PTEN gene transfer suppresses the invasive potential of human malignant gliomas by regulating cell invasion-related molecules

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Abstract. Loss of function of the tumor suppressor gene *PTEN* is more frequently encountered in high-grade malignant gliomas than in low-grade gliomas. High-grade gliomas are characterized by their extremely invasive behavior, suggesting that PTEN is one of the important regulators of cell motility and that alterations of its coding gene contribute to a much more invasive tumor cell phenotype. In order to clarify a role of *PTEN* in glioma invasion, we introduced the wild-type *PTEN* gene into human malignant glioma cell lines and investigated their motile and invasive activity in a brain slice model that presents circumstances analogous to normal brain conditions *in vivo*. In addition, we analyzed biochemical and molecular changes resulting from the transfer of *PTEN* in the glioma cells. Infection of recombinant replication-defective adenovirus vector containing the wild-type *PTEN* cDNA (Ad5CMV-*PTEN*) significantly inhibited the cell migration and invasion activities of *PTEN*-mutated glioma cell lines in *in vitro* migration and chemoinvasion assays. In an organotypic brain slice model, co-culture of glioma spheroids and rat brain slices demonstrated that Ad5CMV-*PTEN* transfected cells failed to invade surrounding normal brain tissues. Ad5CMV-*PTEN* transfer into the glioma cell lines lacking the wild-type gene product decreased the levels of matrix metalloproteinase (MMP)-2 mRNA and inhibited the enzymatic activities of MMP-2 and MMP-9. In contrast, mRNA expression of tissue inhibitor of metalloproteinase (TIMP)-2 was upregulated by the *PTEN* gene transfer. Introduction of *PTEN* gene in glioma cell lines markedly reduced the levels of Rac-GTP and Cdc42-GTP, activated forms of these small GTP-binding proteins, and decreased the phosphorylation levels of focal adhesion kinase. These results suggest that PTEN inhibits glioma cell invasion in two ways: suppressing proteolysis of the extracellular matrix by MMPs and modulating the migratory activity of glioma cells to a less motile nature by inactivating two Rho-family GTP-binding proteins, Rac and Cdc42.

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Introduction

The *PTEN* gene, also known as *MMAC1* or *TEP1*, located on chromosome 10q23.3, was identified in 1997 as a tumor suppressor gene (1,2) that is often altered by somatic mutations in various types of cancer, including malignant gliomas (3). Introduction of *PTEN* gene into tumor cells lacking the wild-type gene product has revealed that PTEN takes part in the modulation of a number of cellular processes such as cell growth, cell survival, cell morphology and cell motility (2,4,5). Transfection of wild-type *PTEN* into a number of different cancer cells has been shown to inhibit their growth and survival. Most of the tumor suppressive properties of PTEN are dependent on its lipid phosphatase acitivity, which inhibits the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway through dephosphorylation of phosphatidylinositol-(3,4,5)-triphosphate (1,5). An additional function of PTEN is its protein phosphatase activity, which may also play a role in regulating the invasive behavior of tumor cells by affecting the phosphorylation levels of focal adhesion kinase (FAK) (7). Tamura *et al* (6,7) reported that PTEN could regulate cell interactions with the extracellular matrix (ECM) by inhibiting both cell migration, and the spreading and formation of focal adhesion. In this context, functional restoration of PTEN in tumor cells may provide a powerful tool not only to control tumor growth but also to inhibit tumor invasion.

Malignant gliomas, which are the most common type of intrinsic central nervous system (CNS) malignant tumors, are characterized by their extremely high invasiveness, which makes them resistant to any kind of treatment including radiation and chemotherapy. It is also well known that malignant gliomas very frequently show a loss of heterozygosity in chromosome 10q and that 30-45% of these tumors have *PTEN* mutations (8). Consequently, up-regulation of *PTEN* in malignant gliomas may open up new targeted molecular therapy perspectives to both overcome glioma invasiveness and control tumor growth.

Tumor invasion is a complex process in which tumor cells initiate migration from the primary site of the tumor, adhere to the ECM, and degrade the ECM with the aid of proteolytic enzymes to invade distant normal tissues (9). Key elements of these tumor invasion processes include tumor cell adhesion, migration, and proteolysis of the ECM. However, biological functions of these elements depend on specific tissue environments, and are substantially affected by the individual target organ ECM. The ECM components of the brain are quite different from those of other organs. It is generally believed that except for the basement membrane of cerebral blood vessels and the glia limitans externa, the CNS extracellular space is filled with an amorphous matrix and largely lacks a well-defined ECM such as that provided by collagen and other fibrous proteins (10,11). Therefore, studies for glioma invasion should require special conditions analogous to those of normal brains *in situ*. We have established a novel brain slice model for glial tumor cell invasion by modifying the organotypic culture of brain tissues (12,13).

In the present study, we used the brain slice model to investigate the effects, on tumor cell migratory and invasive activities, of *PTEN* gene introduction into human malignant glioma cells lacking the wild-type gene product. In addition, to understand molecular and biochemical mechanisms underlying the suppressive effects of *PTEN* gene on glioma cell invasion, we examined the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMP) and their enzymatic activities. Furthermore, we studied the levels of FAK dephosphorylation and small GTP-binding proteins, Rho family (Cdc42/Rac/Rho) that are directly related to the motile activity of cells.

Materials and methods

Recombinant adenovirus. Ad5CMV-*PTEN*, a recombinant adenoviral vector, was constructed by following the previously published procedure (14). The Ad5CMV-*PTEN* contains the cytomegalovirus promoter, wild-type *PTEN* cDNA, which was obtained from the human recombinant Ad5CMV-*PTEN* (harboring the human *PTEN* gene), which was manufactured using the Clontech Adeno-X Tet-off system. This was transfected with virus into human embryonic kidney 293 cells to amplify and isolate recombinant adenovirus, which expresses *PTEN*. The control virus Ad5CMV-*LacZ* was generated in the same way. The adenovirus titer was determined by ß-gal staining.

Cell cultures and PTEN gene transfer. Human malignant glioma cell lines U251 and U373 were generously provided by Dr N. Arita (Hyogo College of Medicine, Hyogo, Japan). Both glioma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and antibiotic agents (penicillin G, 100 U/ml; and streptomycin, 100 μg/ml) and incubated at 37˚C under standard conditions of 100% humidity, 95% air and 5% CO_2. Viral stocks were diluted in serum-free medium to obtain the desired multiplicities of infection (MOI) and added to cell monolayer or tumor cell spheroids and incubated at 37˚C for 1 h. The culture medium with 10% fetal calf serum (FCS) was then added, and the cells were incubated for the desired time periods. U251 and U373 cells were infected with Ad5CMV-ß-gal (Ad5CMV-*LacZ*) at various MOI and infected-efficiency curves at 48 h were constructed by counting positive cells after ß-gal staining. In general, the cell infection using Ad5CMV-ß-gal was dose-dependent in the range of 25-200 MOI. More than 80% of infected cells were obtained at 50 MOI in U251 and U373 cells. Six experimental groups were used: U251 not infected with adenovirus (mock), Ad5CMV-*LacZ*-U251, Ad5CMV-*PTEN*-U251, U373 not infected with adenovirus, Ad5CMV-*LacZ*-U373 and Ad5CMV-*PTEN*-U373 groups infected with corresponding adenovirus at 50 MOI. Mock and Ad5CMV-*LacZ* groups were considered to be control groups.

MTT assay. The MTT assay was performed according to the Mosmann method (15). In brief, $5x10^3$ cells (U251 and U373) were seeded in each well in a 96-well plate. Twenty-four hours later, serum-free medium, containing medium alone (mock), Ad5CMV-*LacZ (LacZ)* and Ad5CMV-*PTEN (PTEN)*, were added. At 24, 48 and 96 h after incubation, 20 μl MTT (5 mg/ml) was added, and the plates were incubated for an additional 3 h. Culture medium was removed, the formazan crystals were dissolved in 100 μl DMSO, and the absorbance value was read on a microplate reader (SPECTRAmax, Molecular Device Corp.) at the dual wavelengths of 540 nm and 620 nm.

In vitro migration assay. Migratory responses of glioma cells to their own conditioned medium were assessed using the modified Boyden chamber method with 48-well microchemotaxis chambers (Nucleopore, Pleasanton, CA) as previously described (16,17). In brief, glioma cells transfected with Ad5CMV-*LacZ* or Ad5CMV-*PTEN* were harvested and resuspended in DMEM containing 0.1% BSA at a density of $8x10^5$ cells/ml. Cell suspensions (30 μl) were placed in the upper well of the chamber and serum-free conditioned medium was placed in the lower well as a chemoattractant. The filter was a polyvinylpyrolidone-free polycarbonate membrane with 8.0-μm pores. The chamber was incubated for 4 h at 37˚C under the standard conditions described above, and cells that had migrated to the lower surface of the filter were fixed and stained with Diff-Quik (Scientific Products, Harleco, Gibbstown, NJ, USA). Cells were counted in 4 independent fields (0.25 mm^2/well).

In vitro invasion assay. The invasive activity of glioma cells was assayed *in vitro* using Falcon cell culture inserts (Becton-Dickinson Biosciences, USA) and a reconstituted basement membrane, Matrigel (BD Biosciences, USA), as previously described (18-21). Briefly, glioma cells treated with Ad5CMV-*LacZ* or Ad5CMV-*PTEN* were seeded onto the upper sides of insert filters coated with Matrigel (60 μg/insert) at a density of $1x10^5$ cells/insert. The lower compartment of the plates contained 500 μl of conditioned medium as a chemoattractant. After 2 days at 37˚C under standard conditions, the lower surfaces of the culture inserts were fixed and stained with Diff-Quik. Invasive activities were quantified as described above for migration assays. *In vitro* cell invasiveness was evaluated using the method of Albini. Each Boyden chamber (BD

Biosciences, USA) consists of a BD Falcon TC Companion Plate with Falcon cell culture inserts containing an 8-μm pore polyethylene terephthalate membrane with a thin layer of Matrigel basement membrane matrix. First, the interiors of the inserts were rehydrated for 2 h with warm (37˚C) culture medium. The upper chambers were then filled with 0.5 ml of cell suspension ($5x10^4$ cells/ml). Mixture of 100 ml of cell suspension (cells cultured with serum-free medium for 2 days), lower chamber were conditioned medium. Cells were counted in 4 independent fields (0.25 mm^2/well).

Invasion assay in the brain slice model. All animal experiments were conducted in accordance with the 'Guidelines for Animal Experiments of Ehime University Committee for Ethics of Animal Experimentation'. To evaluate *PTEN* effects on cell motility under more physiological conditions, we produced rat brain slice cultures which mimic *in vivo* central nervous system conditions.

Brain slice culture. A slice culture of rat whole cerebrum was produced by modifying an organotypic culture method described previously (12,22,23). Brain slices were prepared from 2-day-old neonatal Wister rats (male, SLC Inc., Japan). After brief anesthesia with diethyl ether, the rats were plunged into a 10% povidone-iodine solution, and the heads were cutoff with scissors. The whole brains were quickly removed and placed in Hanks Balanced Salt Solution (HBSS; Life Technologies, Inc., Rockville, MD). Each brain was then cut vertically to the base, 1 mm inward from both rostral and caudal ends of the cerebrum, and mounted on the stage of a microslicer (Dosaka EM Co. Ltd., Kyoto, Japan). Slices of 450-μm thickness were cut and transferred onto double-layered membranes, which consisted of two different membrane types. The upper membrane was a polycarbonate membrane with 8-μm pores (Nucleopore, Pleasanton, CA) and was placed on a membrane with 0.4-μm pores (Millicell-CM, Nihon Millipore Ltd., Tokyo, Japan), with the latter functioning as a lower membrane affixed to the bottom of an upper chamber. These double-layered membranes were then placed in the wells of 6-well culture plates filled with PBS. After the brain slices had been placed on the membranes, the PBS was aspirated and 1 ml of culture medium was added to each well of the plate but without submerging the brain slices. The slice culture medium consisted of 50% Eagle's MEM with HEPES, 25% HBSS, 25% heat-inactivated horse-serum (Life Technologies), 6.5 mg/ml glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Fungizone). The brain slice cultures were incubated at 37˚C under standard conditions of 100% humidity, 95% air, and 5% CO_2. Half of the medium was replaced with fresh medium three times a week. The volume of the medium after the second change was reduced to allow the slices to remain well exposed to the air. This was critical for long-term survival of the neuronal cells in these slices.

Glioma cell spheroids. U251 and U373 human glioma cells were grown as a monolayer culture in DMEM supplemented with 10% FBS. The U251 and U373 cells were labeled with the PKH2 fluorescent cell staining kit (ZYNAXIS Cell Science, Inc., Malvern, PA) as described previously (24). Briefly, the U251 and U373 cells were harvested by trypsinization, washed twice, and resuspended in labeling diluent 'C' to make a 4 μM solution. This solution was added to the cell/diluent suspension and mixed by gentle agitation. After incubating the cells at room temperature for 5 min, the labeling reaction was stopped by adding a double volume of the medium containing 10% FBS and four times the volume of FBS into the sample tubes. The cells were then washed and resuspended in DMEM containing 10% FBS. The labeled U251 and U373 glioma cells ($5x10^6$) were seeded onto a 1.25% agar-coated culture dish (100 mm in diameter, Iwaki Glass, Chiba, Japan). The cells were incubated under continuous agitation at a speed of 40 rpm on a reciprocating shaker (Taitec, Saitama, Japan) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air for 2-3 days. U251 and U373 glioma cell spheroids obtained with this method had a diameter ranging from 300-400 μm.

Invasion assay in brain slices. One rhodamine-labeled spheroid of glioma cells was pipetted, placed on a brain slice as close to the corpus callosum as possible, and co-cultured at 37˚C under standard conditions for 3 days. Cell invasion was observed by a fluorescence microscopy on day 0 and 3.

Western blot analysis. The U251 and U373 cells cultured with serum-free medium were infected with Ad5CMV-*PTEN* at a MOI of 50. After 24 or 48 h, the cells were washed twice with PBS and harvested in lysis buffer. After the cells were homogenized and centrifuged at 10,000 g for 30 min, the supernatants were standardized for protein concentration using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Protein (10 μg) from each sample was resolved by 10% sodium dodecyl sulfate (SDS)-Tris glycine gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were incubated with primary monoclonal antibodies against the PTEN, FAK and phospho-FAK (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), followed by horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham, Piscataway, NJ). The membranes were developed according to the Amersham enhanced chemiluminescence (ECL) protocol.

Non-radioactive Rac/Cdc42 Activation Assay Kit (Chemicon International, Inc., Temecula, CA) utilizes a specific probe to detect the active conformation of Rac or Cdc42 GTPase, the downstream effector: p21 activated kinase (PAK1). PAK1 contains in the N-terminal regulatory region a specific interaction site for the active forms of Rac or Cdc42 GTPase. Following either GTP loading of purified Rac/Cdc42 or preparation of endogenous Rac/Cdc42, PAK1 is added and pulls down GTP-bound Rac or Cdc42. PAK1 will only bind the active GTP-Rac/Cdc42 state, it does not bind the inactive GDP-Rac/Cdc42 form. Subsequently, an immunoblot was performed and PAK-precipitated GTP-Rac/Cdc42 is detected by probing the membrane with a Rac or Cdc42 specific monoclonal antibody, followed by HRP-conjugated secondary antibody and the ECL Detection System.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extracted from four human malignant glioma cell lines using acid guanidinium isothiocyanate phenol chloroform were used as templates for cDNA synthesis (25). Each RT-PCR reaction consisted of 30 cycles at 94˚C for 30 sec and 72˚C for 1 min, followed by incubation at 72˚C for 5 min. The products

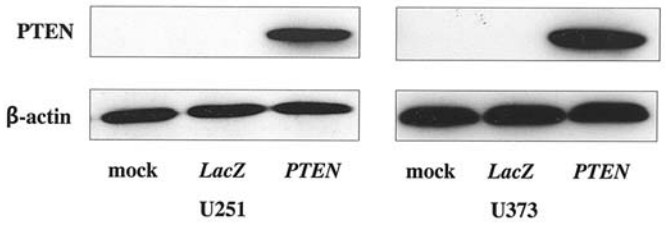


Figure 1. Exogenous expression of PTEN in adenovirus transfected glioma cells. U251 and U373 cells were transfected with medium alone (mock), Ad5CMV-*LacZ (LacZ)*, or Ad5CMV-*PTEN (PTEN)*. Cellular extracts were prepared 24 h after transfection, and an equal amount of protein from each lysate was subjected to Western blot analysis. Endogenous and exogenous PTEN were detected with a monoclonal antibody against wild-type PTEN. Top panels show the PTEN protein, and ß-actin control is shown in the bottom panels.

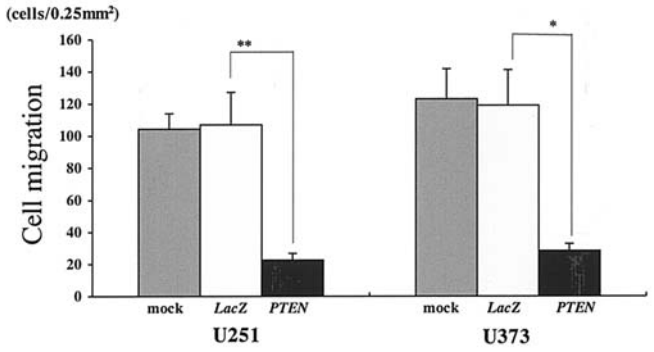


Figure 3. Effects of *PTEN* gene transfer on migration activity of glioma cells. Migration activity of the glioma cells was evaluated by the modified Boyden chamber assay method. Glioma cells transfected with medium alone (mock), Ad5CMV-*LacZ (LacZ)*, or Ad5CMV-*PTEN (PTEN)* were placed in the upper well and the conditioned medium of each treated cell type served as chemoattractant in the lower well. Cells that migrated to the lower surface of the membrane with 8-μm pores were stained with Diff-Quik and counted. Values represent means ± SD of five experiments. $^{*}p<0.005$; $^{**}p<0.0005$ versus *LacZ*.

were electrophoresed on 1% agarose gels including 0.1 μg/ml ethidium bromide. PCR was performed with human MMP-2, MMP-9, human TIMP-1, TIMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (as an internal standard)-specific primers. Primers (Hokkaido Biosystems, Sapporo, Japan) used were 5'-CTCTCCTGACATTGACCTTGGCAC-3' and 5'-AAAAAGCTTACTCGCTGGACATCAGGG-3' for MMP-2, 5'-GGCATCCGGCACCTCTATGGTCC-3' and 5'-GCCACTTGTCGGCGATAAGGAAGG-3' for MMP-9, 5'-TTCCTGGGGACACCAGAAGTCAAC-3' and 5'-TGG ACACTGTGCAGGCTTCAGTTC-3' for TIMP-1, 5'-CTCG GCAGTGTGTGGGGTC-3' and 5'-CGAGAAACTCCTGCT TGGGG-3' for TIMP-2 and 5'-CAAAGTTGTCATGGATG ACC-3' and 5'-CCATGGAGAAGGCTGGGG-3' for GAPDH.

Gelatin zymography. Gelatin zymography (26) was carried out by analyzing conditioned media of glioma cells infected with either Ad5CMV-*PTEN* or the controls (mock and Ad5CMV-LacZ) using 10% SDS-PAGE containing 1 mg/ml of gelatin (Sigma, USA). Cells ($5x10^5$ cells) were placed in 500 μl of serum-free DMEM and incubated for 24 h. The supernatants were mixed with the solution buffer (2% w/v SDS, 10% glycerol, 50 mmol/l Tris-HCl pH 6.8, and 0.005% bromophenol blue) and then 25 μl aliquots of this mixture were loaded on the gel. After electrophoresis, the gels were washed twice in the washing buffer (50 mmol/l Tris, 0.1 mmol/l NaCl, 2.5% Triton X-100) for 1 h and incubated for 24 h at 37˚C in the activating buffer (50 mmol/l Tris-HCl, 5 mmol/l $CaCl_2$, 1 μmol/l $ZnCl_2$, 0.02% Brij-35, pH 7.5). Gels were then stained with 0.1% Coomassie brilliant blue G-250 diluted in 40% methanol and 10% acetic acid, and destained in 10% methanol and 5% acetic acid until clear proteolytic bands were obtained on a homogeneous blue background.

Statistical analysis. The results were representative of experiments repeated at least three times and the values were expressed as means ± standard deviation (SD). Statistical comparisons between groups were carried out using the Student's t-test (StatView, SAS institute Inc. NC). $p<0.05$ were considered statistically significant.

Results

Expression of PTEN protein and cell viability after Ad5CMV-PTEN transfection. Expression of PTEN protein following

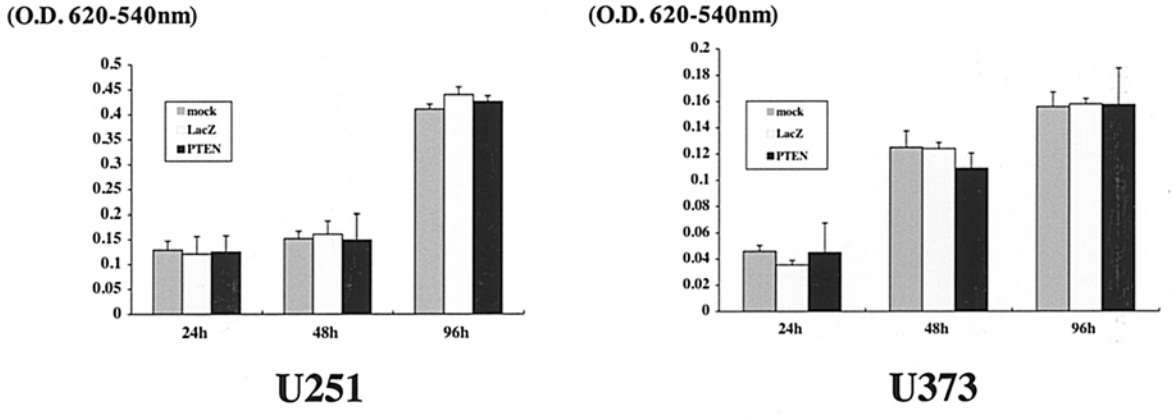


Figure 2. Cell viability assessed by MTT assay. The glioma cells were seeded at $2x10^4$ cells/well 24 h before transfection with medium alone (mock), Ad5CMV-*LacZ (LacZ)* or Ad5CMV-*PTEN (PTEN)*. At the indicated times (24, 48 and 96 h after transfection), the absorbance value was read on a microplate reader at the dual wavelengths of 540 and 620 nm. Values represent means ± SD of eight experiments.

infection with Ad5CMV-*PTEN* at MOI of 50 was confirmed by Western blot analysis. Endogenous PTEN protein was not detected in U251 or U373 cells. Infection with Ad5CMV-*PTEN* produced PTEN proteins at significant levels in both cell lines (Fig. 1). Cell viability after treatment with Ad5CMV-*PTEN* was assessed by MTT assay. At 24, 48 and 96 h after infection with Ad5CMV-*PTEN*, there were no differences in cell viability for either glioma cell type infected with Ad5CMV-*PTEN*, compared with mock- or Ad5CMV-*LacZ*-infected cells (Fig. 2). Therefore, we carried out all subsequent experiments within 96 h after transfection with adenoviral vectors.

PTEN gene transfer inhibits glioma cell migration and invasion in vitro. The *in vitro* migration assay demonstrated that *PTEN* gene introduction significantly decreased cell migration in both U251 and U373 glioma cells compared to the mock- and LacZ-treated groups (Fig. 3).

The *in vitro* invasion assay showed that U251 and U373 glioma cells treated with medium alone (mock) or Ad5CMV-*LacZ* invaded to the lower side of the insert filter after 2 days incubation. By contrast, Ad5CMV-*PTEN* transfer virtually suppressed cell invasion through the Matrigel (Fig. 4A). In U251 cells, the mean values of invasive cells were 55.3, 58.3 and 23.0 respectively for mock-, Ad5CMV-*LacZ*- or Ad5CMV-*PTEN*-infected groups. In U373, the mean values of invasive cells were 50.3, 49.6 and 21.4 respectively for mock-, Ad5CMV-*LacZ*- or Ad5CMV-*PTEN*-infected groups. The invasive potential of glioma cells transfected with Ad5CMV-*PTEN* was significantly reduced compared with that of cells from the mock- or Ad5CMV-*LacZ*-treated groups (Fig. 4B).

Restoration of PTEN suppresses glioma cell invasion in the brain slice model. To evaluate the effects of Ad5CMV-*PTEN* on cell motility under more physiological conditions, we examined how far Ad5CMV-*PTEN*-tranfected glioma cells could migrate on organotypic rat brain slices. The brain slice model enables us to observe the invasion of glioma cells into the surrounding brain tissue, in conditions analogous to those of normal brains *in situ*. The glioma cells treated with medium alone or Ad5CMV-*LacZ* actively migrated around the tumor spheroids, whereas cells from both cell lines treated with Ad5CMV-*PTEN* were markedly inhibited in their migration (Fig. 5).

PTEN expression reduces phosphorylation of FAK. To examine the effects, in glioma cells, of *PTEN* restoration on the expression of FAK and phosphorylated FAK, Western blot analysis was carried out using anti-FAK monoclonal antibodies and anti-phosphorylated FAK monoclonal antibodies. FAK protein expression in glioma cells in the *PTEN*-transfected group was not different from expression levels in control groups. On the other hand, the expression levels of phosphorylated FAK protein in both glioma cell types from the *PTEN*-transfected group were markedly decreased compared with levels in the cells from control groups (Fig. 6).

PTEN expression reduces activation of Rac/Cdc42. To examine the effects of *PTEN* infection to glioma cells on the expression of total Rac/Cdc42 and Rac-GTP/Cdc42-GTP, we used the Chemicon Rac/Cdc42 activation assay kit. Western blot analysis was carried out using anti-Rac monoclonal antibodies and anti-Cdc42 monoclonal antibodies. Expression of total Rac or total Cdc42 protein was not different in U251 glioma cells from *PTEN*-transfected or control groups. On the other hand, the expression levels of Rac-GTP or Cdc42-GTP protein were markedly decreased in U251 glioma cells from the *PTEN*-transfected group compared with levels in cells from the control groups (Fig. 7).

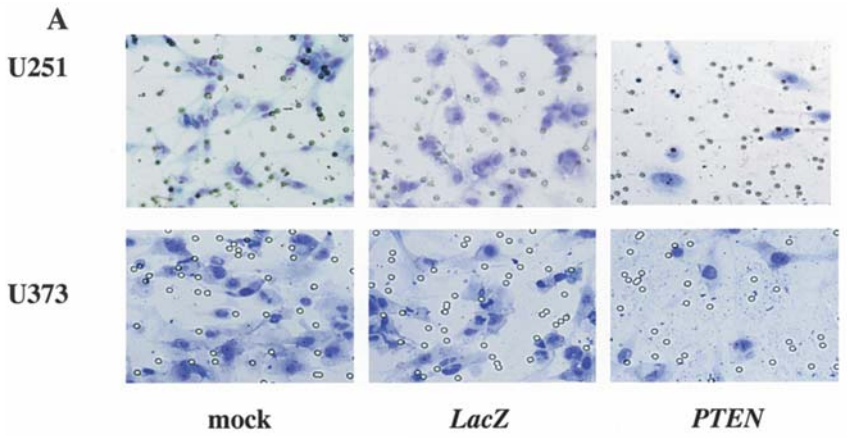
PTEN gene transfer downregulated MMP-2 expression but upregulated TIMP-2 expression. Since MMP-2 and TIMP-2 cooperate in glioma cells to regulate the ECM degradation, we analysed both MMP-2 and TIMP-2 mRNA expression in glioma cell lines that were treated with Ad5CMV-*PTEN*. In addition, we also analysed MMP-9 and TIMP-1 mRNA expression. Fig. 7 shows the constitutional mRNA expression of MMP-2, 9 and TIMP-1, 2. We detected MMP-2, MMP-9 and TIMP-1 in all samples. However, MMP-2 mRNA levels were extremely low in glioma cells treated with Ad5CMV-*PTEN*. TIMP-2 mRNA levels were very low in glioma cells from the untreated and Ad5CMV-*LacZ*-treated groups. However, TIMP-2 mRNA levels were high in glioma cell treated with Ad5CMV-*PTEN*. On the other hand, both MMP-9 and TIMP-1 mRNA levels in both glioma cell types were not affected by *PTEN* gene transfection (Fig. 8).

Enzymatic activities of MMP-2, -9 are downregulated by PTEN introduction. We examined the enzymatic activities of MMPs in the conditioned medium from each cell line by gelatin zymography. Conditioned medium from U251 and U373 cells transfected with medium alone (mock) or Ad5CMV-*LacZ* revealed 72 and 92 kDa lytic bands, demonstrating the secreted enzymatic activity of MMP-2 and MMP-9, respectively. In contrast, the intensity of this lytic zone was markedly reduced in the conditioned medium from Ad5CMV-*PTEN* transfected cells, indicating the inhibitory effect of *PTEN* on the proteolytic activity of MMPs (Fig. 9).

Discussion

In the present study, we demonstrated that forced expression of PTEN in human malignant glioma cells significantly suppressed their strong migratory and invasive activity in the organotypic brain slice culture model as well as in conventional *in vitro* migration and chemoinvasion assay methods. Since the brain slice is non-immunogenic and preserves both intrinsic ECM structures and normal brain cytoarchitecture for relatively long periods of more than one month, co-culture of human glioma cells in rat brain slice tissues is thought to be a good model that mimics biological and biochemical events which actually occur between brain tumor cells and normal brains *in vivo*. Introduction of *PTEN* gene in glioma cells lacking the wild-type gene product inhibits the diffuse spreading of the tumor cells around the tumor spheroid in the brain slice, thus creating a well-demarcated tumor mass which is the same as the intitial tumor spheroid for at least one week.

It is known that introduction of wild-type *PTEN* gene suppresses growth of malignant glioma cells deficient in the



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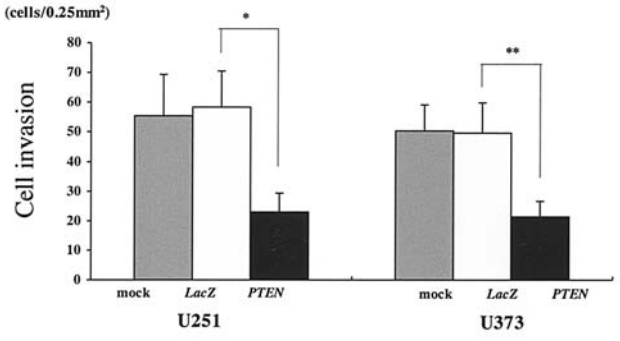

Figure 4. Effects of *PTEN* gene transfer on glioma cell invasion *in vitro*. A, Microphotographs show that U251 (upper) and U373 (lower) glioma cells with each gene transfection invaded Matrigel to the lower side of the insert filter with 8-μm pores 48 h after incubation. Conditioned medium from glioma cells with each transfection served as chemoattractants in the lower wells of Falcon 24-well plates. Original magnification, x200. Left, medium alone (mock). Middle, Ad5CMV-*LacZ (LacZ)*. Right, Ad5CMV-*PTEN (PTEN)*. B, The number of invading cells was counted after a 2 day incubation. Values represent means ± SD of three experiments. $^{*}p<0.0002$; $^{**}p<0.0001$ versus *LacZ*.

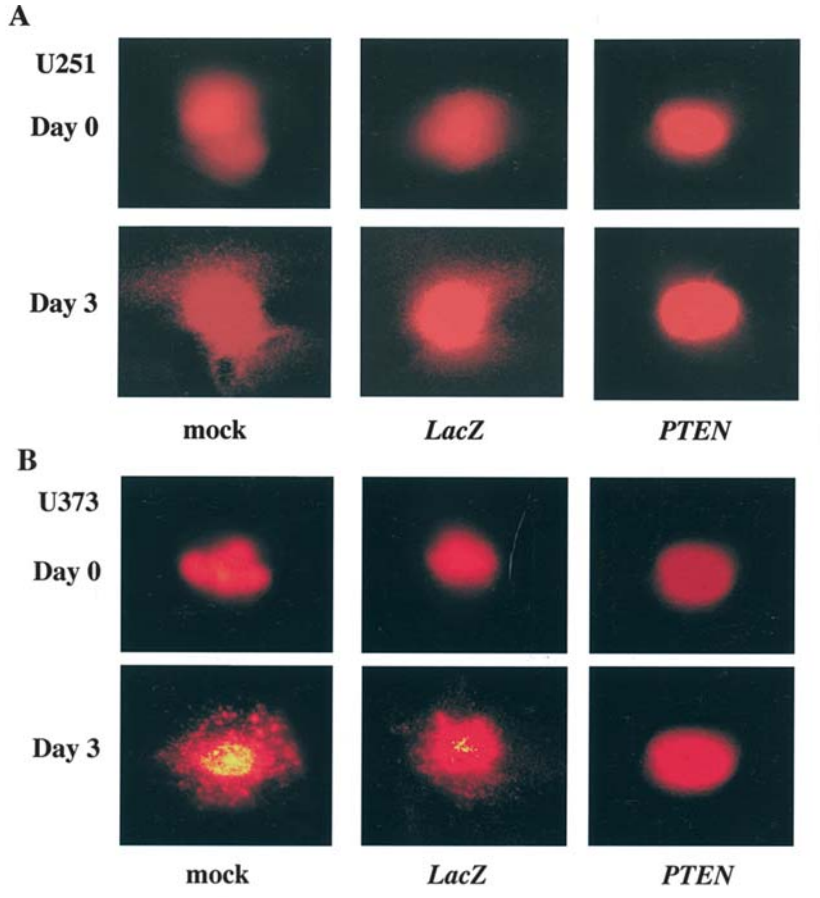

Figure 5. Invasive behavior of glioma cells *PTEN* gene transfer on rat brain slices. Rhodamine labeled spheroids of glioma cells that had been transfected with medium alone (mock), Ad5CMV-*LacZ (LacZ)* or Ad5CMV-*PTEN (PTEN)* were co-cultured with rat brain slices. Fluorescent microphotographs show U251 (A) and U373 (B) cell invasion around the original glioma spheroids. Top panels show the invasion at day 0, and the invasive patterns at day 3 are shown in the bottom panels. Original magnification, x40.

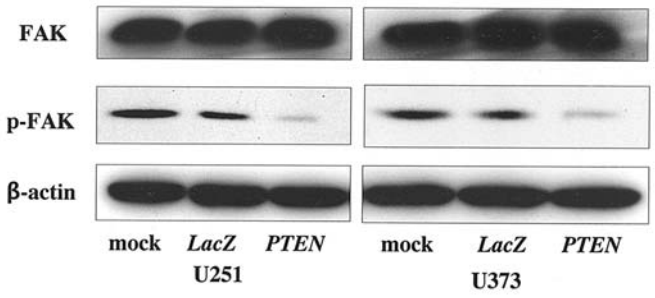

Figure 6. Expression of FAK and phosphorylated FAK proteins. Cellular extracts of glioma cells were prepared 48 h after transfection with medium alone (mock), Ad5CMV-*LacZ (LacZ)* or Ad5CMV-*PTEN (PTEN)* and subjected to Western blot analysis using a monoclonal antibody against FAK (upper) or phosphorylated FAK (middle). ß-actin were used as an internal control (lower).

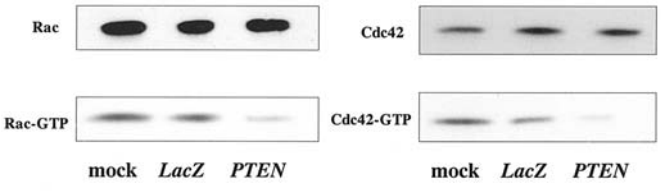

Figure 7. Expression of total Rac and Rac-GTP proteins, and total Cdc42 and Cdc42-GTP proteins. Cellular extracts of U251 cells were prepared 48 h after transfection with medium alone (mock), Ad5CMV-*LacZ (LacZ)* and Ad5CMV-*PTEN (PTEN)* and subjected to Western blot analysis using a monoclonal antibody against Rac or Cdc42 to assess total Rac and Cdc42 proteins. To detect active forms of Rac or Cdc42, cellular extracts of U251 cells after precipitating with PAK1 were subjected to Western blot analysis using the monoclonal antibody against Rac or Cdc42. Top panels show total Rac (left) and total Cdc42 (right) protein expression. Lower panels show active Rac-GTP (left) and Cdc42-GTP (right) protein expression.

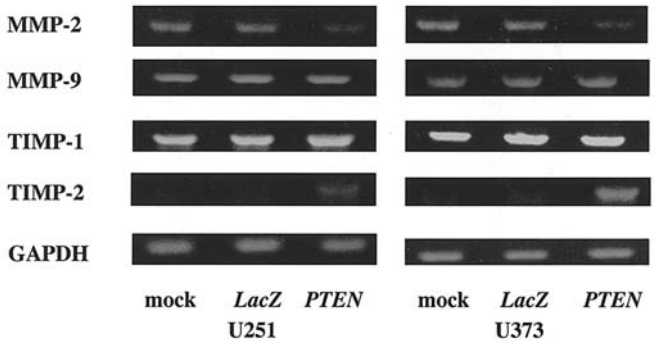

Figure 8. Expession of MMP-2, -9 and TIMP-1, -2 mRNAs in glioma cells transfected with medium alone (mock), Ad5CMV-*LacZ (LacZ)* or Ad5CMV-*PTEN (PTEN)*. Total mRNA extracted from glioma cells with each transfection was amplified by RT-PCR. PCR products resolved by electrophoresis on 1% agarose gels were visualized by ethidium bromide staining. GAPDH was used as an internal control.

functional gene product, indicating a tumor suppressor role of PTEN in malignant gliomas (5). Furthermore, stable expression of PTEN in U251 glioma cells inhibits their growth and induced anoikis, which is also supported by the observation that knockout of the *PTEN* locus results in cells with an increased anchorage-independent growth (27). In the present study, temporary expression of the *PTEN* gene in glioma cell

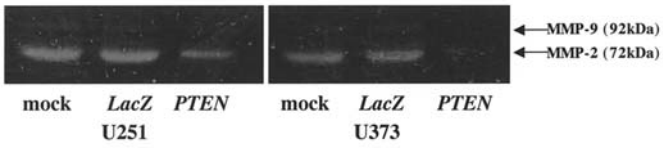

Figure 9. The enzymatic activity of MMPs assessed by gelatin zymography. The culture supernatant from glioma cells (U251 and U373) that were transfected with medium alone (mock), Ad5CMV-*LacZ (LacZ)*, or Ad5CMV-*PTEN (PTEN)* was used for gelatin zymography. Clear lytic zones at the molecular weight of 72 and 92 kDa demonstrate the secreted enzymatic activity of MMP-2 and MMP-9, respectively.

lines through Ad5CMV-*PTEN* transfection did not inhibit cell growth at least during the 96-h period after initiating the culture. All experiments in the present study employing a gene transfection were performed within 96 h after introduction of *PTEN* or *LacZ* genes. Consequently, we think that the growth-inhibitory effects of *PTEN* can be excluded in the experiments on glioma cell migration and invasion.

On the basis of current knowledge in the field, PTEN has several functions as a tumor suppressor. PTEN participates in regulating apoptosis and growth through its lipid phosphatase activity, which regulates the levels of PIP3, from which the activation of Akt, and the processes of apoptosis and anoikis are then promoted (7). On the other hand, PTEN contributes to the regulation of cell adhesion, migration, and invasion by possibly controlling the ERK (MAP kinase) signaling pathway through its FAK-targeting protein tyrosine phosphatase activity (28). In the present study, *PTEN* transfection decreased the expression levels of phosphorylated FAK in glioma cells. Cai *et al* (29) reported that PIP3K inhibitors could not decrease the glioma cell invasion. Collectively these results suggest that inhibitory effects of PTEN on glioma cell migration and invasion depend on its protein phosphatase activity, particularly in terms of FAK dephosphorylation. Furthermore, introduction of PTEN to glioma cells markedly decreased the expression of both Rac-GTP and Cdc42-GTP, the activated forms of these GTP-binding proteins. It is well known that small GTP-binding proteins of Rho family (Cdc42/Rac/Rho), regulate cell motility in malignant tumors. Liliental *et al* (30) reported that immortalized fibroblasts from *PTEN*-knockout mice showed increased cell motility compared with wild-type control cells. The increased cell motility of *PTEN*-knockout fibroblasts was associated with strongly elevated Rac1-GTP and Cdc42-GTP levels. Although further studies are required to elucidate the details of the signaling pathway between *PTEN* restoration in glioma cells and the phosphorylation status of Rho family-members, it is most probable that PTEN-mediated inhibition of glioma cell migration and invasion occurs as a consequence of inactivation of Cdc42 and Rac inactivation, thus suppressing the formation of filopodia and lamellipodia, respectively, the morphological equivalents of active cell locomotion.

One of the common features in the tumor invasion process is the degradation of the surrounding ECM by an array of proteolytic enzymes secreted by the infiltrating tumor cells. Matrix metalloproteinases (MMPs), capable of degrading almost all ECM components, have been suggested to play an important role in mediating tumor invasion in many kinds of

cancer cells (31). It has been demonstrated that malignant gliomas showing strong tumor invasive activities expressed high levels of MMPs (32). Several groups have shown that introduction of *PTEN* reduced activation of MMPs and inhibited glioma invasion (28,33,34). In the present study, we found that PTEN reduced not only expression of MMP-2 in glioma cells, but also the enzymatic activities of MMP-2 and MMP-9 as determined by gelatin zymography. As *PTEN* transfection in glioma cells also up-regulated the expression levels of TIMP-2, introduction of the *PTEN* gene resulted in high anti-proteolytic conditions in the glioma cells, thereby strongly inhibiting the tumor invasion in the brain slice. Although the molecular mechanisms by which PTEN inhibits the expression and activity of MMPs are not known, inactivation of integrin receptors may be important in the biochemical reaction. We found that *PTEN t*ransfection reduced the expression of αvß3 integrin (unpublished data) in glioma cells. The inside-out signaling pathway of the integrins may be affected through the PTEN-mediated dephosphorylation of FAK.

In conclusion, the present results show that *PTEN* gene transfer to human malignant glioma cells lacking functional PTEN can reduce tumor cell migratory and invasive activities in both *in vitro* migration/invasion assays and co-culture systems using organotypic brain slices. Although detailed mechanisms of the inhibitory effects of PTEN on the glioma cell migration and invasion are not clear, down-regulation of MMPs levels and the inhibition of the enzymatic activities along with the suppression of TIMP-2 expression are thought to play a crucial roles in the biological process. Also, PTEN-mediated inactivation of Rac and Cdc42 may inhibit the motility of the glioma cells, thus reducing tumor invasion. More precise studies on PTEN functions and related signaling pathways in tumor invasion would ensure the development of an effective anti-invasion therapy for patients with malignant gliomas.

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