Silencing hypoxia-inducible factor-1α inhibits cell migration and invasion under hypoxic environment in malignant gliomas

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Abstract. Malignant gliomas are characterized by active invasiveness, necrosis, and vascular proliferation. These pathological features have been speculated to be caused by tissue hypoxia. Hypoxia-inducible factor-1 (HIF-1), which is controlled by rapid stabilization of the HIF-1α subunit, is a pivotal transcriptional factor in the cellular response to hypoxia. Although many studies have described the relationship between tumor angiogenesis and hypoxic environment, the roles of HIF-1 in cell invasion have been barely elucidated in malignant gliomas. We investigated the role of HIF-1α in the motile and invasive activities of human glioma cells under hypoxia. Four malignant glioma cell lines, U87MG, U251MG, U373MG, and LN18, were cultured under 21 and 1% oxygen concentration. Expression of HIF-1α under hypoxia was observed to be much higher than that under normoxia in all cell lines. Introducing HIF-1α-targeted small interfering RNA (HIF-1α siRNA) into the glioma cell lines resulted in downregulation of HIF-1α expression, and significantly suppressed glioma cell migration *in vitro*. Furthermore, invasiveness was significantly reduced in the cells transfected with HIF-1α siRNA compared with those transfected with the control siRNA. Co-culture of glioma spheroids and rat brain slices showed that HIF-1α siRNA-transfected glioma cells failed to invade the surrounding normal brain tissue in an organotypic brain slice model. These effects of HIF-1α siRNA were more conspicuous under hypoxia than under normoxia. In addition, under hypoxic conditions, the level of matrix metalloproteinase (MMP)-2 mRNA was upregulated, and that of tissue inhibitor of metalloproteinase (TIMP)-2 was downregulated in all glioma cell lines. Treatment with HIF-1α siRNA resulted in downregulation of MMP-2 mRNA and upregulation of TIMP-2 mRNA. Furthermore, the enzyme activities of MMP-2 and MMP-9, both of which were activated by hypoxia, decreased with the introduction of HIF-1α siRNA. These findings suggest that overexpression of HIF-1α induced by hypoxic stress is an essential event in the activation of glioma cell motility through alteration of invasion-related molecules. Targeting the HIF-1α molecule may be a novel therapeutic strategy for malignant gliomas.

Introduction

Almost all primary brain tumors of glial cell origin, especially malignant gliomas, are characterized by high invasiveness. This usually prevents complete remission, as infiltrative growth into the surrounding normal brain is responsible for the ineffectiveness of traditional therapeutic modalities such as surgery, radiotherapy, and chemotherapy. To overcome glioma invasion, better understanding of its molecular and cellular mechanisms is required.

It is well known that hypoxia affects tumor cells both genetically and epigenetically, and contributes to tumor heterogeneity and aggressive growth patterns. Like other malignant solid tumors, malignant gliomas proliferate under the hypoxic conditions with reduced supplies of oxygen and nutrients that occur after cell proliferation. Tumor progression under the adverse conditions of hypoxia requires an effective adaptation to the environment. Hypoxia-inducible factor-1 (HIF-1) is a master transcription factor of oxygen-regulated genes and plays an essential role in adaptation to hypoxia (1,2). HIF-1 recognizes a hypoxia response element consensus sequence in the promoter of a broad range of target genes related to various aspects of tumor biology, including cell proliferation, cell survival, angiogenesis, and extracellular matrix (ECM) metabolism. HIF-1 is a heterodimeric, basic, helix-loop-helix protein composed of HIF-1α and HIF-1ß subunits. HIF-1α, which is rapidly degraded in normoxia, becomes stabilized

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and activated under hypoxic conditions and forms complexes with constitutively expressed HIF-1ß (3). Therefore, HIF-1 accumulates under hypoxic stimuli, and HIF-1α is detected in large amounts in solid tumors containing hypoxic regions. Previous studies have shown that overexpression of HIF-1α in surgical specimens is positively related to tumor aggressiveness and poor prognosis in human neoplasms (4-7). Significant upregulation of HIF-1α expression has been demonstrated in high-grade gliomas compared with low-grade gliomas, and is associated with treatment failure and increased mortality (8,9). Recently, the role of HIF-1α in tumorigenesis has attracted much attention for the development of novel anti-cancer therapies, although most studies have focused on the oncological effects of HIF-1α on cell growth and angiogenesis. As mentioned above, tumor invasion is an important prognostic factor in patients with glioma. Therefore, elucidation of the relation between HIF-1α expression and glioma cell invasion would provide useful information for the establishment of HIF-1-targeted therapy for malignant glioma. Moreover, little is known about the influence of HIF-1α expression induced by hypoxia on glioma cell invasion.

In this study, we investigated whether downregulation of HIF-1α synthesis alters glioma cell migratory and invasive activities under hypoxic condition using a small interfering RNA for HIF-1α (HIF-1α siRNA). To analyze cell motility under normal living conditions, we examined changes in the motile capacity of glioma cells provoked by inhibition of HIF-1α expression in a brain slice model analogous to normal brain conditions *in vivo*. To determine the molecular and biochemical mechanisms of the suppressive effects of HIF-1α siRNA on glioma cell invasion, we examined the expression and enzymatic activities of matrix metalloproteinase (MMP)-2 and -9 and tissue inhibitors of metalloproteinase (TIMP)-2.

Materials and methods

Cell cultures and hypoxic treatment. Human malignant glioma cell line U87MG was obtained from the American Type Culture Collection (Manassas, VA), U251MG and U373MG were generously provided by Dr N. Arita (Hyogo College of Medicine, Hyogo, Japan), and LN18 was generously donated by Dr M. Tada (Hokkaido University School of Medicine, Sapporo, Japan). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (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 (normoxia). For hypoxic treatment, the cells were incubated in an atmosphere of 1% O_2, 5% CO_2, and 94% N_2 under intermittent flushing with nitrogen, then sealed and incubated at 37˚C for 2, 8, 16, and 24 h. To evaluate the gene silencing effect of RNA interference by Western blot analysis, cells were incubated for 2 h under hypoxic conditions.

Treatment of cells with siRNA. HIF-1α siRNA was designed by searching the coding sequence of HIF-1α (GenBank accession no. NM_001530) for two adenines followed by nineteen nucleotides that had a GC content of ~50% and did not contain more than three thymines or adenines in a row. According to this rule, the target sequence for human HIF-1α mRNA was localized at a position of 373 bases downstream of the start codon. The forward and reverse RNA strands (CUA ACU GGA CAC AGU GUG U *dtdt*) and (ACA CAC UGU GUC CAG UUA G *dtdt*) with two deoxythymidine overhangs were synthesized and annealed (Dharmacon Research, Inc.). These sequences were checked for nonhomology to any human gene by BLAST. For transfection, 2 μl siLentFect Lipid Reagent (Bio-Rad Laboratories, Hercules, CA) was mixed with 98 μl of Opti-MEM (Invitrogen, San Diego, CA, USA), and HIF-1α siRNA was diluted with Opti-MEM to obtain 200 μl of 60 nM solution. These two solutions were combined, gently mixed, and incubated at room temperature for 15 min to form the transfection mixture. All glioma cell lines ($1x10^5$ in 2 ml of medium containing serum) were seeded in 6-well plates and incubated for 24 h before the transfection. Then, DMEM was removed and replaced with 1 ml of the same fresh medium. The transfection mixture was added to all cultures, making a final siRNA concentration of 10 nM, and the cells were incubated at 37˚C for 6 h under normoxic conditions. The mixture was replaced with fresh medium containing serum, and glioma cells could be assayed from 16 h thereafter. As a control for HIF-1α siRNA, we used a corresponding random siRNA sequence (AUU GUA UGC GAU CGC AGA C *dtdt*). These sequences were tested for possible homology to other human genes using BLAST.

Western blot analysis. After normoxic or hypoxic treatment, the culture dishes were immediately placed on ice and washed twice with ice-cold PBS and harvested in lysis buffer. Protein concentrations were determined with a BCA protein assay kit (Pierce Biotechnology, Inc. Rockford, IL). Protein (10 μg) from each sample was separated 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 blocked with 1% dry milk and 0.1% Tween-20 in Tris-buffered saline and incubated with primary antibodies, mouse anti-human HIF-1α monoclonal antibody (Transduction Laboratories), and human anti-ß-actin monoclonal antibody (Sigma-Aldrich Fine Chemical, St. Louis, MO), followed by horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham, Piscataway, NJ). The membranes were developed according to the Amersham enhanced chemiluminescence protocol.

Cell viability assay. To assess the effect of hypoxic conditions and siRNA transfection on glioma cell viability, an MTT assay was performed according to the method of Mosmann (10). In brief, $3x10^3$ cells (U87MG, U251MG, U373MG, and LN18) were seeded in each well of 96-well flat-bottom plates. For the effect of hypoxia, the culture dishes were incubated at the same time under the normoxia and hypoxia. For the effect of siRNA transfection, 24 h after incubation, HIF-1α siRNA and control siRNA were transfected as described. At 72 h after incubation under 21 and 1% oxygen concentration, 10 μl MTT (5 mg/ml) was added, and the plates were incubated for an additional 3 h. The culture medium was removed, the formazan crystals were dissolved in 100 μl isopropanol with 0.04 N HCl, and the absorbance was read on a microplate reader (SpectraAmax, Molecular Device Corp.) with a test wavelength of 540 nm and a reference wavelength of 620 nm.

In vitro migration assay. The migratory responses of glioma cells to their own conditioned medium were assessed using the modified Boyden chamber method with 48-well micro-chemotaxis chambers (Nucleopore, Pleasanton, CA) as previously described (11,12). In brief, glioma cells transfected with HIF-1α siRNA or control siRNA 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 6 h at 37˚C under normoxic or hypoxic conditions at the same time, and cells that had migrated to the lower surface of the filter were fixed and stained with Diff-Quik (Scientific Products, Harleco, Gibbstown, NJ). Cells were counted in three 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 (Becton Dickinson Biosciences), as previously described (13-16). Briefly, glioma cells treated with HIF-1α siRNA or control siRNA in DMEM containing 0.1% BSA 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 Falcon 24-well plates contained 500 μl of conditioned medium as a chemoattractant. Two days after simultaneous incubation at 37˚C under normoxic or hypoxic conditions, the lower surfaces of the culture inserts were fixed and stained with Diff-Quik. *In vitro* cell invasive activities were quantified as described for the migration assays.

Invasion assay in brain slice model. All animal experiments were conducted in accordance with the guidelines of the Ehime University Committee for Ethics of Animal Experimentation. To assess the effect of HIF-1α suppression on cell motility under more physiological conditions, we used rat brain slice cultures that mimic *in vivo* central nervous system conditions. A slice culture of rat whole cerebrum was produced by modifying a previously described organotypic culture method (17-19). 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 were decapitated using scissors. The whole brains were quickly removed and placed in Hanks' balanced salt solution (HBSS; Life Technologies, Inc., Rockville, MD). The brains were mounted on the stage of a microslicer (Dosaka EM Co. Ltd., Kyoto, Japan) and cut into 500-μm coronal slices, which were transferred onto 30-μm Millicell-CM inserts with 0.4-μm pores (Millipore) in 6-well culture plates. The wells contained 1.0 ml of 50% Eagle's minimal essential medium with HEPES, 25% HBSS, 25% heat-inactivated horse-serum (Life Technologies Inc.), 6.5 mg/ml glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Fungizone). Brain slices were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was replaced with fresh medium twice per week before the experiment.

Glioma cell spheroid implantation. All human glioma cells transfected with HIF-1α siRNA and control siRNA were fluorescently labeled with PKH26 (rhodamine) using a kit (Zynaxis Cell Science, Inc., Malvern, PA) as described previously (20). Briefly, $1.0x10^7$ harvested glioma cells in serum-free DMEM were resuspended in 1 ml of diluent C, and then PKH26 dye in 1 ml of diluent C ($2x10^{-6}$ M) was added. After incubation of the cells at room temperature for 5 min, the labeling reaction was stopped by adding 2 ml of FBS. The cells were then thoroughly washed to remove free PKH26, and resuspended in DMEM containing 10% FBS. The labeled glioma cells ($5x10^5$) were seeded into non-treated 60-mm culture dishes, and incubated under continuous agitation at a speed of 30 rpm on a reciprocating shaker (Taitec, Saitama, Japan) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 for 1 day. The glioma cell spheroids obtained by this method were of 300-400 μm in diameter. One rhodamine-labeled glioma cell spheroid was pipetted, placed on a brain slice as close to the corpus callosum as possible, and co-cultured at 37˚C under normoxic or hypoxic conditions for 3 days. Cell invasion was observed by fluorescence microscopy on days 0 and 3.

Expression of MMP-2 and TIMP-2. After 24 h of normoxic or hypoxic treatment, total RNA extracted from four human malignant glioma cell lines transfected with HIF-1α siRNA, control siRNA, and non-treated controls (mocks) using acid guanidinium isothiocyanate phenol chloroform, were used as templates for cDNA synthesis (21). Each RT-PCR reaction consisted of 30 cycles at 94˚C for 30 sec, 53˚C for 30 sec, and 68˚C for 1 min, followed by incubation at 72˚C for 5 min. The products were electrophoresed on 1.5% agarose gels including 0.1 μg/ml ethidium bromide. PCR was performed with human HIF-1α, MMP-2, TIMP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; internal standard)-specific primers. The primers (Hokkaido Biosystems, Sapporo, Japan) used were: 5'-CCCCAGATTCAGGATCAGACA-3' and 5'-CCA TCATGTTCCATTTTTCGC-3' for HIF-1α, 5'-CTCTCCT GACATTGACCTTGGCAC-3' and 5'- AAAAAGCTTAC TCGCTGGACATCAGGG-3' for MMP-2, 5'-CTCGGCAG TGTGTGGGGTC-3' and 5'-CGAGAAACTCCTGCTTGG GG-3' for TIMP-2, and 5'-CAAAGTTGTCATGGATGAC C-3' and 5'-CCATGGAGAAGGCTGGGG-3' for GAPDH.

Gelatin zymography. To quantify MMP-2 and MMP-9 activities, gelatin zymography (22) was performed by analyzing the conditioned media of glioma cells transfected with HIF-1α siRNA and of the controls (mock and control siRNA) using 10% SDS-PAGE containing 1 mg/ml of gelatin (Sigma, USA). Cells ($5x10^5$) were placed in 5 ml of serum-free DMEM and incubated for 24 h under 21 and 1% oxygen. The supernatants were mixed with the solution buffer (2% w/v SDS, 10% glycerol, 50 mmol/l Tris-HCl, pH 7.5, and 0.005% bromophenol blue) and then 25 μg aliquots of this mixture were loaded onto the gel. After electrophoresis, the gels were washed for 1.5 h at room temperature in washing buffer (50 mM Tris-HCl, pH 7.5, containing 0.1 mM NaCl and 2.5% Triton X-100) to remove SDS, transferred to a buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$), and incubated for 18 h at 37˚C. The gels were then stained with 0.1% Coomassie

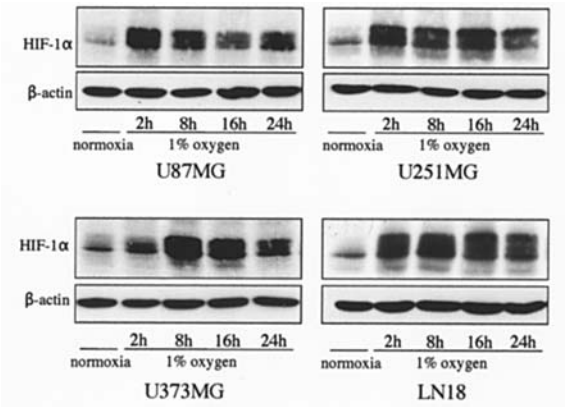

Figure 1. Expression of HIF-1α protein in human glioma cell lines U87MG, U251MG, U373MG, and LN18. After hypoxic treatment for 0 h (normoxia), 2, 8, 16, and 24 h, cellular extracts from each lysate were subjected to Western blot analysis. HIF-1α protein expression was detected with a monoclonal antibody against HIF-1α. Top panels show HIF-1α protein (120 kDa), while ß-actin is shown as an internal control in the bottom panels. Overexpression of HIF-1α protein was observed by 2 h of hypoxic treatment in all cell lines. HIF-1α expression was clearly augmented by hypoxic treatment in all cell lines.

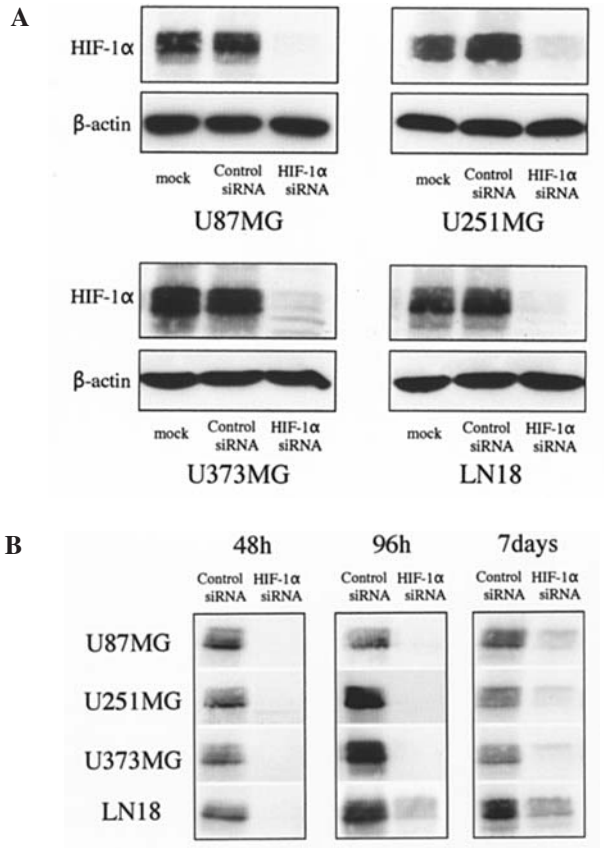

Figure 2. Effects of HIF-1α siRNA on HIF-1α protein expression were examined by Western blot analysis. (A) HIF-1α protein expression after 2 h of hypoxic treatment in non-treated cells (mocks), cells treated with control siRNA, and cells treated with HIF-1α siRNA are shown. Transfection with HIF-1α siRNA repressed HIF-1α protein synthesis in all glioma cell lines. (B) Effects of HIF-1α siRNA on HIF-1α expression at 48 h, 96 h, and 7 days after transfection in cells treated with control siRNA and cells treated with HIF-1α siRNA. The HIF-1α band was not clearly seen until 48 h after transfection, whereas HIF-1α protein was detected at day 7 in all cell lines.

brilliant blue R-250 diluted in 45% ethanol and 10% acetic acid, and destained in 20% ethanol and 5% acetic acid until clear proteolytic bands were obtained on a homogeneous blue background.

Statistical analysis. The results are representative of experiments repeated at least 3 times and the values are expressed as the means ± standard deviation (SD). Statistical comparisons between groups were performed using Student's t-test (Stat View, SAS Institute Inc., NC). p-values <0.05 were considered statistically significant.

Results

Effects of HIF-1α siRNA on HIF-1α expression under hypoxia in glioma cells. To assess the change of HIF-1α synthesis under hypoxic conditions in glioma cells, amounts of HIF-1α protein in nuclear extracts were assayed by Western blot analysis after 2, 8, 16, and 24 h of hypoxic treatment. In this study, a concentration of 1% O_2 was used to induce hypoxia. Weak HIF-1α expressions was detected under normoxia in all cell lines. After hypoxic treatment, HIF-1α proteins were markedly overexpressed at each processing time, compared with those under normoxic conditions (Fig. 1). Next, we examined the effects of HIF-1α siRNA on HIF-1α protein expression. Transfection with HIF-1α siRNA markedly inhibited the over-expression of HIF-1α after 2 h hypoxic treatment compared with that of control siRNA and mocks in all glioma cell lines (Fig. 2A). To examine the duration of inhibition of HIF-1α synthesis by HIF-1α siRNA, glioma cells were transfected with HIF-1α siRNA and cultured for 48 h, 96 h, and 7 days under hypoxic conditions. Although clear bands indicating HIF-1α were not seen until 96 h of incubation, HIF-1α proteins was apparently expressed at 7 days (Fig. 2B). Moreover, we examined the influence of siRNA on cell viability and cell growth. After a 96 h incubation under normoxia and hypoxia, cell viability, as assessed by the MTT assay, did not differ between cells treated with HIF-1α siRNA and those with control siRNA compared with that without treatment (Fig. 3). Cell growth suppression was not seen in HIF-1α siRNA and control siRNA cells (data not shown). Therefore, we performed all subsequent experiments within 96 h after transfection with siRNA.

Downregulation of HIF-1α expression inhibits glioma cell migration and invasion in vitro. To evaluate the relation between cell migratory ability and HIF-1α expression under hypoxic conditions, we performed a migration assay using a modified Boyden chamber method under conditions of normoxia and hypoxia. HIF-1α siRNA-transfected cells showed a tendency to suppressed migratory activity under normoxia; however, there was no significant difference compared with the effect of control siRNA. Under hypoxic conditions, migration of glioma cells transfected with HIF-1α siRNA was significantly reduced compared with the mocks and control siRNA-transfected cells in all cell lines (Fig. 4).

We investigated the influence of HIF-1α protein under hypoxia on the glioma cell invasive activity using an *in vitro* invasion assay. The microphotographs in Fig. 5A show

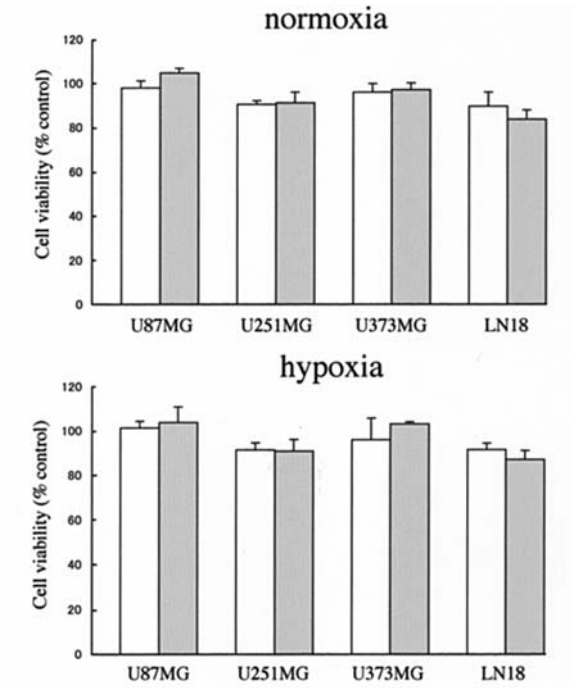

Figure 3. Cell viability after introduction of siRNA was assessed by MTT assay. The glioma cells were seeded at $3x10^3$ cells/well in 96-well flat-bottom plates. At the indicated times, the absorbance was read on a microplate reader at the dual wavelengths of 540 and 620 nm. The cells were incubated for 72 h under the normoxia and hypoxia after siRNA transfection. Cell growth and viability did not differ between treatment with HIF-1α siRNA (gray) and control siRNA (white). Values represent the means ± SD of four experiments.

U373MG cells invading through the matrigel after 48 h of incubation with or without siRNA treatment under the conditions of normoxia and hypoxia. Except for the U87MG cells, there were no differences in invasiveness between HIF-1α siRNA-treated cells and control siRNA-treated cells under normoxia. In contrast, under hypoxic conditions, the invasiveness of glioma cells transfected with HIF-1α siRNA was significantly suppressed compared with that of the cells treated with control siRNA (Fig. 5B).

Suppression of HIF-1α expression inhibits glioma cell invasion in an organotypic brain slice model. To evaluate the effects of HIF-1α siRNA on cell motility under more physiological conditions, we investigated the distance migrated by the glioma cells treated with HIF-1α siRNA and control siRNA on organotypic rat brain slices under normoxic or hypoxic conditions. The brain slice model enabled us to observe the invasion of glioma cells into surrounding brain tissue in conditions analogous to those of normal brain *in situ*. We implanted rhodamine-labeled spheroids of each glioma cell line into the corpus callosum of the cerebral hemisphere (23). Under normoxic conditions, glioma cells transfected with HIF-1α siRNA and control siRNA migrated diffusely around the spheroids. Under hypoxia, glioma cells transfected with HIF-1α siRNA showed repressed migration and invasion of the surrounding normal brain tissues compared with those transfected with control siRNA in all cell lines (Fig. 6A and B).

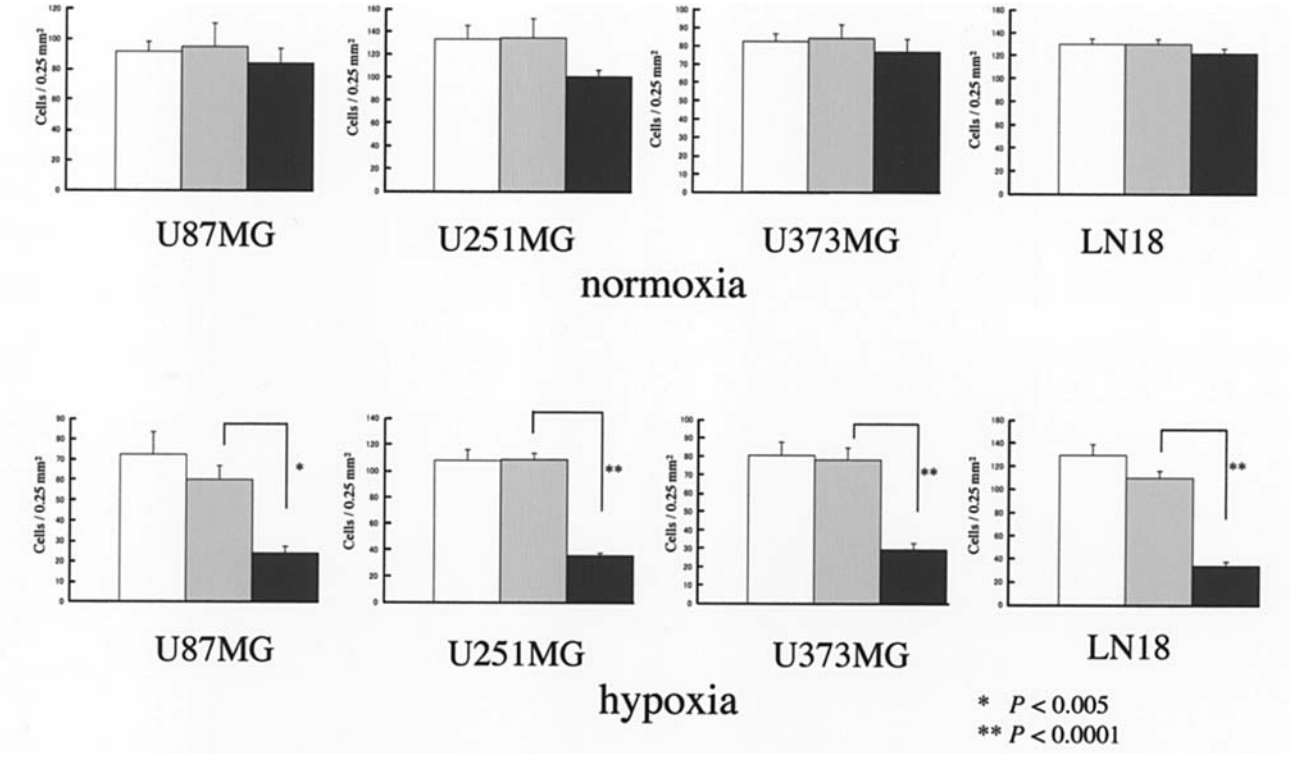

Figure 4. Effects of HIF-1α siRNA on migration activity of glioma cells. Migration activity was evaluated by a modified Boyden chamber assay method. Non-treated cells (mocks), cells treated with control siRNA, and cells treated with HIF-1α siRNA were placed in the upper well and the conditioned medium of each group of cells served as a chemoattractant in the lower well. Cells that migrated to the lower surface of the membrane, which had 8-μm pores, were stained with Diff-Quik and counted. Migration of glioma cells transfected with HIF-1α siRNA (black) was significantly reduced compared with that of nontreated cells (white) and control siRNA-transfected cells (gray) under hypoxic conditions. Under normoxic conditions, introduction of HIF-1α siRNA did not altered the migratory ability of any of the cell lines. Upper panel, normoxia; lower panel, hypoxia. Values represent the mean ± SD of three experiments. $^{*}p<0.005$, $^{**}p<0.0001$.

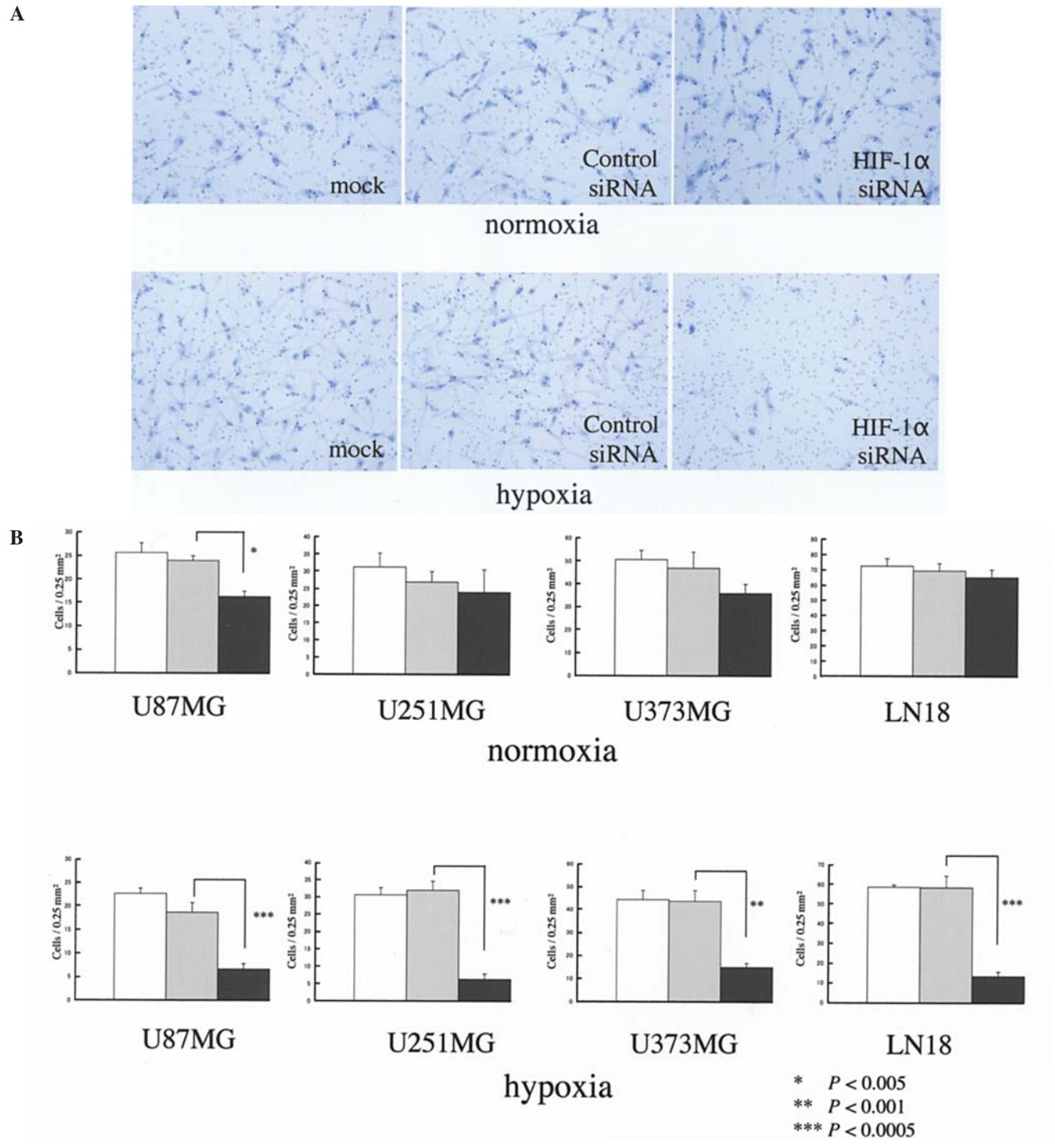

Figure 5. Effects of HIF-1α siRNA on glioma cell invasion *in vitro*. (A) Microphotographs show that, after 48 h of incubation, U373MG glioma cells had invaded the Matrigel to the lower side of an insert filter with 8-μm pores. Conditioned medium from glioma cells transfected with siRNA served as a chemoattractant in the lower wells of Falcon 24-well plates. Original magnification, x200. Upper panel, normoxia; lower panel, hypoxia; left, nontreated (mocks); middle, control siRNA; and right, HIF-1α siRNA. (B) The number of invading cells was counted after 2 days of incubation. The invasiveness of glioma cells transfected with HIF-1α siRNA (black) was significantly suppressed compared with cells treated with control siRNA (gray). Under normoxic conditions, only U87MG glioma cells treated with HIF-1α siRNA showed less invasiveness than the cells treated with control siRNA. Values represent the mean ± SD of three experiments. $^{*}p<0.005$, $^{**}p<0.001$, $^{***}p<0.0005$.

Reduction in HIF-1α synthesis decreases MMP-2 expression but increases TIMP-2 expression. We investigated whether expression of MMP-2, TIMP-2, and MMP-9 was affected by hypoxia and HIF-1α siRNA treatment. MMP-2 mRNA, which was constitutively expressed, was apparently upregulated under the hypoxic conditions in all cell lines. However, MMP-2 mRNA levels were significantly lower in cells treated with HIF-1α siRNA compared with those treated with control siRNA. Although TIMP-2 mRNA levels were downregulated under hypoxia in all glioma cell lines, introduction of HIF-1α

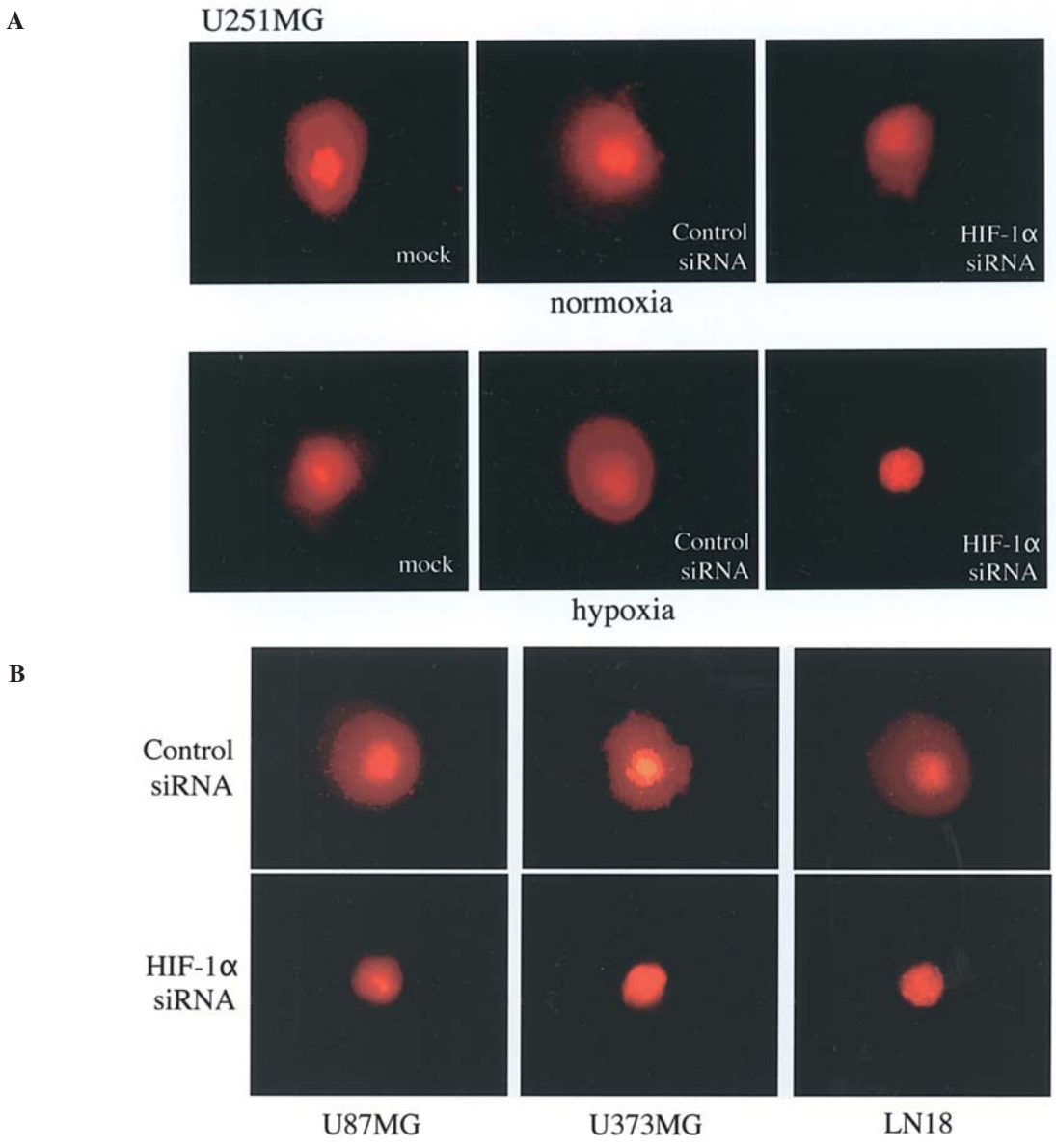

Figure 6. Invasive behavior of glioma cells transfected with HIF-1α siRNA on rat brain slices. Rhodamine-labeled glioma cell spheroids were co-cultured with rat brain slices. (A) Fluorescence microphotographs show U251MG glioma cell invasion around the original glioma spheroids under normoxic conditions (upper panel) and hypoxic conditions (lower panel). U251MG glioma cells transfected with HIF-1α siRNA showed much less invasion of the surrounding normal brain tissues compared with those transfected with control siRNA under hypoxic conditions. There was no difference in the spread of tumor cells between nontreated cells, cells treated with control siRNA, and cells treated with HIF-1α siRNA under normoxic conditions. Left, nontreated (mocks); middle, control siRNA; and right, HIF-1α siRNA. (B) Fuluorescence microphotographs show invasion of U87MG, U373MG, and LN18 glioma cells treated with control siRNA (upper panel) and HIF-1α siRNA (lower panel) under hypoxic conditions. Glioma cells treated with control siRNA actively migrated around the tumor spheroids, whereas the migration of cells treated with HIF-1α siRNA was inhibited. Original magnification, x40.

siRNA elevated them (Fig. 7A and B). MMP-9 mRNA levels were not affected by hypoxia or HIF-1α siRNA transfection (data not shown).

Effect of HIF-1α siRNA on enzymatic activities of MMP-2 and MMP-9 under hypoxia. We examined the enzymatic activities of MMP-2 and MMP-9 in the conditioned medium from each cell line using gelatinolytic zymography. MMP-2 and MMP-9 were revealed as 72 and 92 kDa lytic bands, respectively. Both bands were observed in non-treated cells under normoxic conditions, and these bands were more prominent under hypoxic conditions. These results indicate that MMP-2 and MMP-9 were activated under low-oxygen conditions. The intensities of these gelatinolytic bands were clearly decreased in the conditioned medium from HIF-1α siRNA-transfected cells. This indicates that downregulation of HIF-1α expression reduced the enzyme activities of MMP-2 and MMP-9, especially under hypoxic conditions in glioma cells (Fig. 8).

Discussion

In the present study, we showed that HIF-1α is an important regulator of glioma cell motility under hypoxia. Hypoxia selects for more aggressive and metastatic phenotypes that are associated with poor prognosis (24). Since HIF-1α is induced by a hypoxic stimulus, it is inevitable that HIF-1α

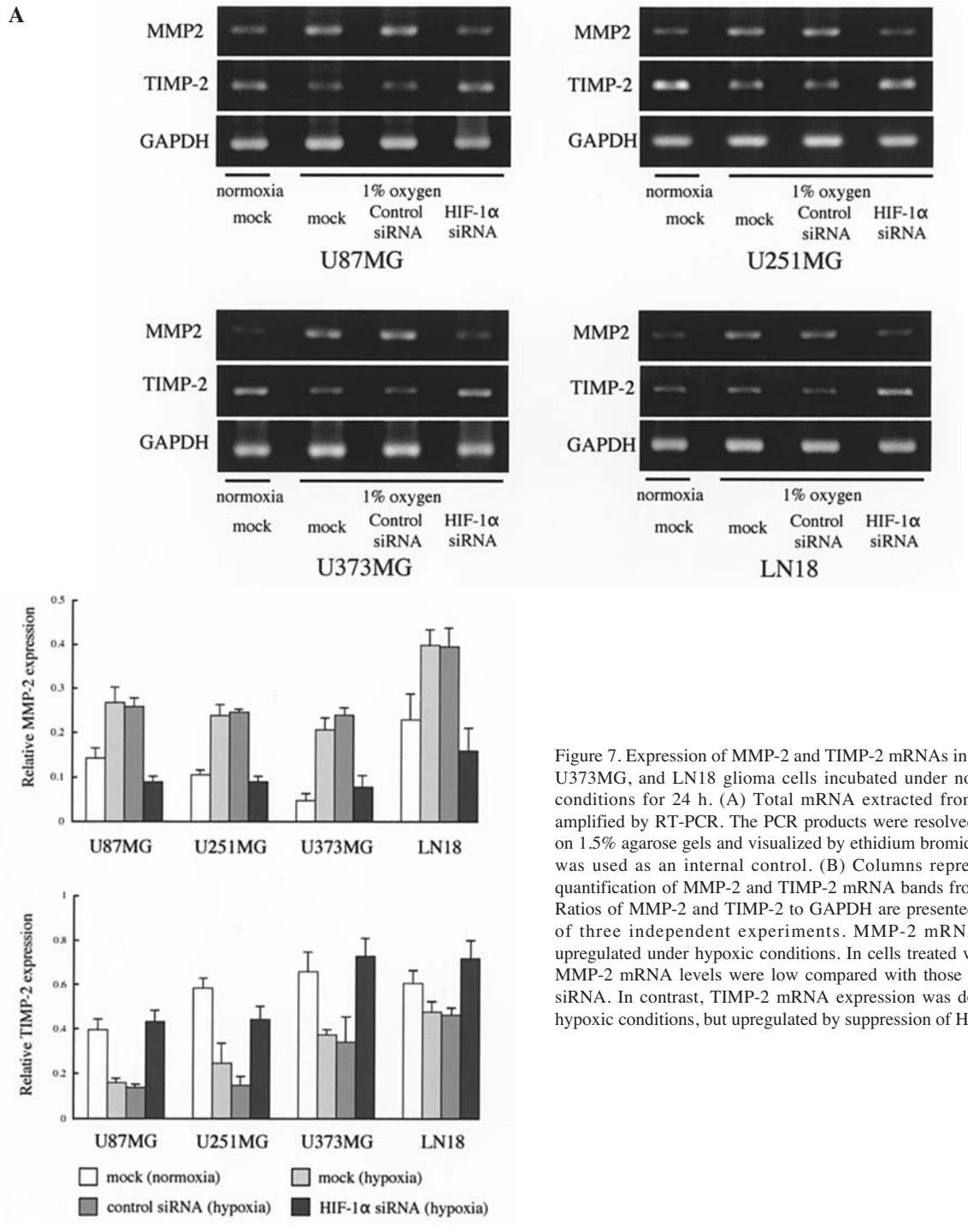

Figure 7. Expression of MMP-2 and TIMP-2 mRNAs in U87MG, U251MG, U373MG, and LN18 glioma cells incubated under normoxic or hypoxic conditions for 24 h. (A) Total mRNA extracted from glioma cells was amplified by RT-PCR. The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. GAPDH was used as an internal control. (B) Columns represent densitometric quantification of MMP-2 and TIMP-2 mRNA bands from each glioma cell. Ratios of MMP-2 and TIMP-2 to GAPDH are presented as the mean ± SD of three independent experiments. MMP-2 mRNA expression was upregulated under hypoxic conditions. In cells treated with HIF-1α siRNA, MMP-2 mRNA levels were low compared with those treated with control siRNA. In contrast, TIMP-2 mRNA expression was downregulated under hypoxic conditions, but upregulated by suppression of HIF-1α expression.

protein is highly expressed in malignant solid tumors containing hypoxic regions. Intense diffuse expression of HIF-1α protein has been observed in tumor tissues, especially areas of necrosis surrounding malignant gliomas (8,9). Malignant gliomas are characterized by their extremely invasive behavior, leading to the hypothesis that HIF-1 may play a crucial role in glioma cell motility. Therefore, we investigated whether silencing HIF-1α by HIF-1α-specific siRNA inhibited the migration and invasion of human glioma cells. Recent approaches to the inhibition of targeted molecules include transcription factor decoy, antisense, oligonucleotide and dominant negative mutants, and RNA interference. We used siRNA to downregulate HIF-1α expression, because siRNA methods can almost abrogate expression of RNA and protein, and enable the consequences to be observed in various tumor cell lines.

In the glioma cell lines used in this study, HIF-1α protein levels were low under normoxic conditions, but were highly upregulated by hypoxic treatment. Transfection with HIF-1α siRNA inhibited HIF-1α protein expression. However, neither HIF-1α siRNA nor control siRNA affected cell growth or cell viability under normoxic or hypoxic conditions. Some studies have shown that treatment with HIF-1α siRNA under normoxia and hypoxia suppressed constitutive HIF-1α as well as hypoxia-induced HIF-1α, attenuated cell proliferation, and induced apoptosis (25). The significance of HIF-1α expression remains

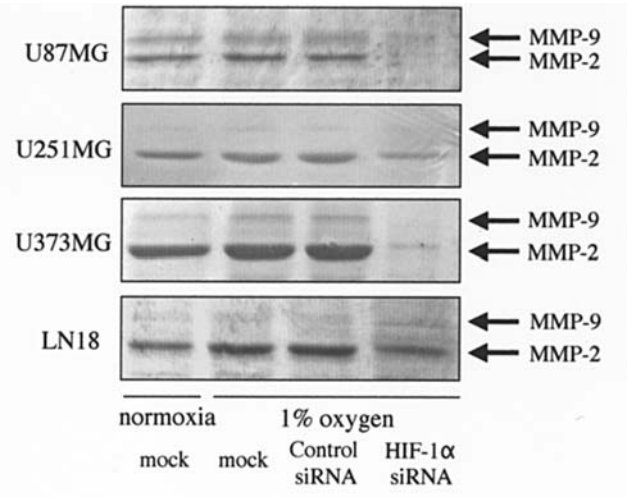

Figure 8. Enzymatic activities of MMP-2 and MMP-9 assessed by gelatin zymography. The culture supernatant from glioma cells was used for gelatin zymography. Clear lytic zones at a molecular weight of 72 kDa demonstrate the enzyme activity of MMP-2. Both MMP-2 and MMP-9 were activated under low-oxygen conditions. The intensities of these lytic zones were clearly reduced in the conditioned medium from HIF-1α siRNA-transfected cells. HIF-1α siRNA decreased the enzymatic activities of MMP-2 and MMP-9 in all cell lines.

unclear and is dependent on the type of cancer and the presence or absence of genetic alterations that affect the balance between pro- and anti-apoptotic factors (26).

In the present study, we provided evidence that knockout of HIF-1α expression by RNA interference led to a dramatic decrease in *in vitro* cell migration and invasion under hypoxia. However, 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 it with the aid of proteolytic enzymes to invade distant tissues (27). Key elements of these processes include tumor cell adhesion, migration, and proteolysis of the ECM. However, the biological functions of these elements depend on specific tissue environments and are substantially affected by the ECM of the target organ. The ECM components of the brain are different than those of other organs (28,29). Therefore, *in situ* studies of glioma invasion require special conditions analogous to those of normal brain. We have established a novel brain slice model of glial tumor cell invasion by modifying an organotypic culture of brain tissues (17,30). In the experiments using the brain slice model, we were concerned whether the normal histological structure of the slice would be maintained under hypoxic stress. To avoid this problem, brain slices incubated for 3 days under the normoxia and hypoxia were stained with hematoxylin and eosin and observed under a microscope. We confirmed that the brain slices retained their normal morphologic features <1% oxygen for 3 days (data not shown). Introduction of HIF-1α siRNA to glioma cells under hypoxic conditions inhibited the spread of the tumor cells around the tumor spheroid in the brain slice, thereby creating a well-demarcated tumor mass similar to the intitial tumor spheroid. In contrast, control siRNA-treated cells diffusely invaded the surrounding normal brain tissue in hypoxia. Collectively, these results indicated that hypoxia-induced HIF-1α plays an essential role in cell motility in malignant gliomas.

MMPs are implicated in tumor cell invasion because they degrade almost all components of the ECM (31). It has been suggested that high levels of expression of MMPs are closely related to the malignant progression observed in gliomas (32). MMP-2 and MMP-9 are expressed and secreted by malignant gliomas *in vivo* and can modulate the invasive phenotype *in vitro* (33,34). Therefore, we investigated whether hypoxia affected the enzyme kinetics of MMP-2 and MMP-9 in addition to TIMP-2 expression. We showed that MMP-2 mRNA and not MMP-9 mRNA, was increased under hypoxic conditions, while TIMP-2 mRNA was reduced in all glioma cell lines tested. Moreover, we found that the enzymatic activities of MMP-2 and MMP-9 were increased under the hypoxic conditions, as assessed by gelatin zymography. The hypoxia-induced activation of MMP-2 and MMP-9 was counteracted by HIF-1α siRNA treatment. It has been reported that, using *in situ* hybridization and immunohistochemistry, MMP-2 expression is localized most prominently in tumor cells with very little signal in the vasculature, and MMP-9 is not only expressed prominently in vascular structures but also expressed in tumor cells (35). The discrepancy between mRNA levels and enzyme activity of MMP-2 and MMP-9 may be caused by the difference in their histological localization.

It should be noted that expression of HIF-1α is not restricted to hypoxic cells alone in many tumors, but is also regulated by genetic alterations that activate oncogenes and inactivate suppressor genes (36-40). We have reported that *PTEN* gene transfer suppressed glioma cell invasion (41). In addition, we examined the relation between HIF-1α expression and *PTEN* gene function in U87MG, which has a mutated *PTEN*. Transfection of the *PTEN* gene apparently reduced expression of HIF-1α protein under hypoxic conditions (unpublished data). We believe that the context of genetic alterations shape the role of HIF-1 in the susceptibility of cells to adaptation and progression towards malignancy.

In conclusion, our results show that suppression of HIF-1α inhibits the motility of human malignant glioma cells. HIF-1α protein appears to be a key factor in glioma cell migration and invasion under hypoxic conditions. Although the mechanism of the effect of HIF-1α on hypoxic glioma cell motility is not clear, downregulation of MMP-2 mRNA expression and inhibition of the enzymatic activities of MMP-2 and -9, along with upregulation of TIMP-2, are considered to play crucial roles in the process. These findings indicate that targeting HIF-1α may be a promising therapeutic strategy against the invasion of malignant gliomas.

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