CXCL12-Mediated Induction of Plasminogen Activator Inhibitor-1 Expression in Human CXCR4 Positive Astrogliaoma Cells

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Received October 7, 2008; accepted January 17, 2009

Glioblastoma is the most malignant and common brain tumor. To promote their growth, these glioma cells secrete a variety of soluble factors including plasminogen activator inhibitor-1 (PAI-1), which functions as an inhibitor of plasminogen activators. We report here with the basis of microarray gene expression analysis that CXCR4 expressing glioma cells are capable of expressing PAI-1 mRNA and protein upon CXCL12 stimulation. Pretreatment with U0126, an inhibitor of mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2, abrogated CXCL12-induced PAI-1 expression. Pertussis toxin (PTX), an inhibitor of Gα proteins, also had inhibitory effects, indicating that the activation of Gα and ERK MAPK are required for this response. Interestingly, CXCL12 showed additive effects with another PAI-1 inducers, tumor necrosis factor (TNF)-α and/or tumor growth factor (TGF)-β1, in increasing PAI-1 expression. These results indicate that CXCL12/CXCR4 signaling in glioma cells may be another mechanism for these cells to express PAI-1, which may be involved in angiogenesis and tumor invasion in brain tumors.

Key words plasminogen activator inhibitor-1; chemokine; cytokine; astroglioma

Glioblastoma Multiform (GBM) is the most common and invasive brain tumor differentiated by the existence of necrosis and vascular proliferation in tumor tissues.1,3 These malignant gliomas produce various soluble factors important in angiogenesis.2,4 Interestingly, one of the known functions of these factors is the induction of plasminogen activator inhibitor-1 (PAI-1).3 PAI-1 is well known as a primary physiological inhibitor of plasminogen activator (PA), which has been implicated in numerous physiological processes including tumor growth and angiogenesis.5,6

Recent studies have shown that chemokines, including CXCL12, may have diverse effects including angiogenesis, tumor metastasis and invasion.5,7 The biological function of CXCL12 is mediated through the CXCR4 which is expressed by astrocytes.5,6 Recently, a new CXCL12 receptor named CXCR7 has been discovered,7 and may also participate in the regulation of tumor angiogenesis.

Upon engagement of CXCR4 with CXCL12, a wide array of intracellular signaling events occur through these complexes; activation of phosphatidylinositol 3-kinase (PI3-K),5 G-protein signaling,5 and mitogen activated protein kinases (MAPKs).5 In addition, these CXCR4-mediated signaling pathways eventually lead to various biological effects including gene induction.5,6

Recent findings have shown that the level of CXCL12/CXCR4 and PAI-1 expression in brain tumors is increased with increasing tumor grade, and correlated with the invasive and metastatic potential of brain tumors.10,11 The induction of PAI-1 expression in astrocytes has been occurred by cytokines such as TNF-α and TGF-β.12,13 However, the modulation of PAI-1 expression by chemokine, especially CXCL12, has not been reported. In this work, we report that CXCL12 stimulation alone or with TNF-α or TGF-β leads to the induction of PAI-1 in CXCR4 expressing human astrogliaoma cells through G-protein/ERK MAPK-mediated signaling, but not through the PI3-kinase pathway.

MATERIALS AND METHODS

Cell Culture and Reagents CRT-J13 cells and U87-T37 cells, stable CXCR4 positive transfectants, were maintained in RPMI and HAM’S-F-12 DMEM containing 0.5 mg/ml G-418,9 respectively. CXCR4 expression was confirmed regularly by FACS analysis and by measurement of [Ca2+]i upon CXCL12 stimulation. Recombinant human (rh) CXCL12, mouse anti-hCXCL12 Ab, mouse anti-hCXCR4 Ab, rhTNF-α, and rhTGF-β1 were purchased from R & D Systems (Minneapolis, MN, U.S.A.). Anti-ERK2 antibodies (Abs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Phospho-ERK1/2, phospho-AKT, ERK1/2, and AKT Abs were purchased from Cell Signaling (Beverly, MA, U.S.A.). The MEK1/2 inhibitor U0126 was from Promega (Madison, WI, U.S.A.). Pertussis toxin (PTX) and cholera toxin (CTX) were purchased from Sigma (St. Louis, MO, U.S.A.). LY294002 were purchased from Calbiochem (San Diego, CA, U.S.A.).

cRNA Target Preparation and Affymetrix GeneChip Hybridization CRT-J13 cells were incubated in the absence or presence of CXCL12 (50 ng/ml) for 3 h, then total RNA was isolated by using TRIZOL (Life Technologies, MD, U.S.A.). Ten micrograms of RNA were converted to cDNA by using SuperScript Choice (Gibco-BRL), substituting T7-oligo(dT24) for random primers. Second-strand synthesis was performed using T4 DNA polymerase, and the resultant cDNA was isolated by phenol-chloroform extraction. cDNA was in vitro transcribed by using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem, NY, U.S.A.) supplied with biotin-labeled UTP and CTP to produce biotin-labeled cRNA. Labeled cRNA was isolated by using a QIAIEN RNeasy Mini column Kit (Qiagen). cRNA was fragmented in 500 mm potassium acetate, 150 mm magnesium acetate, 200 mm Tris-acetate (pH 8.1) for 35 min at 94°C, and hybridization performance was analyzed by using Test 2 arrays (Affymetrix, CA, U.S.A.). Target cRNA was
hybridized to the U95A microarray. Microarrays were washed and stained with streptavidin-conjugated phycoerythrin (SAPE) by using the Affymetrix GeneChip Fluidic Station 400. Staining intensity was antibody amplified by using a biotinylated anti-streptavidin Ab at a concentration of 3 μg/ml followed by a second SAPE stain and was visualized at 570 nm. All microarrays were scanned using fluorescent scanners (Hewlett Packard Gene Array Scanner; CA, U.S.A.). Data were extracted and analyzed using Affymetrix Microarray Suite, MicroDB and Data Mining Tool software.

**RNase Protection Assay (RPA)** RNA was extracted with TRIZOL directly in the cell culture dish. A pGEM-4Z vector containing a fragment of the hPAI-1 cDNA (bp 76—1682) was linearized with HincII, which digests within the hPAI-1 cDNA insert. In vitro transcription of this linearized vector with Sp6 RNA polymerase generates a 495-bp antisense riboprobe. A pAMP-1 vector (Gibco) containing a fragment of the human GAPDH cDNA (bp 43—531) was linearized with Nco1. In vitro transcription of this linearized vector with T7 RNA polymerase generates a 290-bp antisense riboprobe. RPA was carried out as previously described.9)

**Immunoblotting** Immunoblotting was performed for AKT and ERK1/2 MAPK analysis. Cells were lysed in lysis buffer [50 mM Tris–HCl, 2.5 mM EGTA, 1 mM EDTA, 10 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF) and 2 mM Na3VO4]. Cell lysates (20 μg) were run on 12% SDS-PAGE, transferred to nitrocellulose membranes (Hybond ECL, Amersham), and incubated with phospho-AKT or phospho-ERK1/2 Abs at 4 °C overnight. The membranes were then incubated with rabbit HRP-conjugated Ab (Zymed), and incubated with phospho-AKT or phospho-ERK1/2 Abs at 4 °C overnight. The membranes were washed and stained with streptavidin-conjugated phycoerythrin. RP A was carried out as previously described.9)

**Measurement of PAI-1 Protein Expression** CRT-J13 and U87-T37 cells were plated at 2×10^5/well in six-well plates, and incubated with serum-free medium, CXCL12, TNF-α without or with CXCL12, TGF-β1 without or with CXCL12, or all three for 24 h. PAI-1 proteins from the collected supernatants were quantitated using ELISA system (American Diagnostica Inc., CT, U.S.A.).

**Statistical Analysis** One-way analysis of variance (ANOVA) was used to determine the significance of differences between treatment groups. The LSD method among post-hoc tests was used for multi-group comparisons. Statistical significance was accepted for p values of <0.05.

**RESULTS**

**PAI-1 mRNA Expression Is Enhanced by CXCL12** We have previously shown that CXCR4 expressing human glioma cells produce chemokines such as IL-8, MCP-1 and IP-10 upon CXCL12 stimulation.9) In parallel, microarray gene expression analysis was done to test whether CXCR4 expressing glioma cells, upon CXCL12 stimulation, express genes that play important roles related to brain tumor growth. Comparing gene expression profiles in unstimulated and CXCL12 (50 ng/ml) stimulated CRT-J13 cells for 3 h revealed upregulation of 25 genes out of 2043 on a selected human cancer array (Table 1). Interestingly, we found PAI-1 mRNA expression among these upregulated genes is induced (4.6-fold) by CXCL12 stimulation. To confirm this result, CRT-J13 cells were stimulated with CXCL12 (50 ng/ml) for 0—24 h, then PAI-1 mRNA was analyzed by RPA. As shown in Fig. 1A, low constitutive PAI-1 mRNA was detected (lane 1), and CXCL12 treatment caused a time-dependent increase in PAI-1 mRNA expression, with optimal expression detected at 8 h (2.8-fold, lane 4). Comparable results were observed in CXCR4 positive U87-T37 cells (data not shown). Dose-response curves were obtained after treatment with CXCL12 in the range of 0.1—500 ng/ml for 6 h (Fig. 1B). PAI-1 mRNA expression increased with increasing CXCL12 concentrations, reaching maximum 2.8—3.1-fold induction using 50—500 ng/ml. Two protected PAI-1 mRNA bands were detected; the upper band likely corresponds to a fragment of the 3.0 kb PAI-1 mRNA species and the lower band to the 2.2 kb PAI-1 mRNA as reported previously by Yamamoto et al.14) To establish the specificity of this effect, neutralizing Abs to CXCL12 and CXCR4 were used, which abolished CXCL12-induced PAI expression (50% and 58%; Fig. 1C). Mouse IgG had no effect in this system (4%), indicating that the increase in PAI expression was induced by CXCL12 through CXCR4.

**The MEK1/2 Inhibitor U0126 and Pertussis Toxin (PTX) Abrogate CXCL12 Induction of PAI-1 Expression and ERK MAPK Signaling Is Mediated by Gα Proteins**

**Table 1. Up-Regulated Genes in CXCR4 Positive CRT-J13 Cells upon CXCL12 Stimulation**

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<thead>
<tr>
<th>Accession No.</th>
<th>Description</th>
<th>(fold induction)</th>
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<tr>
<td>S82297</td>
<td>Beta 2-microglobulin (4.8)</td>
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<td>X04409</td>
<td>G(s) alpha (alpha-S1) (Gs component of adenyyl cyclase) (4.6)</td>
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</tr>
<tr>
<td>J04164</td>
<td>Interferon-inducible protein (IP-10) (4.6)</td>
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<td>J03764</td>
<td>Plasminogen Activator Inhibitor-1 (PAI-1) (4.6)</td>
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<tr>
<td>U37146</td>
<td>Silencing mediator of retinoid and thyroid hormone (SMRT) (4.5)</td>
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<tr>
<td>M62831</td>
<td>Transcription factor ETR101 (4.5)</td>
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<tr>
<td>X95735</td>
<td>Zyxin (4.5)</td>
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<tr>
<td>AL031670</td>
<td>Ferritin, light polypeptide-like 1 (4.5)</td>
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<tr>
<td>M14199</td>
<td>Laminin receptor, 67 kDa (4.7LIR) (4.5)</td>
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<tr>
<td>M29093</td>
<td>Transactivator (jun-B) (4.5)</td>
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<tr>
<td>M28225</td>
<td>JE gene encoding a monocyte secretory protein (MCP-1) (4.4)</td>
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<td>X95404</td>
<td>Non-muscle type cofilin (4.4)</td>
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<tr>
<td>M26252</td>
<td>Cytosolic thyroid hormone-binding protein (3.8)</td>
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<td>M60278</td>
<td>Heparin-binding EGF-like growth factor (3.7)</td>
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<td>Y15915</td>
<td>Collagen type 1 α1 and platelet derived growth factor-β (3.6)</td>
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<td>Z23090</td>
<td>28 kDa heat shock protein (3.6)</td>
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<td>Z54367</td>
<td>Plectin (3.6)</td>
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<td>G(i) alpha (adenylate cyclase inhibiting GTP-binding protein) (3.5)</td>
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<td>c-myc binding protein (MBP-1) (3.3)</td>
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<tr>
<td>M28130</td>
<td>Interleukin-8 (IL-8) (3.3)</td>
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<td>D00017</td>
<td>Lipocortin II (3.0)</td>
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<tr>
<td>M17733</td>
<td>Thymosin beta-4 (3.0)</td>
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a) CXCL12 (50 ng/ml) for 3 h. b) >3-fold (fold induction compared to unstimulated cells). The 25 highest up-regulated genes derived from the gene chip analysis are ranked.

(14) To establish the specificity of this effect, neutralizing Abs to CXCL12 and CXCR4 were used, which abolished CXCL12-induced PAI expression (50% and 58%; Fig. 1C). Mouse IgG had no effect in this system (4%), indicating that the increase in PAI expression was induced by CXCL12 through CXCR4.

The MEK1/2 Inhibitor U0126 and Pertussis Toxin (PTX) Abrogate CXCL12 Induction of PAI-1 Expression and ERK MAPK Signaling Is Mediated by Gα Proteins
CXCL12 has been shown to activate various signaling pathways through CXCR4. In particular, activation by CXCL12 of the ERK MAPK-/PI3-kinase-/G protein-mediated pathways leads to cell proliferation and gene induction.6,9) To examine the signaling pathways involved in CXCL12-induced PAI expression, CRT-J13 cells were stimulated with CXCL12 for 0—60 min, cells were lysed, and immunoprecipitated with anti-human ERK2 Ab, and then used for an in vitro kinase assay. Result is representative of two separated experiments.

CXCL12 has been shown to activate various signaling pathways through CXCR4. In particular, activation by CXCL12 of the ERK MAPK-/PI3-kinase-/G protein-mediated pathways leads to cell proliferation and gene induction.5,9) To examine the signaling pathways involved in CXCL12-induced PAI expression, CRT-J13 cells were stimulated with CXCL12 for 0—60 min, cells were lysed, and subjected to 12% SDS-PAGE. As shown in Fig. 2A, phosphorylation of AKT and ERK1/2 was seen after 5 min of CXCL12 stimulation (lane 3), and was sustained for 60 min (lane 7). Next, we deter-
mined if these signaling pathways were involved in CXCL12-induced PAI-1 expression. CRT-J13 cells were pretreated with U0126 (20 μM), LY294002 (10 μM) or PTX (0.1 μg/ml), then incubated with CXCL12 (50 ng/ml) for an additional 6 h. PAI-1 mRNA was analyzed by RPA. As shown in Fig. 2B, U0126 (lane 4) and PTX (lane 6) inhibited CXCL12-induced PAI-1 expression (66% and 73%), indicating that activation of ERK MAPK and Gαi are required for this response. The PI3-kinase inhibitor LY294002 had a minimal inhibitory effect (lane 5), suggesting that PI3-kinase-mediated signaling is not involved in this response. The DMSO had a negligible effect (lane 3).

Since the ERK MAPK-/Gαi-mediated signaling pathways are involved in the CXCL12-induced PAI-1 expression, we further assessed whether activation of ERK MAPK is dominated by G-protein signaling in these response. The PI3-kinase inhibitor LY294002 had a minimal inhibitory effect (lane 5), suggesting that PI3-kinase-mediated signaling is not involved in this response. The DMSO had a negligible effect (lane 3).

Since the ERK MAPK-/Gαi-mediated signaling pathways are involved in the CXCL12-induced PAI-1 expression, we further assessed whether activation of ERK MAPK is dominated by G-protein signaling in these response. CRT-J13 were stimulated with CXCL12 for 5 min, cells were lysed, and subjected to immunoprecipitation with anti-ERK2 Ab, and then an in vitro kinase assay was performed. MBP was used as the substrate for ERK2. Figure 2C illustrates that MBP phosphorylation by ERK2 was seen after CXCL12 stimulation for 5 min (lane 2). Pretreatment of cells with PTX (lane 3) and U0126 (lane 5) but not CTX (lane 4) or LY294002 (lane 6), inhibited CXCL12-induced ERK2 activation, indicating that ERK MAPK activation involves Gαi proteins.

Effect of TNF-α and TGF-β1 on PAI-1 mRNA and Protein Expression  TNF-α and TGF-β1 stimulation in astrocytes have been reported to increase PAI-1 mRNA expression.[12,13] We tested whether TNF-α or TGF-β1, in conjunction with CXCL12, affected PAI-1 mRNA expression. CRT-J13 cells were treated with CXCL12 (50 ng/ml), TNF-α (2 ng/ml) without or with CXCL12, TGF-β1 (2 ng/ml) without or with CXCL12, or with all three for 6 h. As shown in Fig. 3A, PAI-1 mRNA expression was increased 2.1-fold by CXCL12 (lane 2); 1.9-fold by TNF-α (lane 3); and 3.7-fold by TGF-β1 (lane 4). Co-stimulation led to an additive effect; CXCL12 plus TNF-α (4.3-fold, lane 5); CXCL12 plus TGF-β1 (4.9-fold, lane 6); and to a 7.1-fold increase by all three stimuli (lane 7). These results demonstrate that CXCL12, TNF-α and TGF-β1 can function in an additive manner to increase PAI-1 mRNA expression.

Accumulation of a specific mRNA in cells is not always accompanied by comparable induction of protein expression. Therefore, CXCR4 expressing CRT-J13 cells were treated with CXCL12 (50 ng/ml), TNF-α (2 ng/ml) without or with CXCL12, TGF-β1 (2 ng/ml) without or with CXCL12, or all three for 24 h, then supernatants were collected and analyzed for PAI-1 protein production by ELISA. PAI-1 protein expression increased by 1.8-fold upon CXCL12 stimulation, 2.1-fold induction by TNF-α, and by 3.9-fold by TGF-β1. Co-stimulation led to a 2.8-fold induction by CXCL12 plus TNF-α, a 4.8-fold induction by CXCL12 plus TGF-β1, and a 7.2-fold induction by all three when compared with unstimulated cells (Fig. 3B). Similar results were observed using U87-T37 cells (data not shown). These results demonstrate that CXCL12, TNF-α and TGF-β1 have a similar additive effect on PAI-1 protein expression as that observed at the mRNA level.

**DISCUSSION**

In this study, we have obtained evidence that CXCL12 stimulation of CXCR4 positive astroglial cells leads to the induction of PAI-1 mRNA and protein expression; U0126, an inhibitor of the ERK1/2 MAPK signaling, blocks CXCL12-induced PAI-1 expression, and PTX, an inhibitor of Gαi proteins, abrogates CXCL12 induction of PAI-1 expression.
However, we observed that the PI3-kinase-mediated signaling pathway is not involved in this response, although this pathway is known to be involved in cell proliferation in glioma cells.19

The literature amply demonstrates that PAIs are involved in tumor growth, invasion and angiogenesis.20 The data for PAI-1 involvement in angiogenesis is contradictory, with the observations that PAI-1 can either enhance or inhibit tumor growth and angiogenesis.4,15 However, a recent study suggests that the dual effects of PAI-1 in angiogenesis are dose-dependent,16 which may explain the observed functional differences. We observed that CXCL12 has additive effects with either TNF-α or TGF-β1 in increasing PAI-1 expression in glioma cells. Glioma cells produce TNF-α and TGF-β1,2 thus these cytokines are present in the microenvironment of GBMs and, in conjunction with CXCL12, may potentiate PAI-1 expression. To promote their growth, malignant glioma stimulate the formation of new blood vessels through processes driven primarily by VEGF.17 It has been recently demonstrated that CXCL12 increases IL-8 production16) that are up-regulated by CXCL12 stimulation have been implicated in the angiogenic process, including IL-8, PAI-1, 67LR, collagen type-1 and TGF-β1,2, suggesting that the dual effects of PAI-1 in angiogenesis are dose-dependent,16 which may explain the observed functional differences. We observed that CXCL12 has additive effects with either TNF-α or TGF-β1 in increasing PAI-1 expression in glioma cells. Glioma cells produce TNF-α and TGF-β1,2 thus these cytokines are present in the microenvironment of GBMs and, in conjunction with CXCL12, may potentiate PAI-1 expression. To promote their growth, malignant glioma stimulate the formation of new blood vessels through processes driven primarily by VEGF.17 It has been recently reported that PAI-1 stimulates expression of vascular endothelial growth factor (VEGF) in the glioma cells,18 and thereby endothelial cell migration and proliferation through upregulation of CXCR4.19 Thus, PAI-1 released from glioma cells may contribute to angiogenesis of tumors.

CXCL12 and CXCR4 are detected at extremely low levels in normal brain, however, both are overexpressed in astroglial tumor tissue, and the expression levels are correlated with tumor grade.11 It has been demonstrated that introduction of CXCR4 monoclonal Abs in glioblastoma cells causes inhibition of cell proliferation.6 We have previously demonstrated that CXCL12 increases IL-8 production in astroglialoma cells and IL-8 has potent angiogenic properties, and elevated levels of IL-8 may be associated with GBMs.20 Therefore, these observations demonstrate that the CXCL12/CXCR4 complexes, and angiogenic factors such as IL-8 and PAI-1, are aberrantly expressed in GBM tumor tissue, and may contribute in part to tumor growth and angiogenesis.

We employed cDNA microarray analysis to compare gene expression patterns in CXCL12 stimulated astroglialoma cells. A listing of the 25 most highly expressed genes is shown in Table 1. This list contains several genes such as thymosin beta-4 and c-myc binding protein-1 that have been reported to be associated with angiogenesis.21,22 Overexpression of the 67 kDa lamin receptor (67LR) is an independent prognostic factor in primary breast cancer, and correlates with the dissemination of tumor cells.23 Thus, a number of the genes that are up-regulated by CXCL12 stimulation have been implicated in the angiogenic process, including IL-8, PAI-1, 67LR, collagen type-1 α1, and PDGF-β1,17,24 suggesting that CXCL12 stimulation in glioma cells may contribute to these processes.

In summary, we demonstrate that PAI-1 mRNA and protein expression are increased upon CXCL12 stimulation in CXCR4 positive astroglialoma cells. In addition, we demonstrate that the CXCL12 insult with TNF-α and TGF-β1 has an additive effect to in increase PAI-1 expression. Taken together, our results point to links between chemokines, inflammatory cytokines, and the PAI-1 system in the glioma cells.

Acknowledgements This work was supported in part by National Institute of Health Grants to E.N.B. and M.A.O., and by a Veterans Administration MERIT Award to M.A.O.

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