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Laboratory Investigation

Hypoxia inducible factor 1-α regulates of platelet derived growth factor-B in human glioblastoma cells

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Summary

Hypoxia inducible factors (HIF) are transcription factors regulating expression of several genes related to oxygen homeostasis in response to hypoxic stress. Although HIF1-α and platelet derived growth factor-B (PDGF-B) are expressed in glioma tissue and closely related to tumor angiogenesis mediating vascular endothelial growth factor (VEGF) activity, their direct relationship has not yet been clarified. The aim of this study is to investigate whether HIF1-α regulates PDGF-B expression. The human glioblastoma cell lines, U87MG, U251MG, and A172, were exposed to 1–21% oxygen for 24 h. PDGF-B mRNA expression were quantitatively analyzed by real time RT-PCR, their intracellular protein levels were determined by computerized image analysis supported by flow cytometry to detect intracellular PDGF-B, and the concentration of secreted PDGF-B protein was assayed by ELIA. We also assayed following transfection of the cells with short interference RNA (siRNA) targeting HIF1-α mRNA. Relative PDGF-B mRNA and secretion of PDGF-B protein were significantly elevated at 1% oxygen. Following transfection of HIF1-α siRNA at 1% oxygen, PDGF-B expression was significantly suppressed at mRNA level. Our findings indicated that HIF1-α up-regulated expression of PDGF-B in human glioblastoma cells and showed the feasibility of siRNA technology in glioblastoma cell lines.

Introduction

During tumor growth cells experience low oxygen tension. To restore an adequate supply of oxygen, either improved oxygen transport or new vessel formation is required [1]. HIF1-α subunit protein has been shown to be overexpressed in many types of human cancer, as well as in regional and distant metastases [2]. In glioblastoma multiforme (GBM) tissues, HIF1-α is highly expressed in areas of the tumor adjacent to those undergoing necrosis, including pseudopalisading cells [3], indicating that the pattern of HIF1-α expression in GBM may be modulated, at least in part, by tumor oxygenation [4]. In addition, HIF1-α expression is associated with angiogenesis and invasion, encoding vascular endothelial growth factor (VEGF) [5]. Then, hypoxia in regions of GBM probably leads to tumor angiogenesis [6]. Despite enhanced expression of PDGF-B mRNA has also been described in these highly malignant and well vascularized gliomas [6], the past literature did not show whether PDGF-B is up-regulated by HIF1-α in GBM. In hypoxia, accumulated evidences indicate that HIF1-α is activated to up-regulate VEGF transcriptionally [7], while expression of PDGF-B is also elevated to stimulate VEGF expression [8]. The purpose of the current study is to elucidate direct evidence of transcriptional regulation of PDGF-B by HIF1-α.

Material and methods

Cell lines

The human malignant glioma cell lines, U87MG and U251MG (American Type Culture Collection, Manassas, VA), and the human glioblastoma multiforme cell line, A172, kindly supplied by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan, were each cultured in plastic flasks (Falcon 100 cm^2, PGC Scientifics, Frederick, MD) in DMEM (Sigma, St. Louis, MO) containing 10% heat inactivated fetal calf serum (FCS; Biocell, Carson, CA), 0.05% (w/v) glutamine, 100 μg/ml gentamicin, and 100 IU/ml penicillin (hereafter called culture medium) at 37 °C in a humidified air atmosphere containing 5% carbon dioxide. Culture medium was exchanged twice weekly, and sub-confluent cells were passaged using 0.05% trypsin/0.02% ethylenediamine tetra-acetic acid (EDTA). For normoxic conditions, cells were incubated in an atmosphere of 5% CO_2 and 21% O_2. For hypoxic conditions, 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 kept in a humidified incubator at 37 °C. Oxygen concentration was monitored by oxygen sensors in these incubators.

Real time RT-PCR

Expression levels of HIF1-α and PDGF-B mRNAs in the cells were assayed quantitatively using realtime reverse transcription-polymerase chain reaction (RT-PCR) with the appropriate reagents (TaqMan(®) One-Step RT-PCR Master Mix Reagents Kit; Applied Biosystems, Tokyo, Japan) and thermal cycler (Gene Amp 5700 Sequence Detection Systems; Applied Biosystems). Aliquots of 1×10^6 cells in 2 ml serum free DMEM were placed in each well of a 6-well plate (Nalge Nunc International, Naperville, IL) and exposed for 24 h to normo- (21%) or hypoxic (1%) conditions. Total RNA was prepared using TRIZOL reagent (Gibco-BRL, Tokyo, Japan), and cDNA was synthesized using pd(N)6 Random Hexamer (Amersham Bioscience, Piscataway, NJ). PCR amplification was performed using primers that spanned at least one intron, and monitored with FAM-labeled probes:

HIF1-α : probe, 5′-FAM-AAGGTATTGCACTGC ACAGGCCACA;
forward primer, 5′-CTAGCCGAGGAAGAACTAT GAACAT;
reverse primer, 5′-CTGAGGTTGGTTACTGTTGG-TATCA;
PDGF-B: probe, 5′-FAM-TTGGCCACTCCAGCCA-CAGG;
forward primer, 5′-AGACTCCTTGTGGACTGGCT;
reverse primer, 5′-CTTTTGGGAAATGGAGGTCAT.

Each amplification utilized cDNA derived from 20 μl mRNA solution (10 ng/μl), and the relative amount of PCR product was calculated as threshold cycle (CT value) of the sample divided by that of human *β*-actin (Applied Biosystems, Tokyo, Japan). GAPDH was not used as an internal control since its expression is known to be enhanced under anaerobic conditions, suggesting it may also be increased by hypoxia.

Intracellular detection of PDGF-B

Intracellular PDGF-B was determined by FACS analysis (FACS Calibur, Japan Becton Dickinson Bioscience, Tokyo, Japan). Aliquots of 2×10^6 cells in 2 ml culture medium were seeded onto each well of a 6-well plate (Nalge Nunc) and allowed to adhere overnight. After 24 h exposure to normo-or hypoxic conditions, the cells were harvested by trypsinization and washed twice with phosphate buffer. Non-specific Fc receptors were pre-blocked by incubating cells with 10% normal human serum for 20 min at 4 °C. The cells were washed twice with staining buffer (Mg^{++}/Ca^{++}-free PBS containing 1% heat-inactivated FCS, and 0.09% (w/v) sodium azide, pH 7.4), centrifuged at $250 \times g$, and resuspended in 500 μl of fixative (Cytofix/Cytoperm solution, BD Bioscience Pharmingen, San Diego, CA). The cells were incubated for 20 min at 4 °C to be fixed and permeabilized and subsequently washed twice in 0.1% saponin solution (Perm/Wash solution, BD Bioscience Pharmingen). After centrifugation at $250 \times g$, the pellet was incubated with 1 ml 0.1% saponin solution containing 0.1 μg/ml mouse anti-human PDGF-B monoclonal antibody (I B L, Co., Ltd., Tokyo, Japan) for 30 min at room temperature. The cells were washed twice with 0.1% saponin solution and incubated with FITC-conjugated goat anti-mouse immunoglobulin (1:200, DAKO, Tokyo, Japan) for 30 min at room temperature; cells not incubated with the fluorochrome-conjugated immunoglobulin were utilized as negative control. The cells were washed twice in 0.1% saponin solution and resuspended in staining buffer prior to flow cytometric analysis. The positive control was examined with non-immune mouse immunoglobulin as a primary antibody.

Enzyme immunoassay (EIA) of PDGF-B in the supernatant

Following exposure of cells to normo- or hypoxic conditions, the supernatants were collected, and PDGF-B secretion was assayed by two-step sandwich immunoassays (Chemicon International, Inc., Temecula, CA). Specimens were incubated with an enzyme substrate and with 3,3′,5,5′-tetramethylbenzidine (TMB), and color was analyzed by an ELISA plate reader (BioRad, Richmond, CA) at 450 nm and quantitated relative to a standard curve ($R^2 = 0.996$).

Immmunostaining of cells

Cells (5×10^4 in 1 ml culture medium) were seeded onto chamber slides (Nalge Nunc) and allowed to attach overnight. Following 24 h exposure to normo- or hypoxic conditions, or gene silencing with siRNA, the cells were fixed with 4% paraformaldehyde for 30 min, washed with PBS, and incubated with blocking solution involving non-specific antigens (Block Ace, Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan) for 30 min. After 3 washes with PBS, the samples were incubated with mouse anti-human PDGF-B IgG (1 μg/ml, IBL Co., Ltd.), mouse anti-human Laminin A/C (1:100, Novocastra Labo. Ltd, Newcastle upon Tyne, UK) or mouse anti-human HIF1-α (1:1000, Novus Biologicals, Inc, Littleton, CO) monoclonal antibody for 30 min at room temperature. Samples were subsequently incubated with second antibody, FITC-conjugated goat anti-mouse immunoglobulin (1:200, DAKO, Tokyo, Japan) or Alexa Fluor 568-conjugated anti-mouse IgG (0.1 μg/ml, Molecular Probes, Inc., Eugene, OR), for 30 min at room temperature. The positive control was examined with non-immune mouse immunoglobulin as a primary antibody.

Gene silencing with short interfering ribonucleic acids (siRNA)

Briefly, beginning with the AUG start codon and scanning the length of the gene for AA sequences, the AA and the adjacent 19 nucleotides, potential siRNA target sites, were compared with potential sites, and any target sequence with significant homology to other human genes was eliminated. Each target sequence was selected

to be as close as possible to the medial portions of the mRNA, making it theoretically more susceptible to siRNA degradation [9]. The appropriate double stranded RNA pairs were synthesized (Dharmacon Research, Lafayette, CO), with an RNA duplex of Laminin A/C used as the positive control, and a scrambled RNA duplex used as the negative control. The sequences utilized were:

HIF1-α;
target; 5′-AAGUUGCCACUUCCACAUAAU
sense; 5′-GUUGCCACUUCCACAUAAUdTdT
antisense; 5′-dTdTCAACGGUGAAGGUGUAUUA
Laminin A/C:
target; 5′-CTGGACTTCCAGAAGAACA
sense; 5′-CUGGACUUCCAGAAGAACAdTdT
antisense; 5′-dTdTGACCUGAAGGUCUUCUUGU
Scramble:
target; 5′-GCGCGCTTTGTAGGATTCG
sense; 5′-GCGCGCUUUGUAGGAUUCGdTdT
antisense; 5′-dTdTCGCGCGAAACAUCCUAAGC
PDGF-B:
target; 5′- CCACTCGATCCGCTCCTTTGA
sense; 5′- CCACTCGATCCGCTCCTTTGAdTdT
antisense; 5′-dTdT CAAAGGAGCGGATCGAGTGG

For each duplex, the concentration was adjusted to 25, 50, or 100 nM in 1× universal buffer (20mM KCl, 6 mM HEPES, pH 7.5, 0.2 mM $MgCl_2$) just prior to use.

To assess the transfection efficiency of each siRNA in the glioma cells, each sequence was labeled with the LabelT siRNA Tracker Intracellular Localization Kit (Mirus, Madison, WI), and subcellular localization of HIF1-α, scramble, or Laminin A/C mRNA was monitored. Briefly, the LabelIT siRNA Tracker Reagent was brought to room temperature and spun quickly to collect the pellet. To each was added 50μl LabelIT Reconstruction Solution, and the reaction volume was brought to 100μl with 60μl of molecular biology-grade H_2O, 10μl 10× Labeling Buffer, 20μl of a 40μM stock of siRNA duplex, and 10μl LabelIT siRNA Tracker Reagent (Cy5 dye). Following incubation at 37 °C for 1 h, the unreacted tracking reagent was removed by ethanol precipitation, and the labeled siRNA pellet was centrifuged, washed with 70% ethanol, and resuspended to 10 μM in siRNA Dilution Buffer.

In a sterile Eppendorf tube, 4μl of TransIT-TKO Transfection Reagent was added dropwise to 200μl serum-free medium (Opti-MEM, Invitrogen, Tokyo, Japan), mixed thoroughly by vortexing and incubated at room temperature for 20 min. To it was added 5μl of he labeled siRNA, and the tube was incubated at room temperature for 20 min. Chamber slides onto which 1×10^5 cells in 1 ml of culture medium had been seeded on the previous day and allowed to adhere under normoxic conditions were washed twice with PBS and covered with 800μl of complete growth medium, and the 200μl of TransIT-TKO Reagent/labeled siRNA complex were added dropwise, making a final siRNA concentration of 50 nM. The cells were incubated at 37 °C for 24 h in a humidified CO_2 chamber in 21% oxygen. Transfection efficiency was assessed by inverted laser confocal microscopy.

Similar methods were used for gene silencing of the expression of PDGF-B, Laminin A/C, and HIF1-α mRNA, except that the mRNA was not labeled.

Microscopic analysis

Fluorescently immunostained or labeled cells were observed by inverted laser confocal microscopy at 40 × magnification (FLUOVIEW FV500, Olympus, Tokyo, Japan) with differential interference contrast effects. Expression of HIF1-α protein in each culture was evaluated by determining the relative fluorescence intensity of ten random visual fields and comparing them with that of normoxic cells (cultured in 21% oxygen) or of the control cells in the siRNA assay by public domain image processing and analysis using ImagePro plus (Media Cybernetics, Inc., Silver Spring, MD). Activation of HIF1-α within the nucleus and expression of Laminin A/C in the nucleus were detected alternatively by inverted fluorescence microscopy (I×71-ARCEVA Olympus, Tokyo, Japan). The images obtained were digitized and processed using a digital-image analysis system (Fluorescent Imaging System SZX12-RFL3, Olympus, Tokyo, Japan).

Statistical analysis

Statistical analysis and graph plotting were performed using a commercially available software packages (GraphPad Prism 4, GraphPad Software, Inc., San Diego, CA). Relative fluorescence intensities, expressed as means ± standard deviation, were compared using Student's *t*-test. Statistical significance was defined as a *P*-value less than 0.05

Results

Expression of PDGF- β and HIF1-α after exposure to oxygen concentration gradient

For each of the three cell lines, the relative fluorescence intensity of PDGF-B was significantly higher at 1% oxygen, compared with that at 21% oxygen as 100% (A172 cell line: 100 ± 9.6% at 21% vs. 565.6 ± 36.8% at 1%, $P < 0.01$; U87MG cell line: 100 ± 18.9% at 21% vs. 765.8 ± 28.5% at 1%, $P < 0.01$; U251MG cell line: 100 ± 6.5% at 21% vs. 326.8 ± 22.4% at 1%, $P < 0.01$) (Figure 1(a)). Flow cytometric analysis, which measures intracellular expression, showed similar results (Figure 1(b)). PDGF-B secretion into the supernatant also was significantly higher in cells exposed to 1% oxygen, compared with that at 21% oxygen as 100% (A172 cell line: 54.22 ± 1.26 ng/ml at 21% vs. 168.54 ± 11.23 ng/ml at 1%, $P < 0.005$; U87MG cell line: 32.88 ± 2.90 ng/ml at 21% vs. 126.84 ± 8.85 ng/ml at 1%, $P < 0.001$; U251MG cell line: 21.80 ± 2.93 ng/ml at 21% vs. 156.98 ± 11.88 ng/ml at

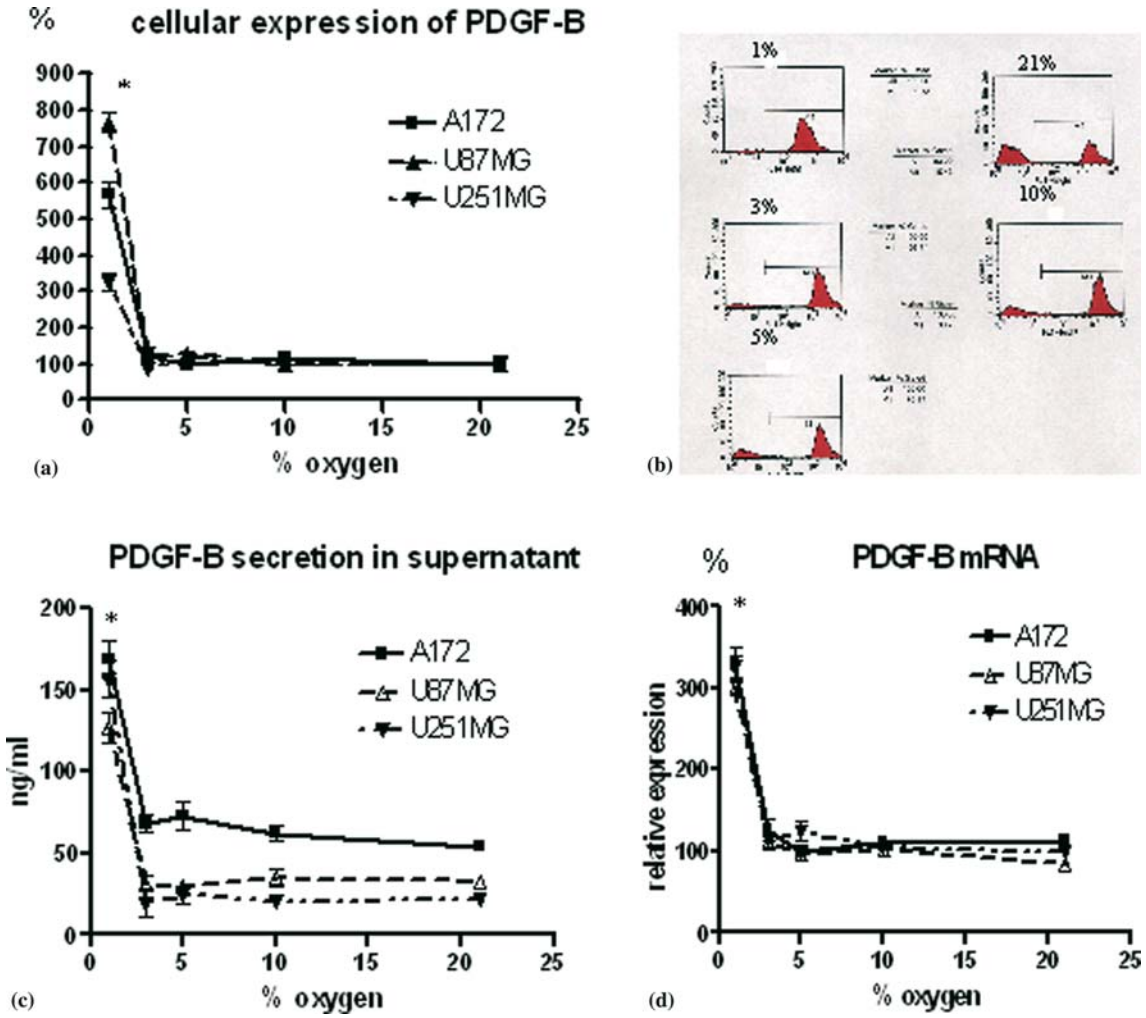

Figure 1. Expression of PDGF-B after exposure to oxygen concentration gradient. (a) The relative fluorescence intensity of PDGF-B does not change significantly over the oxygen concentration range from 21% to 3%, but it is significantly higher at 1% oxygen. $*P < 0.001$; (b) Flow cytometric analysis, indicating intracellular expression of PDGF-B protein, shows similar results, represented by A172. (c) PDGF-B secretion into the supernatant detected by EIA study also is not changed significantly over the oxygen concentration range from 10% to 3%, but it is significantly higher in cells exposed to 1% oxygen. $*P < 0.001$; (d) Percent PDGF-B mRNA expression relative to β-actin is elevated only after exposure to 1% oxygen. $*P < 0.001$.

1%, $P < 0.001$) (Figure 1(c)). Percent PDGF-B mRNA expression relative to β-actin was increased after exposure to 1% oxygen (A172 cell line: 112.8 ± 5.8% at 21% vs. 328.0 ± 21.5% at 1%, $P < 0.005$; U87MG cell line: 84.2 ± 6.8% at 21% vs. 302.2 ± 11.8% at 1%, $P < 0.005$; U251MG cell line: 98.6 ± 5.2% at 21% vs. 325.6 ± 14.5% at 1%, $P < 0.01$) (Figure 1(d)).

While relative intracellular fluorescence intensity of HIF1-α was significantly increased in cells exposed to 1% oxygen, compared with that at 21% oxygen as 100% (A172 cell line: 100 ± 12.2% at 21% vs. 593.7 ± 35.6% at 1%, $P < 0.005$; U87MG cell line: 100 ± 13.2% at 21% vs. 496.4 ± 35.8% at 1%, $P < 0.01$; U251MG cell line: 100 ± 13.6% at 21% vs. 392.8 ± 26.4% at 1%, $P < 0.01$) (Figure 2(a)). Percent HIF1-α mRNA expression relative to that of β-actin was altered significantly at 1% oxygen (A172 cell line: 54.8 ± 6.2% at 21% vs. 134.5 ± 12.8% at 1%, $P < 0.01$; U87MG cell line: 48.8 ± 2.4% at 21% vs. 228.5 ± 7.5% at 1% 02, $P < 0.01$; U251MG cell line: 48.5 ± 7.2% at 21% vs. 156.3 ± 13.8% at 1%, $P < 0.01$) (Figure 2(b)).

Confocal laser microscopy showed similarly enhanced expression of both PDGF-B and HIF1-α protein at 1% oxygen. Fluorescence microscopy clearly revealed enhanced intranuclear accumulation of HIF1-α in cells exposed to 1% oxygen (Figure 3).

Expression of PDGF-B and HIF1-α after gene silencing with siRNA targeting HIF1-α mRNA

When we assayed the transfection efficiency of each Cy5-labeled siRNA, we found that the Laminin A/C and HIF1-α siRNA had very similar (> 95%) transfection efficiency into each of the three cell lines. In contrast, our negative control, without siRNA and TransIT-TKO Transfection Reagent, and mock transfection, using the transfection reagent only, resulted in no autofluorescent activity at the wavelength of Cy5. When we transfected Laminin A/C siRNA into these cells, we found that expression of Laminin A/C, normally localized to the nuclear membrane, was dramatically suppressed. In contrast, transfection of mock (only

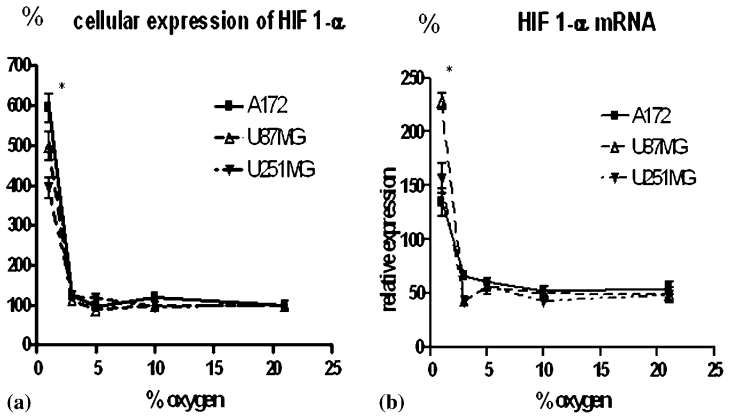

Figure 2. Expression of HIF1-α after exposure to oxygen concentration gradient. (a) While relative intracellular fluorescence intensity of HIF1-α does not change significantly over oxygen concentrations ranging from 21% to 3%, it is significantly increased in cells exposed to 1% oxygen. $*P < 0.001$; (b) Percent HIF1-α mRNA expression relative to that of β-actin is not altered significantly over the entire range of oxygen concentrations examined. $*P < 0.001$.

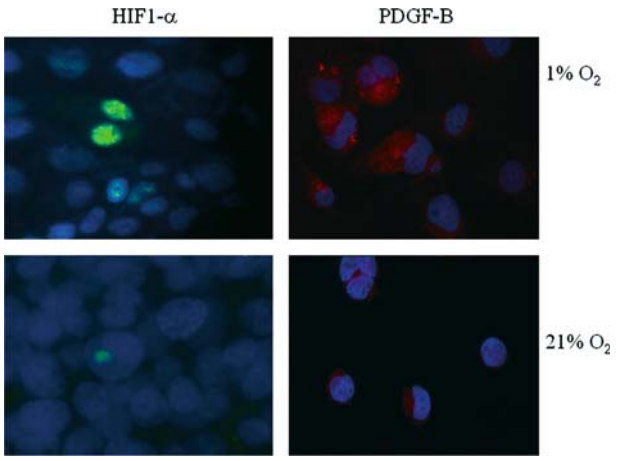

Figure 3. Confocal laser microscopy, represented by U87MG, shows similarly enhanced expression of both PDGF-B and HIF1-α protein at 1% oxygen.

transfectant), scramble or HIF1-α siRNA did not alter the expression of Laminin A/C, compared with control (Figure 4).

When cells transfected with HIF1-α siRNA were exposed to 1% oxygen for 24 h, the percent HIF1-α mRNA, relative to that of β-actin, was significantly reduced compared with their respective control cells (A172 cells: 164.6 ± 15.6% for control vs. 5.8 ± 2.4% for 100 nM HIF1-α siRNA, $P < 0.001$; U87MG cells: 146.8 ± 12.5% for control vs. 12.8 ± 0.8% for 100 nM HIF1-α siRNA, $P < 0.001$; U251MG cells: 268.2 ± 22.4% for control vs. 3.6 ± 0.4% for 100 nM HIF1-α siRNA, $P < 0.001$), whereas mock, scramble, or Laminin A/C siRNA had no effect (Figure 5(a)). The relative intracellular fluorescence intensity of HIF1-α was diminished after exposure to HIF1-α siRNA, compared with control as 100% (A172 cells: 100 ± 5.1% for control vs. 1.8 ± 1.0% for 100 nM HIF1-α siRNA, $P < 0.001$; U87MG cells: 100 ± 6.1% for control vs. 2.4 ± 0.8% for 100 nM HIF1-α siRNA, $P < 0.001$; U251MG cells: 100 ± 6.4% for control vs. 3.2 ± 0.8% for 100 nM HIF1-α siRNA, $P < 0.001$) (Figure 5(b)). Suppression of intracellular HIF1-α expression was also supported by the results of confocal laser microscopy (Figure 5(c)).

We found that the percent PDGF-B mRNA expression relative to that of β-actin was significantly decreased in cells transfected with HIF1-α siRNA and exposed to 1% oxygen (A172 cells: 524.5 ± 33.5% for control vs. 7.8 ± 1.2% for 100 nM HIF1-α siRNA,

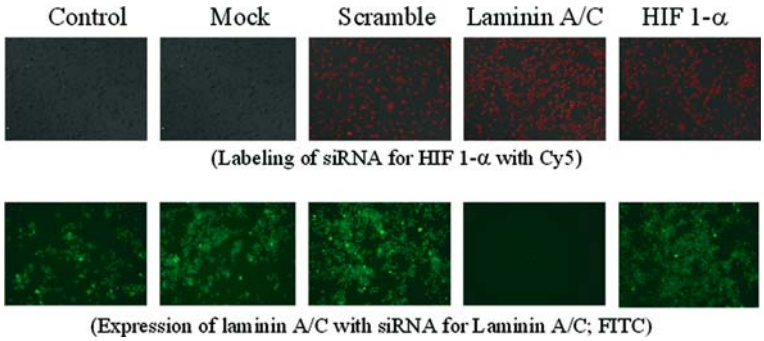

Figure 4. Fluorescence microscopic study for transfection efficiency of siRNA and Laminin A/C as a positive control U251MG) upper; The transfection of HIF1-α siRNA have similar transfection efficiency over 95% (Cy5-labeled siRNA). In contrast, our negative control, without siRNA and TransIT-TKO Transfection Reagent, and mock transfection, using the transfection reagent but no labeled siRNA, result in no autofluorescent activity (× 40). lower; When transfected Laminin A/C siRNA, expression of Laminin A/C (FITC-labeled), normally localized to the nuclear membrane, is dramatically suppressed. In contrast, transfection of mock, scramble or HIF1-α siRNA does not alter the expression of Laminin A/C, compared with control (× 40).

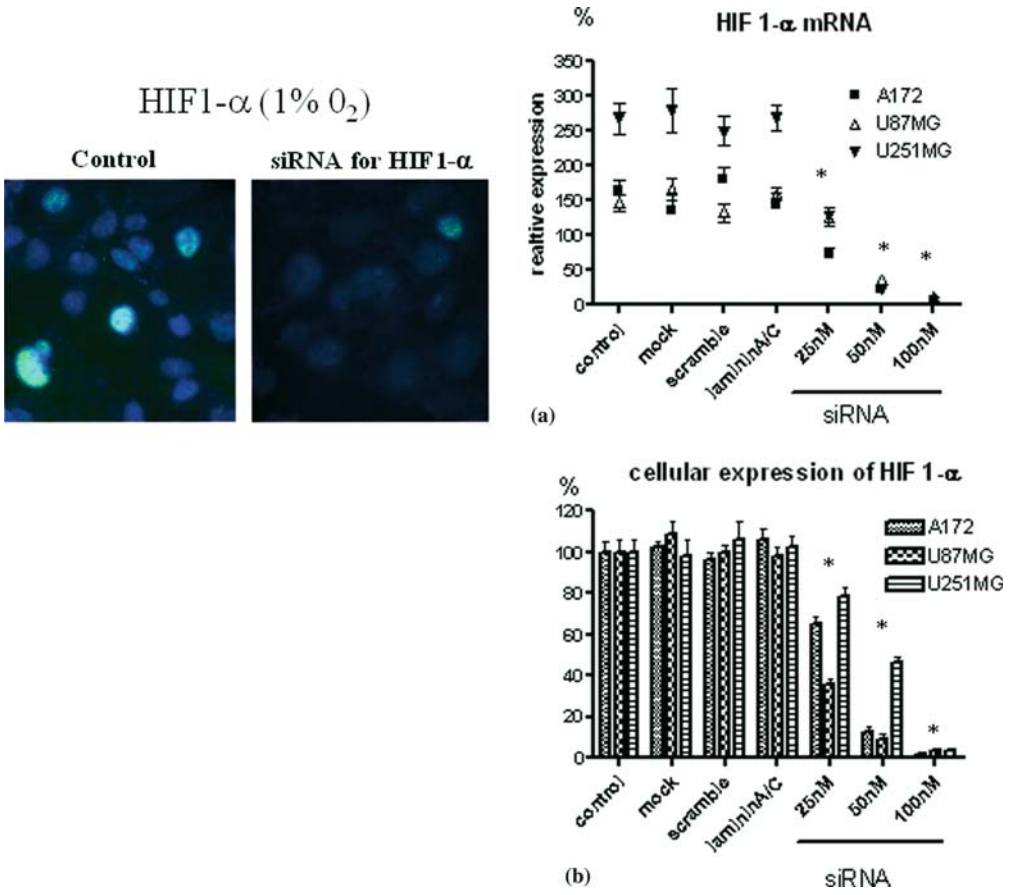

Figure 5. Expression HIF1-α after gene silencing with HIF1-α siRNA. (a) When cells transfected with HIF1-α siRNA are exposed to 1% oxygen for 24 h, the percent HIF1-α mRNA, relative to that of β-actin, is significantly reduced compared with their respective control cells. Whereas mock, scramble, or Laminin A/C siRNA have no effect. *$P < 0.001$; (b) The relative intracellular fluorescence intensity of HIF1-α is diminished after exposure to HIF1-α siRNA. *$P < 0.001$; (c) Suppression of intracellular HIF1-α expression is supported by the results of confocal laser microscopy (U87MG). (× 40).

$P < 0.001$; U87MG cells: 412.4 ± 22.6% for control vs. 66.3 ± 5.8% for 100 nM HIF1-α siRNA, $P < 0.001$; U251MG cells: 580.0 ± 24.5% for control vs. 18.6 ± 2.8% for 100 nM HIF1-α siRNA, $P < 0.001$), whereas mock, scramble, or Laminin A/C siRNA had no effect (Figure 6(a)). Relative intracellular fluorescence intensity of PDGF-β was diminished by exposure to HIF1-α siRNA for 24 h in 1% oxygen, compared with control as 100% (A172 cells: 100 ± 2.4% for control vs. 12.6 ± 1.2% for 100 nM HIF1-α siRNA, $P < 0.001$; U87MG cells: 100 ± 1.2% for control vs. 22.4 ± 1.0% for 100 nM HIF1-α siRNA, $P < 0.001$; U251MG cells: 100 ± 3.2% for control vs. 33.6 ± 0.6% for 100 nM HIF1-α siRNA, $P < 0.001$) (Figure 7(b)). HIF1-α siRNA also inhibited PDGF-B secretion into the supernatant (A172 cells: 45.2 ± 2.36 ng/ml for control vs. 18.0 ± 2.4 ng/ml for 100 nM HIF1-α siRNA, $P < 0.001$; U87MG cells: 134.2 ± 14.5 ng/ml for control vs. 48.4 ± 2.8 ng/ml for 100 nM HIF1-α siRNA, $P < 0.001$; U251MG cells: 68.8 ± 2.5 ng/ml for control vs. 8.8 ± 0.6 ng/ml for 100 nM HIF1-α siRNA, $P < 0.001$) (Figure 6(c)). Confocal laser microscopy showed that HIF1-α siRNA inhibited intracellular expression of PDGF-β (Figure 7(a)), as did flow cytometric assays of intracellular PDGF-B, both in 1% oxygen and in 21% oxygen (Figure 7(b)).

Discussion

We have shown here the ability to quantitatively measure the expression of PDGF-B and HIF1-α in glioblastoma cell lines after exposure to normoxia or hypoxia. We also found that PDGF-B and HIF1-α mRNA and protein were concomitantly expressed under normoxic conditions in these three cell lines and that expression of PDGF-β mRNA and protein were elevated simultaneously and dramatically after exposure to severe hypoxic conditions at 1%. When expression of HIF1-α protein was correspondingly elevated in hypoxia, the expression of HIF1-α mRNA increased. The current study demonstrated that the degradation of HIF1-α mRNA by specific siRNA induced the down-regulation of PDGF-β mRNA and protein, indicating that PDGF-B expression is transcriptionally regulated in severe hypoxic condition mediated by HIF1-α. The transfection efficiencies we obtained, of 95% or better, with both HIF-1α and Laminin A/C siRNA, and the length of continuous labeling, from 1 to 6 days (data not shown), are much better than those obtained with traditional single-stranded antisense oligonucleotides (ASOs). ASOs have been used extensively to determine the functions of a particular gene, both in vivo and in vitro[10]. In neurobiology, ASOs have gained increasing

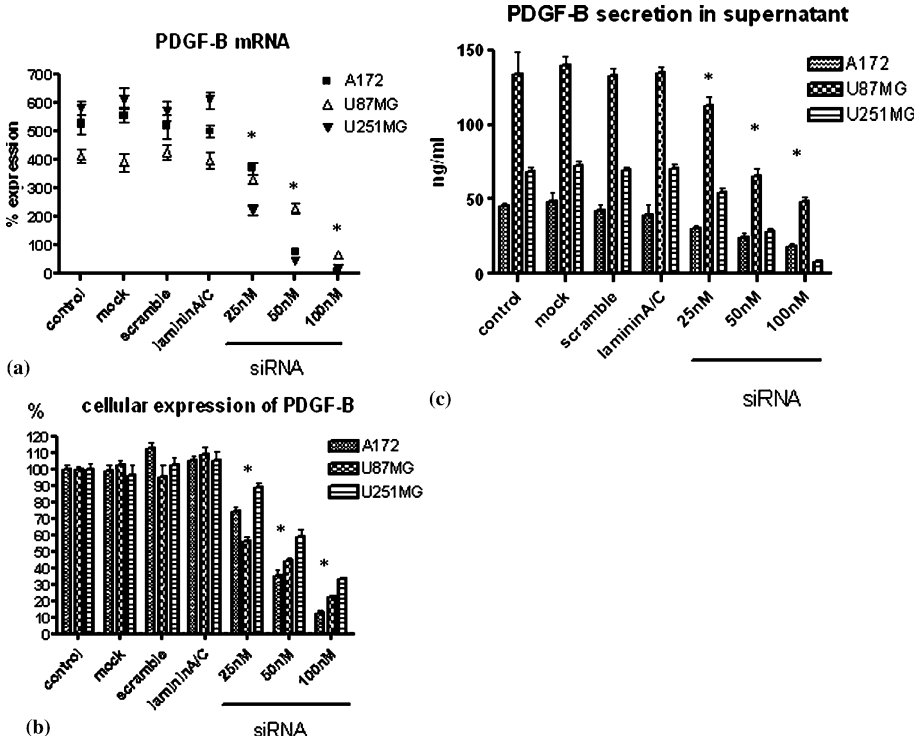

Figure 6. Expression of PDGF-B after gene silencing with HIF1-α siRNA. (a) The percent PDGF-B mRNA expression relative to that of β-actin is significantly decreased in cells transfected with HIF1-α siRNA in 1% oxygen, whereas mock, scramble, or Laminin A/C siRNA has no effect. $^*P < 0.001$; (b) Relative intracellular fluorescence intensity of PDGF-B is diminished by exposure to HIF1-α siRNA for 24 h in 1% oxygen. $^*P < 0.001$; (c) Inhibited PDGF-B secretion by HIF1-α siRNA. $^*P < 0.001$.

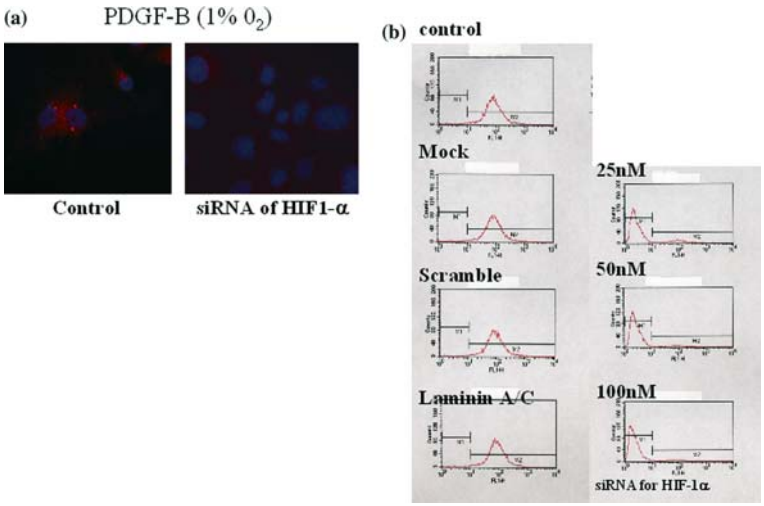

Figure 7. (a) Confocal laser microscopy shows inhibited intracellular expression of PDGF-B by HIF1-α siRNA (A-172) (× 40). (b) Flow cytometric analysis for suppressed intracellular expression of PDGF-B by HIF1-α siRNA at 1% oxygen.

acceptance for studying and possibly ameliorating or treating CNS tumors[11]. However, the well documented non-specific effects and lower transfection efficiencies of ASOs (maximum, about 15%) complicate interpretation of studies employing single-stranded ASOs[12]. As an alternative, sequence-specific degradation of mRNAs can be triggered by the RNA interference (RNAi) process [13]. This method can be used to study the consequences of 'knock-out' and 'knock-down' of gene expression. Comparable with classical genetic deletions, siRNA can lead to the complete and stable degradation of the targeted mRNA. Practical laboratory experience has shown that longer lasting effects of siRNA may be achieved by modifying the method of transfection [14]. In addition to its use in basic molecular neurobiology research, the use of siRNA may be applicable to the future clinical treatment of malignant gliomas [3].

HIF-1 plays a crucial role in oxygen homeostasis during normal vascular development [15]. This protein is a heterodimeric basic-helix-loop-PAS (bHLH-PAS) transcription factor composed of α and β subunits [16]. HIF1-β, also known as the aryl hydrocarbon receptor nuclear translocator, can dimerize with several different

bHLH-PAS transcription factors [17]. By contrast, HIF1-α subunit has unique characteristics, in that its expression is directly affected by cellular oxygen concentration [12]. It has been shown that, as cellular oxygen decreases, the level of HIF-α activity increases. Under these conditions, HIF-1 is stabilized from proteasome degradation and activated by hypoxia, resulting in its stimulating of expression of hypoxia-sensitive genes that mediate oxygen homeostasis in many tissues [18]. The molecular mechanisms associated with hypoxia have been investigated primarily in infarction of normal organs, including myocardial infarction, with a focus on erythrocytes and vascular endothelial cells [6]. In addition, HIF-1 has been shown to be involved in the cellular response to hypoxia in the ischemic testis [10]. In studies with normal cells, HIF1 was observed to activate a large battery of genes whose products function either to increase oxygen availability or to allow metabolic adaptation to oxygen deprivation [19]. Among the genes activated by HIF1-α are those encoding erythropoietin, VEGF, glucose transporters, glycolytic enzymes, insulin-like growth factor 2 (IGF2), and IGF binding proteins 1, 2, and 3 [17]. The effect of HIF1 on PDGF-β gene in glioma cells, however, is not yet known. Hypoxia has recently come to be considered one of the most important factors modifying the malignant properties of cancer cells in vivo [20]. Tumor cells exposed to hypoxia show upregulation of the HIF-signaling pathway, which may promote tumor progression and allow tumor cells to survive under hypoxic conditions [21]. Tumor angiogenesis and anaerobic metabolism, including glycolysis, may also be associated with this mechanism of evacuating tumor cells from hypoxic crisis. Several studies have suggested a positive correlation between hypoxia or HIF-1α expression and malignant phenotype, such as an increase in histological grading, invasion, or metastasis [22]. Adaptation to hypoxic stress in the tumor microenvironment may thus be associated with the malignant progression of tumor cells. Many reports indicate that this transcriptional factor up-regulates VEGF in malignant glioma [21]. In fact, VEGF is co-located with HIF1-α predominantly in the area neighboring necrotic zone which is considered as oxygen supply is much lowered [23]. In hypoxia, PDGF-B is upregulated in several cancer cells [22], but in gliomas whether it is transcriptionally regulated by the specific factor, HIF1-α has not yet been clarified.

PDGF-B is overexpressed in human gliomas and responsible for recruiting per-endothelial cells to vessels [5]. It was also reported that PDGF-B demonstrates dose-dependent effects on glial tumorigenesis [24]. In mice, overexpression of PDGF-B in U87MG cells enhanced intracranial glioma formation by stimulating vascular endothelial growth factor (VEGF) expression in neovessels and by attracting vessel-associated pericytes. When PDGF-B and VEGF were overexpressed simultaneously by U87 MG tumors, there was a marked elevation of capillary-pericyte recruitment, vessels induced by VEGF in tumor vicinity migrated into the central regions of these tumors. These data suggested PDGF-B is a paracrine factor in gliomas, and PDGF-B enhances glioma angiogenesis, at least in part, by stimulating VEGF expression in tumor endothelia an by recruiting pericytes to neovessels [25]. These accumulated evidences have shown that PDGF-B plays an important role in angiogenesis. Meanwhile, mice deficient either in PDGF-B or PDGF-receptor β developed hemorrhages or edema during the later stage of embryogenesis [7]. In hypoxia, HIF1-α is activated to up-regulate VEGF transcriptionally in gliomas [26] and expression of PDGF-B is also elevated to stimulate VEGF expression [9]. The present study indicated clearly that HIF-1α regulates PDGF-B expression.

Cells, including glioma cells, are usually cultured under optimal conditions for cell growth, in an atmosphere of 5% CO_2 and 21% O_2 in a humid incubator. In a study of spatial oxygen distribution in gliomas and the surrounding brain tissue, both glioma tissue and peritumoral brain were observed to contain regions at oxygen tensions less that 2.5 mmHg, considerably lower than that of arterial blood. In addition, the median oxygen concentration was much lower in high grade gliomas than in low grade gliomas [10]. Thus, by culturing glioma cells in 21% oxygen, in vivo conditions would not be duplicated. Moreover, other biological markers relating to hypoxia and angiogenesis may differ between normoxic and hypoxic conditions.

We have shown here that malignant glioma cells have a system to relieve hypoxic stress, mediated by the PDGF-B pathway. Expression of PDGF-B, in turn, is regulated by HIF-1α. This pathway might be a major driving force in HIF-1α expression in tumor angiogenesis of malignant gliomas. Although glioma angiogenesis mediated by may be driven by oncogenic stimuli, it may also be driven, at least in part, by a physiologic stimulus.

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