrich). Subsequently, equal amounts of the proteins (50 µg) were resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell). Polyclonal anti-ERK, anti-IκBα, anti-uPAR, and anti–PAI-1 antisera were purchased from Santa Cruz Biotechnology, Inc., whereas anti–phospho-ERK and anti–phospho-c-jun NH2-terminal kinase were purchased from Cell Signaling Technology. Antigen-antibody complexes were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce).

Down-Regulation with siRNA
S1P2, PAI-1, and uPAR expression was down-regulated using SmartPool siRNA purchased from Dharmacon, Inc. The siRNA (100 nmol/L) was transfected into the cells using Dharmafect 1 (Dharmacon) for 48 h. Nontargeting Pool1 (Dharmacon) was used as a control.

Attachment Assay
Cells were scraped off the dishes in PBS and dissociated by pipetting. The cells were pelleted by centrifugation, counted, and resuspended in fetal bovine serum–free DMEM at 100,000/200 µL. Subsequently, the cells were incubated with 1 µmol/L PAI-1 (EMD Biosciences) or 1 µmol/L bovine serum albumin for 10 min at 37°C and plated onto vitronectin-coated
dishes. The cells were allowed to adhere for 10 min; the medium containing the nonattached cells was removed and centrifuged, and the cells were counted in the presence of trypan blue using a hemocytometer. The attachment assay, done after transfection with PAI-1 or uPAR siRNA, was done as described above with minor differences (50,000 cells/200 μL and the cells were allowed to adhere for 5 min).

**Invasion Assay**

The cells were stimulated with S1P (100 nmol/L) and/or IL-1 (10 ng/mL) for 12 h. The invasion of the cells was subsequently measured in a modified Boyden chamber using polycarbonate filters (25 × 80 mm, 12 μm pore size) coated with Matrigel (BD; ref. 54). Both IL-1 and S1P were added to both the upper and lower chambers, whereas the cells were added to the upper chamber at 5 × 10⁴ per well. After 7 h, nonmigratory cells on the upper membrane surface were mechanically removed, and the cells that traversed and spread on the lower surface of the filter were fixed and stained with Diff-Quik (Fisher Scientific). The invading cells were counted with an inverted microscope and a 10× objective (55). Each data point is the average number of cells in five random fields and is the mean ± SD of three individual wells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


**FIGURE 7.** IL-1 and S1P up-regulate the expression of PAI-1 and uPAR mRNA in primary nonestablished glioblastoma cells and stimulate their invasion. Primary nonestablished glioblastoma GBM6 and GBM12 cells were stimulated without or with 10 ng/mL IL-1 or 100 nmol/L S1P for 18 h. RNA was isolated and the expression of PAI-1 (A), uPAR (B), and uPA (C) mRNA was analyzed by quantitative PCR. The data were normalized to GAPDH mRNA and expressed as a ratio to mRNA levels in untreated cells. D, GBM6 and GBM12 cells (50,000 per well) were stimulated without or with 10 ng/mL IL-1 or 100 nmol/L S1P and allowed to migrate through Matrigel-coated polycarbonate filters for 7 h. The invasion was measured as described in Materials and Methods. Columns, mean from three independent experiments; bars, SD.


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