PDIA6 regulation of ADAM17 shedding activity and EGFR-mediated migration and invasion of glioblastoma cells

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OBJECTIVE In patients with glioblastoma, local invasion of tumor cells causes recurrence and shortens survival. The goal of this study was to determine whether protein disulfide isomerase (PDI) A6 regulates migration and invasion of glioblastoma cells and the associated factors.

METHODS U87MG cells were treated with either PDIA6 or ADAM17 small interfering RNA (siRNA) fragments or with both types of siRNA fragments, and expression was confirmed by reverse transcription–polymerase chain reaction and Western blot. Migration and invasion were assessed using a wound-healing assay, a Matrigel assay, and an organotypic culture system. After the U87MG cells were treated with siRNAs and epidermal growth factor receptor (EGFR) inhibitors, the expression of matrix metalloproteinase–2 (MMP-2), membrane Type 1-matrix metalloproteinase (MT1-MMP), integrin, phosphorylated focal adhesion kinase (pFAK), and phosphorylated EGFR (pEGFR) was detected by Western blotting and zymography.

RESULTS U87MG cell migration and invasion increased significantly after inhibition of PDIA6. The MMP-2 activation ratio and ADAM17 activity (as a sheddase of the proligand) increased, and expression of pEGFR, pFAK, integrin α5β3, and MT1-MMP was induced, compared with control levels. Furthermore, heparin-binding epidermal growth factor (EGFR signaling ligand) was highly expressed in PDIA6-knockdown cells. After siPDIA6-transfected U87MG cells were treated with EGFR signaling inhibitors, expression of pFAK, MMP-2, and MT1-MMP decreased and invasion decreased significantly. Simultaneous double-knockdown of PDIA6 and ADAM17 reduced pEGFR and pFAK expression, compared with control levels.

CONCLUSIONS The authors propose that inhibiting PDIA6 could transduce EGFR signaling by activating and inducing ADAM17 during migration and invasion of U87MG glioblastoma cells. The results of this study suggest that PDIA6 is an important component of EGFR-mediated migration and invasion of U87MG cells. This is the first report of the effects of PDIA6 on migration and invasion in glioblastoma.

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KEY WORDS PDIA6; ADAM17; EGFR; MMP-2; MT1-MMP; glioblastoma; U87MG; oncology

Glioblastoma is the most malignant of primary adult brain tumors and is characterized by a highly localized invasive cell population, abundantly proliferative cells, neoangiogenesis, and necrosis. Despite continuous improvements in glioblastoma treatment, the median survival time does not exceed 15 months.3,32 Local invasion of malignant glioma leads to recurrence and therapeutic failure. Therefore, it is important to understand the molecular events that underlie the biological behavior of invasive tumor cells.

ABBREVIATIONS ADAM = a disintegrin and metalloprotease; A+P = knockdown of both ADAM17 and PDIA6; DMEM = Dulbecco's modified Eagle medium; EGFR = epidermal growth factor receptor; FAK = focal adhesion kinase; F-actin = filamentous actin; HB-EGF = heparin-binding epidermal growth factor; MT1-MMP = membrane Type 1–matrix metalloproteinase; NC = negative control; PBS = phosphate-buffered saline; PDI = protein disulfide isomerase; pEGFR = phosphorylated EGFR; pFAK = phosphorylated FAK; RT-PCR = reverse transcription–polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; siRNA = small interfering RNA.

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Protein disulfide isomerases (PDis) are multifunctional proteins in the thioredoxin superfamily that function as isomerases and as chaperones during protein folding. Members of the PDI family have 3 catalytic components, including thiol-disulfide oxidoreductase, disulfide isomerase, and redox-dependent chaperone, and regulate the formation and rearrangement of disulfide bonds during correct folding of nascent proteins. The PDI family comprises 21 proteins that vary in size, expression, localization, and enzymatic function. Among them, PDIA6 is a 440-amino acid protein. Similar to human PDI, this protein contains 2 thioredoxin-like domains, and these domains share 47% amino acid sequence identity in the 2 proteins (PDI and PDIA6), suggesting that these proteins are functionally related.

Proteome analysis has been used to identify correlations between PDI expression and cancers. According to these analyses, PDI is highly expressed in various tumor types, such as brain, prostate, lymphoma, kidney, prostate, and lung cancer. Overexpression of PDI occurs in lymph node metastases, metastatic tumors of the breast, and colorectal cancer. Nevertheless, the function of PDIA6 in tumors has rarely been reported until now.

PDIA4 and PDIA6 overexpression was detected in a patient with lung adenocarcinoma and in association with resistance to chemotherapy in patients with lung adenocarcinoma and in association with resistance to chemotherapy in patients with lung adenocarcinoma and ischemic brain damage. In proteomic studies reported in 2004, PDIA3 was found to be highly expressed in a low-grade astrocytoma. However, some studies have demonstrated that PDI is associated with the invasive phenotype of glioblastoma and hepatocellular carcinoma using proteomic pattern screening technology. PDI controls the activity of ADAM17 at the cell surface (based on shedding of an epidermal growth factor receptor [EGFR] protein). Williams and colleagues demonstrated that PDI maintains ADAM17 in an inactive conformation and that changes in the local redox environment, such as mobilization of reactive oxygen species, facilitate activation of ADAM17.

ADAMs (with ADAM defined as “a disintegrin and metalloprotease”) are membrane-anchored metalloproteases that process and shed the ectodomains of membrane-anchored growth factors, adhesion molecules, cytokines, and receptors. Because many transmembrane growth and differentiation factors, including members of the ErbB family of receptors, require ectodomain shedding for proper action in vivo, proteolysis is viewed as a regulatory mechanism in the developing embryo. ADAMs are associated with proliferation and invasion of various types of cancer, such as glioblastoma and cancers of the lung, prostate, breast, and tongue. ADAM17 promotes migration and invasion of lymphatic endothelial cells and of cells in the glioblastoma cell line in prostate cancer and in nasopharyngeal carcinoma. ADAM17 is also associated with a poor prognosis in cervical carcinoma.

We investigated whether PDIA6 can regulate cell migration and invasion and determined the correlation between PDIA6 and ADAM17 during migration and invasion of the U87MG human glioblastoma cell line.

Methods

Cell Culture

Cells of the human malignant glioma cell line U87MG were acquired from the American Type Culture Collection and were cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (Gibco) and maintained at 37°C in an incubator with 5% CO₂ and 95% room air. The EGFR tyrosine kinase inhibitor gefitinib and AG1478 were purchased from Sigma-Aldrich. Primary antibodies for actin, PDIA6, integrin alpha, and integrin beta were purchased from Santa Cruz Biotechnology, and membrane Type I–matrix metalloproteinase (MT1-MMP), ADAM17, phosphorylated focal adhesion kinase (pFAK), and phosphorylated epidermal growth factor receptor (pEGFR) were purchased from Abcam.

siRNA Oligonucleotide Transfection

An siRNA oligonucleotide was used for PDIA6 and ADAM17 knockdown. Synthetic PDIA6 siRNA (5′-GA GUGAUAGUUGUGGCUCU-3′), ADAM17 siRNA (5′-C CAGGAGG CGAUUAUGCU-3′), and scrambled RNA (as the negative control [NC]) were purchased from Bio- neer. Approximately 2 × 10⁵ U87MG cells were seeded on a 60-mm culture plate and transfected with the siRNA oligonucleotide using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instructions. Gene silencing by siRNA was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The respective cell lines were renamed siPDIA6 (PDIA6 siRNA), siADAM17 (ADAM17 siRNA), and siNC.

Wound-Healing Migration Assay

U87MG cells transfected with PDIA6 siRNA and scrambled RNA were maintained until nearly 100% confluence, and then a linear scratch was made using a 200-μl pipette tip. After being washed twice with phosphate-buffered saline (PBS), the cells were incubated for 12 hours, and images were obtained at 40× magnification.

Matrix Gel Assay

The Matrigel assay was performed as described previously. Briefly, Matrigel was coated on a PET (polyethylene terephthalate) membrane of a cell culture insert (8-μm pore size) (Corning Corp.). Cells (5 × 10⁴) were added to the membrane coated with Matrigel and were incubated for 24 hours at 37°C in a humidified atmosphere of 95% room air and 5% CO₂. Cells that traversed the Matrigel layer and attached to the filter were stained with the Diff-Quik Stain Set (Dade Diagnostics) and were counted in 5 randomly selected fields. Results are expressed as mean ± standard error of 3 independent experiments.

Brain Slice Invasion Assay

We performed organotypic cultures to recapitulate the matrix macromolecules normally encountered by infiltrating glioblastoma cells, as described previously by Jung et al. The cells were stained with DiI (1,1'-dioctadecyl-
Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from 3 cultured glioma cell lines using TRIzol reagent. One microgram of RNA was reverse-transcribed to synthesize cDNA. Reverse transcription (RT) was followed by polymerase chain reaction (PCR). For first-strand synthesis, purified total RNA (1 μg) was incubated with oligo(dt) (0.5 μg/μl, Promega) for 5 minutes at 70°C, followed by the addition of buffer containing 4 μl of 5x reaction buffer (Promega), 1 μl of dNTP (10 mM each), 3.5 μl of 25 mM MgCl₂, 2 μl of RNase inhibitor (40 U/μl, Promega), and 1 μl of reverse transcriptase (200 U/μl, Promega). The reaction volume was 20 μl, and the mixture was incubated for 1 hour at 42°C. Complementary DNA was amplified by Ex Taq polymerase (TaKaRa) (0.5 μl of single-stranded cDNA solution/25-μl reaction volume) using specific human primers (Table 1).

Cell Adhesion Assay

Human fibronectin (10 μg/ml, Asahi Techno Glass) was coated on a 96-well plate for 1 hour at room temperature and washed with PBS. Then 1 × 10⁴ cells/well were seeded and allowed to adhere to the dishes for 50 minutes at 37°C. Adherent cells were stained with the MTT reagent, and absorption was measured at 590 nm using an Infinite M200 PRO spectrophotometer (Tecan Group).

Preparation of Total Protein, Membrane Proteins, and Conditioned Media

Cells were lysed in a protein extraction buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS (sodium dodecyl sulfate), 1% NP-40, 1 mM PMSF, and 1 mg/ml protease inhibitor cocktail) to prepare total protein. The cell membrane fraction was prepared using a Mem-PER membrane protein extraction kit (Pierce Biotechnology), according to the manufacturer’s protocol.

The cells were treated with siRNA or EGFR inhibitors to prepare the conditioned media. The cells were washed with PBS, serum-free medium (500 μl) was added to each plate, and the cells were incubated at 37°C for 48 hours. The conditioned media were clarified by centrifugation. The concentrations of all protein samples were determined using the Bio-Rad protein assay kit.

Western Blot Analysis

Whole cell lysates (5–10 μg) were separated by 8%–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Pall Corp.). The membranes were incubated for 1 hour at room temperature with 5% nonfat dry milk, probed overnight at 4°C with each of the primary antibodies, and incubated with horseradish peroxidase–labeled goat anti–rabbit or anti–mouse antibody (AbFrontier). The bound secondary antibody was detected by enhanced chemiluminescence (Amersham Biosciences), and protein levels were determined by autoradiography using the LAS-4000 instrument (Fuji).

Gelatin Zymography

Prior to electrophoresis, 10 μg of proteins in conditioned media were mixed with sample buffer (50 mM of Tris-HCl, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Aliquots were electrophoresed on 8% SDS-PAGE containing 1 mg/ml Type A gelatin (Sigma-Aldrich). Each gel was washed 3 times for 30 minutes in 2.5% Triton X-100 and then incubated for 20 hours at 37°C in incubation buffer (50 mM Tris-HCl [pH 7.5], 10 mM CaCl₂, and 200 mM NaCl). The gels were stained with Coomassie Brilliant Blue R-250 (0.2% Coomassie Brilliant Blue R-250, 20% methanol, and 10% acetic acid in water) and de-stained in 20% methanol and 10% acetic acid in water.

F-Actin Immunofluorescence Staining

Cells (5 × 10⁵) were seeded on an 8-well noncoated chamber slide using rhodamine-conjugated phalloidin (Molecular Probes), according to the manufacturer’s instructions. Fluorescence photomicrographs were taken using a confocal microscope (LaserSharp 2000 version 5.1) equipped with a Plan-Apochromat 63×/1.40 oil objective. Confocal images were acquired using LSM 5102.3 software. The wavelength of each antibody was the Alexa 568 emission spectrum at 603 nm.

Statistical Analysis

Significant differences between the 2 groups of treated cells were determined using the paired t-test, with p < 0.05 considered significant. The statistical analysis was performed using SPSS for Windows, version 12.0 (SPSS Inc.).

Results

Endogenous PDIA6 Expression

U87MG cells were transfected with siPDIA6 and

### Table 1. RT-PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
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<tr>
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<td>5′-tactctgaggccagt-3′</td>
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<td>ADAM17</td>
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</tr>
<tr>
<td></td>
<td>5′-gactgaccagcatcaaa-3′</td>
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scrambled siRNA, and expression was confirmed by RT-PCR and Western blotting. As shown in Fig. 1A and B, PDIA6 expression decreased significantly, compared with that of the control, and we confirmed that PDIA6 silencing was maintained for at least 6 days (Fig. 1C).

**Inhibiting PDIA6 Induces Migration and Invasion of U87MG Cells**

A wound-healing assay and organotypic culture were used to determine the effect of PDIA6 on migration and invasion of U87MG cells. PDIA6-knockdown in U87MG cells resulted in a high level of motility and complete wound closure after 12 hours (Fig. 2 left), whereas the control cells showed less motility and incomplete wound closure. These results indicate that PDIA6 may negatively regulate cell motility. We confirmed the effect on invasion of U87MG cells using an organotypic culture system that used normal rat brain as a substrate. The siPDIA6 cells showed extensive invasion of the surrounding normal brain, whereas the control cells did not (Fig. 2 right).

Dynamic regulation of the filamentous actin (F-actin) cytoskeleton is critical to numerous physical cellular processes, including cell adhesion, migration, and division. The change in F-actin filaments between siNC and siPDIA6 cells were visualized by rhodamine-labeled phalloidin staining. The pseudopodia observed in the siPDIA6-treated cells were thinner and more elongated than those in the control cells (Fig. 3).

**PDIA6 Knockdown Induces Expression of MMP-2 and ADAM17 and Enhances MMP-2 Activity**

PDI has been described as a negative regulator of ADAM17, and ADAM17 regulated MMP-2 and MMP-9 expression in a study of prostate cancer invasion. We sought to determine whether PDIA6 affected expression of these proteins in U87MG glioblastoma cells using RT-PCR and Western blot analyses. MMP-2 activity in the supernatant was evaluated by gelatin zymography. ADAM17 and MMP-2 expression increased after inhibition of PDIA6 in U87MG cells (Fig. 4A and B). The MMP-2 activation ratio increased significantly compared with that in control cells (Fig. 4C).

**ADAM17 Knockdown Decreases MMP-2 Expression and Activity**

To determine whether ADAM17 has an effect on MMP-2 expression and activity in U87MG cells, we transfected the cells with ADAM17 siRNA and assessed MMP-2 expression and activity by Western blotting and zymography. As shown in Fig. 4B and C, ADAM17 knockdown in U87MG cells decreased MMP-2 expression and activity but did not affect PDIA6 expression, compared with the control. These results suggest that PDIA6 acts upstream of ADAM17 and that there is a correlation between PDIA6 and ADAM17 during migration and invasion of U87MG cells.
Next, we determined the factors associated with inducing MMP-2 activity. The MT1-MMP–TIMP-2–proMMP-2 complex forms to activate MMP-2, and a second MT1-MMP forms a dimer with the MT1-MMP in the complex and cleaves proMMP-2 to produce active MMP-2. Active MMP-2 then processes the extracellular matrix proteins not cleaved by MT1-MMP. The increase in MMP-14 activity was not due to an increased quantity of MMP-14 mRNA or protein but relies on appropriate localization at the plasma membrane, in accordance with earlier findings.

We examined the MT1-MMP expression in total protein and the membrane fraction of U87MG cells transfected with PDIA6 or ADAM17 siRNA. As shown in Fig. 4D, MT1-MMP expression in total protein and the membrane fraction was increased in siPDIA6-treated cells but was decreased in siADAM17-treated cells, in comparison with expression in control cells.

These results show opposite effects of PDIA6 and ADAM17 on MMP-2 and MT1-MMP, suggesting that PDIA6 may negatively control ADAM17 function.

**PDIA6 Is Associated With the EGFR, Integrin, and FAK Signaling Pathways Through Regulation of ADAM17 Shedding Activity**

To identify whether PDIA6 regulates ADAM17 activity, ADAM17 shedding activity was indirectly examined by detecting heparin-binding epidermal growth factor (HB-EGF) secreted in conditioned media after treatment with PDIA6 siRNA. HB-EGF is a transmembrane protein,
and ectodomain shedding by ADAM leads to secretion of HB-EGF, which acts as an EGFR signaling ligand.\textsuperscript{23,35,46} In addition, increased HB-EGF expression plays a significant role in the development of malignant phenotypes, thus contributing to the metastatic and invasive behaviors of tumors.\textsuperscript{22} In our study, HB-EGF expression was increased in the total protein of siPDIA6, compared with the control (Fig. 5A, upper panel), and HB-EGF secretion in conditioned media of siPDIA6 was also increased significantly, whereas none was detected in the controls (Fig. 5A, lower panel). Then to investigate whether the EGF induced by siPDIA6 activates EGFR signaling, pEGFR expression was determined by Western blotting. The level of expression of pEGFR was significantly greater in siPDIA6-treated cells than in the control cells.

MT1-MMP is recruited to sites of focal adhesion by FAK, and FAK and MT1-MMP must interact to degrade the matrix at focal adhesions.\textsuperscript{43} FAK also mediates the correlation between EGFR and integrin signaling. Thus, we assessed integrin α5β3 expression and FAK activation by Western blotting. The expression of both factors was significantly higher in siPDIA6 cells than in control cells (Fig. 5B). Integrin α5β3 in the membrane fraction was also induced in siPDIA6 cells (Fig. 5C). These results suggest that activation of ADAM17 by silencing PDIA6 could cleave HB-EGF, and the secreted HB-EGF then activated EGFR signaling.

To confirm whether the correlation between PDIA6 and ADAM17 stimulates EGFR signaling, both PDIA6 and ADAM17 (A+P) were simultaneously knocked down in U87MG cells, and expression of pEGFR, pFAK, integrin, MMP-2, and MT1-MMP was assessed by Western blotting. After double siRNA transfection, silencing of each gene was confirmed by RT-PCR (Fig. 6 left). Expression of pEGFR, pFAK, and MT1-MMP expression decreased in the double siRNA-treated cells compared with that in the control cells, but expression of integrin α5 and MMP-2 in A+P cells was similar or slightly higher than that in the control cells (Fig. 6 right).

PDIA6 Affects Cell-Matrix Adhesion

We expected that integrin induced by PDIA6 knockdown would affect cell-matrix adhesion. Fibronectin is an extracellular matrix glycoprotein that binds to integrin. Therefore, we examined the effect of PDIA6 on cell-matrix interaction on fibronectin-coated and uncoated plates. No difference in adhesion ability was observed between siPDIA6 and control cells on the uncoated plate (p = 0.542, Fig. 7 left), but siPDIA6 cells showed significantly greater adhesion to the coated plate than did the control cells (p < 0.05, Fig. 7 right).

AG1478 and Gefitinib Reduce the Factors Increased by siPDIA6 in U87MG Cells

To verify that EGFR signaling induced by PDIA6 knockdown affects the expression of downstream factors, we treated control and siPDIA6 cells with AG1478 and gefitinib (EGFR inhibitors) and performed a Western blot analysis. After treatment, MMP-2, MT1-MMP, and pFAK expression (Fig. 8A), as well as MMP-2 activity (Fig. 8B),

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**FIG. 5.** Western blot images demonstrating that PDIA6 is associated with the EGFR, integrin, and FAK signaling pathways through regulation of ADAM17 shedding activity. HB-EGF expression was examined in total protein and conditioned media by Western blot (A). Western blot analysis was performed to detect pEGFR, pFAK, and integrin α5 and β3 expression in total protein (B), and integrin α5 and β3 in the membrane fraction (C).

**FIG. 6.** Western blot images demonstrating that the association between PDIA6 and ADAM17 is related to EGFR and FAK signaling. Double PDIA6 and ADAM17 siRNA was transfected into U87MG cells and the knockdown effect was confirmed by RT-PCR (left). EGFR, FAK, MT1-MMP, MMP-2, and integrin α5 expression was detected by Western blot (right). A+P indicates knockdown of both ADAM17 and PDIA6.
decreased compared with expression in untreated cells. Fibronectin was highly expressed in total protein and conditioned media of the PDIA6 siRNA-treated cells compared with expression in control cells, and expression decreased significantly after treatment with the 2 EGFR inhibitors. Integrin α5 and β3 were not affected by the EGFR inhibitors (Fig. 8A).

**AG1478 and Gefitinib Reduce Cell Invasion That Increases in Response to siPDIA6 in U87MG Cells**

To verify whether the EGFR signaling transduced by inhibiting PDIA6 in U87MG cells affects cell invasion, a Matrigel assay was performed using U87MG cells treated with siNC, siPDIA6, and the EGFR inhibitors gefitinib and AG1478. The invasive ability of siPDIA6 cells increased more than 2-fold compared with that of control cells (Fig. 8C, p < 0.01), whereas the invasive ability of the siPDIA6 cells treated with the EGFR inhibitors decreased about 70% (p < 0.05) and 46% (p < 0.01), respectively, compared with that of untreated siPDIA6 cells.

**Discussion**

It has previously been reported that PDI may be positively associated with tumor invasion according to proteome analyses of some types of tumors. In our study, we used wound-healing and Matrigel assays to determine that PDIA6 gene silencing enhanced migration and invasion of U87MG human glioblastoma cells. Studying invasion of brain tumors in a 3D brain-slice model system is more relevant and reliable than using other in vitro mod-

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**FIG. 7.** Graphs showing effect of PDIA6 on cell-matrix adhesion. The adhesion assay was performed using an uncoated plate (left) and a fibronectin-coated plate (right). Cells were incubated for 1 hour, and adherent cells were detected by the MTT assay.

**FIG. 8.** Western blot (A), gelatin zymography (B), and graph of invasion assay results (C). EGFR inhibitors decreased cell invasion and invasion-associated factors, which were induced by inhibition of PDIA6, EGFR tyrosine inhibitors, AG1478 (10 μM) and gefitinib (1 μM), were applied to siPDIA6 transfected U87MG for 24 hours. The graph’s y-axis label indicates the total number of cells invading through Matrigel. C = siRNA negative control; P = PDIA6 siRNA; P+Ge = siPDIA6 siRNA and 1-μM gefitinib; P+AG = PDIA6 siRNA and 10-μM AG1478. *p < 0.05, **p < 0.01.
Some thiol isomerases are located in the endoplasmic reticulum, whereas others are on the cell surface where they are involved in receptor activation and remodeling. For example, ERP5 is recruited to the cell surface and plays a major role in platelet aggregation in response to platelet agonists. ERP5 becomes physically associated with the integrin β3 subunit during platelet stimulation and mediates adhesion to the α2β1-specific peptide by reorganizing the disulfide bonds of integrin. Some studies have reported that PDIA6 negatively regulates ADAM17 shedding activity. The ADAM domain appears to be involved mostly in shedding cell-surface molecules, and ADAM17 induces migration and invasion in various tumors. Cleavage allows cells to send and/or receive signals and change their adhesion to surrounding cells or substrates.

Based on these reports, we considered that the correlation between PDIA6 and ADAM17 might be important for regulating migration and invasion of U87 human glioblastoma cells. We found that ADAM17 and MMP-2 expression increased significantly and that the active form of MMP-2 was induced in PDIA6-silenced cells. In addition, inhibiting ADAM17 led to decreased MMP-2 expression and activity. These results indicate that the association between PDIA6 and ADAM17 is involved in regulating MMP-2 in U87 glioblastoma cells.

Next, we confirmed whether PDIA6 could also regulate ADAM17 shedding activity. ADAM is considered to be a key component in EGFR signaling through ectodomain shedding of EGFR ligands, such as ephiregulin, transforming growth factor alpha, amphiregulin, and heparin-binding epidermal growth factor (HB-EGF). ADAM17 is a primary sheddase for multiple EGFR proligands. EGFR is an important mediator responsible for the invasiveness of malignant gliomas. We hypothesized that if PDIA6 negatively modulates ADAM17 function as a sheddase that EGFR signaling could be activated in U87MG cells. HB-EGF, among many EGFR signaling ligands, is well known to bind EGFR after shedding by ADAM. We examined the expression of pEGFR and HB-EGF secreted in conditioned media of PDIA6-silenced U87MG and control cells. HB-EGF and pEGFR were highly expressed and HB-EGF secretion in the conditioned media was also induced, whereas the control cells showed no or only a minimal signal band. In addition, inhibiting both PDIA6 and ADAM17 also reduced the expression of pEGFR and pFAK but not that of MMP-2, compared with control cells. These results suggest that PDIA6 regulates the shedding activity of ADAM17 and that HB-EGF secreted by ADAM17 transduces EGFR-FAK signaling.

In our study, MT1-MMP, pFAK, and integrin α5β3 expression in total protein and the membrane fraction increased significantly after inhibiting PDIA6 in U87MG cells, and adhesion of siPDIA6 cells was enhanced on fibronectin-coated plates. Finally, we confirmed that EGFR signaling induced by inhibiting PDIA6 affected these factors. After treatment with gefitinib, AG1478, and EGFR tyrosine inhibitors, pFAK, MMP-2, MT1-MMP, and fibronectin expression (induced in PDIA6 cells) decreased but that of integrin α5β3 did not. MMP-2 activity also decreased in the inhibitor-treated cells. Based on the Matrigel assay results, we verified that blocking pEGFR signaling decreased cell invasion induced by siRNA PDIA6 in the U87MG glioblastoma cell line.

Conclusions

Inhibiting PDIA6 enhanced migration and invasion of U87MG glioblastoma cells, and PDIA6 gene silencing increased expression and shedding activity of ADAM17. ADAM17 activation by inhibiting PDIA6, in turn, activated EGFR signaling by shedding HB-EGF. Blocking both PDIA6 and ADAM17 resulted in decreased pEGFR and pFAK expression. Furthermore, after treatment with EGFR inhibitors, pFAK, MMP-2 and MT1-MMP over-
expressed by inhibiting PDIA6 decreased along with cell invasion.

We propose that inhibiting PDIA6 transduces EGFR signaling by activating and inducing ADAM17, a sheddase of EGFR ligands, during migration and invasion of U87MG glioblastoma cells and then regulates downstream factors as follows. 1) EGFR signaling activated by HB-EGF could affect FAK–MT1-MMP–MMP-2 signaling. 2) Induction of MT1-MMP activates proMMP-2 and may act as a sheddase of other EGFR signaling ligands. 3) Induced fibronectin may act as an integrin ligand. 4) FAK signaling may be associated with cytoskeletal reorganization.

MMP-2 and MT1-MMP induced by inhibiting PDIA6 enhanced cell migration and invasion. Our results suggest that PDIA6 is an important component of EGFR-mediated cell migration and invasion of U87MG glioblastoma cells and could be a molecular target for therapy. Although more studies are needed to confirm PDIA6 function, this is the first report of the effect of PDIA6 on migration and invasion in glioblastoma.

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References


Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Jung, Kim, Ryu. Analysis and interpretation of data: Jung, Kim, Ryu, Lim. Administrative/technical/material support: Ryu, S Li, C Li, Lim, Jung. Study supervision: Jung.

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