Downregulation of GFAP, TSP-1, and p53 in Human Glioblastoma Cell Line, U373MG, by IE1 Protein From Human Cytomegalovirus

KARIM LEE,[1†] KIPYOUNG JEON,[2†] JONG-MOOK KIM,[3] VIC NARRY KIM,[2] DONG HEE CHOI,[4]
SEUNG U. KIM,[5,6] AND SUNYOUNG KIM[1,2*]
[1]*Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Korea*
[2]*School of Biological Sciences, Seoul National University, Seoul, Korea*
[3]*ViroMed Co. Ltd., Seoul, Korea*
[4]*Department of Obstetrics and Gynecology, Bundang Cha General Hospital, Pochon Cha University, Kyungki-do, Korea*
[5]*Brain Disease Research Center, Ajou University School of Medicine, Suwon, Korea*
[6]*University of British Columbia, Vancouver, Canada*

KEY WORDS
HCMV; IE1; GFAP; TSP-1; p53

ABSTRACT
Human cytomegalovirus (HCMV) is a member of the β-herpesvirus family, which has tropism for glial cells. It was recently reported that HCMV might play important roles in the pathogenesis of malignant glioma. In this study, we investigated the effects of the HCMV IE1 protein on the gene expression profile in the human glioblastoma cell line, U373MG by employing cDNA microarray technology. Using DNA chips containing approximately 1,000 human cDNAs, RNA samples from U373MG cells stably expressing IE1 were compared with those from the control cells lacking IE1 cDNA. Fluorescence intensities of 13 genes were significantly decreased in IE1-expressing cells, while one gene was found to be upregulated. Among these 14 genes, we chose to work further on glial fibrillary acidic protein (GFAP), thrombospondin-1 (TSP-1), and p53, because of their previously known involvement in tumorigenesis. The mRNA levels of all these genes were found to be decreased in IE1-expressing glioblastoma cells by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) as well as Northern blot analysis. The decreased expression of these genes was also observed at protein levels as measured by immunocytochemistry or fluorescence-activated cell sorting (FACS) analysis. Our data strongly suggested that HCMV IE1 could modulate the expression of cellular genes that might play important roles in the pathogenesis of glial tumors. © 2005 Wiley-Liss, Inc.

INTRODUCTION

Human cytomegalovirus (HCMV) is a member of β-Herpesvirinae endemic in the human population that can cause life-threatening complications in immunocompromised or immunosuppressed individuals (Mocarski, 1996). Various HCMV-infected cells were detected in the histological sections of affected brains. Indeed, endothelial, astroglial and neuronal cells in the human brain have been infected by HCMV in vitro (Schmidbauer et al., 1989; Poland et al., 1990; Plachter et al., 1996). Recently, a high percentage of malignant glioma samples were shown to have been infected by HCMV, and HCMV gene products including IE1 were indeed expressed in these tumors, suggesting that HCMV might play active roles in the pathogenesis of glioma (Cobbs et al., 2002).

Similar to other herpesviruses, HCMV gene expression occurs in three temporal phases designated immediate-early (IE), early, and late (Mocarski, 1996). The IE genes encode the first set of proteins expressed upon infection, some of which are involved in fine-tuning of the virus life cycle. One of the most intensively characterized IE genes of HCMV is IE1. This 72-kDa phosphoprotein is abundantly expressed immediately after infection (Plachter et al., 1996). In transient transfection assays, IE1 has been shown to be involved in the autoregulation of its own promoter and also in the transactivation of viral and cellular promoters, alone or in concert with other viral or cellular proteins (Stenberg and Stinski, 1985; Davis et al., 1987; Tevethia et al., 1987; Cherrington and Mocarski, 1989; Sambucetti et al., 1989; Stenberg et al., 1989; Iwamoto et al., 1990; Hagemeier et al., 1992; Monick et al., 1992; Michelson et al., 1994; Margolis et al., 1995; Yurochko et al., 1995; Kim et al., 1999; Murayama et al., 2000). It was also reported that the IE1 gene product could cooperate with another IE gene product, IE2, and adenoviral E1A to transform primary baby rat kidney (BRK) cells (Shen et al., 1997). Despite the implication of such data in a possible involvement of IE1 in tumorigenesis, the actual role of this viral protein in the real situation remains to be elucidated.

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*Correspondence to: Sunyoung Kim, Laboratory for Virology, Institute of Molecular Biology and Genetics, Building-105, Seoul National University, Kwanak-gu, Seoul 151-742, Korea. E-mail: sunyoung@plaza.snu.ac.kr
†Karim Lee and Kipyoung Jeon contributed equally to this work.

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In this study, we investigated the effects of the IE1 protein on gene expression profile in glioblastoma cell line U373MG by cDNA microarray analysis. We found that human cytomegalovirus IE1 protein regulates several cellular genes that might play important roles in the pathogenesis of glial tumors.

MATERIALS AND METHODS

Cell Cultures and Plasmids

Human glioblastoma cell line U373MG (HTB 17) and A172 (CRL1620) were obtained from American Type Culture Collection (Rockville, MD). U251MG and U343MG were kind gifts from Dr. Yong-Kil Hong (Catholic University of Korea). These cell lines were used to generate constant stable cell lines expressing IE1. Cells were grown in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 U of penicillin per ml, and 100μg of streptomycin per ml in a 5% CO_2 humidified incubator at 37°C.

pEQ273 contains the full genomic sequence of IE1, including the HCMV major immediate-early promoter, while pEQ336 having only the promoter and lacking the coding sequence as described (Biegalke and Geballe, 1991). The retroviral vector, MT5-cIE1, was constructed by inserting the cDNA sequence of IE1 into retroviral vector MT5 (Lee et al., 2004). The reporter plasmid, pCN-chloramphenicol acetyl transferase (CAT), were constructed by inserting the CAT gene into expression vector pCN (Lee et al., 2000).

Construction of IE1-Expressing Cell Lines

U373MG cells expressing IE1 and containing the control vector lacking the IE1 cDNA sequence are collectively called U373MG-IE1 and U373MG-X, respectively. Construction of U373MG-X and U373MG-IE1 has been described elsewhere (Kim et al., 1999). Other glioma cell lines, such as U251MG, U343MG, and A172, were manipulated in the same manner using retroviral vectors. Stable transduction of U373MG cells by LNC-gIE1 was confirmed by genomic reverse transcription-polymerase chain reaction (RT-PCR) and DNA PCR, using primers specific for IE1. The primer sequences are as follows: IE1-1: 5′ ATGGAGTCCTCTGCCAAGAG 3′ IE1-5: 5′ TTACTGGTCAGCCTTGCTTC 3′.

Transient Transfection and CAT Assays in U373MG Cells

For transient expression assays, U373MG cells were transfected by the DEAE-dextran method as previously described (Ikeuchi et al., 1990). Briefly, cells were washed with serum-free medium, and exposed to a DNA/DEAE-dextran mixture in serum-free medium (400μg DEAE-dextran/ml) for 1 h at 37°C. The cells were then harvested, suspended in fresh media, and grown for 40–48 h. CAT activity was determined according to a standard method (Gorman et al., 1982). Equal amounts of protein were incubated with acetyl coenzyme A (1 mM) (Sigma, St. Louis, MO) and [^{14}C]-chloramphenicol at 37°C. Acetylated products were separated by thin layer chromatography (Silica gel; Merck, Darmstadt, Germany). The amounts of acetylation were quantified by phosphoimager (Fuji, Japan).

cDNA Microarray Analysis

The cells were washed once with phosphate-buffered saline (PBS) and lysed with guanidine buffer. Then total RNA was extracted from the lysate using the CsCl density-gradient centrifugation method. In this study, 20 μg of each RNA sample was reverse transcribed into cDNA by using a Fluorescence Labelling Core Kit (TaKaRa Bio, Ostsu, Shiga, Japan), which contains oligo-dT primer, AMV reverse transcriptase, Cy3-UTP, and Cy5-UTP. cDNAs were purified to remove unincorporated ribonucleotides and then melted into hybridization buffer. The fluorescent dye-labeled cDNAs were hybridized for 16 h at 65°C to a DNA chip arrayed and immobilized with approximately 1,000 cDNA fragments of human cellular genes on a slide glass (TaKaRa Intelligene Human 1k CHIP Version 2.0, TaKaRa Bio). The arrays were washed according to the manufacturer's instructions. Scanning was performed using an Affymetrix scanner (Affymetrix, Santa Clara, CA). Data obtained from scanning were analyzed using the Imagene 4.2 software system (Biodiscovery, Marina del Rey, CA). Gene expression was considered to be significantly altered if the level changed by threefold or greater. If the signal intensity of a gene was found to be comparable to the background, it was removed from the list of genes whose levels were changed, even though its fold change was greater than three.

Real-Time Quantitative RT-PCR

cDNAs were synthesized from 1μg of each RNA sample by reverse transcription. Real-time quantitative PCR analysis was performed using SYBR Green dye and the primers specific for β-actin or GFAP. The sequences of primers are as follows, in which F is a forward primer and R is a reverse primer: β-actin F: 5′ TCACCCACACTGTGCCCATCTACGA 3′, β-actin R: 5′ CAGCGGAACCGCTC ATTGCCAATGG 3′, GFAP F: 5′ CGAGATCGCCACCTACAGGA 3′, GFAP R: 5′ GATTCGAGAAACCAGCCTGG 3′ TSP-1 F: 5′ CCTCAGGAACAAAGGCTGCTC 3′, TSP-1 R: 5′ GCCAATGTAGTTAGTGCGGATG 3′, p53 F: 5′ CCCAGCCAAAGAAGAAACCA 3′, p53 R: 5′ TTCCAAGGCCTCATTCAGCT 3′.

From each PCR reaction, the C_T value was obtained from the amplification curve. The difference in mRNA levels of cellular genes between IE1-expressing and control cell lines was calculated from ΔC_T value as described (Martell et al., 1999; Yu et al., 2000).

Northern Blot Analysis

In this study, 20 μg of total RNA, prepared as described above, was subjected to 1% formaldehyde-agarose gel electrophoresis, blotted to nylon membrane (Hybond-N; Amersham, Piscataway, NJ), and hybridized with ^{32}P-labeled GFAP or β-actin probe.

Immunocytochemistry

IE1-expressing and control U373MG cell lines were grown on glass cover slips in DMEM supplemented with 10% FBS. Cells were fixed with 2% formaldehyde, permeabilized with cold acetone, and then double-stained with anti-GFAP (NeoMarkers, Fremont, CA) and anti-IE1 (MAB810; Chemicon, Temecula, CA) monoclonal antibodies.

Western Blot Analysis

To confirm the expression of IE1 in U373MG: LNC-IE1 cell lines, whole cell lysates were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Hybond-C extra; Amersham). Immunoblotting was performed with an anti-IE1 monoclonal antibody (Chemicon). Blots were visualized by chemiluminescence, using horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Pierce, Rockford, IL).

siRNA Preparation and Transfection

Four siRNA duplexes—siIE1-1, siIE1-2, siIE1-3, and siIE1-4—were synthesized by Dharmacon Research (Lafayette, CO). Their target sequences are 5′-TGGTGCGGCATAGAATCAA-3′, 5′-AAGGATGAACTTAGGAGAA-3′, 5′-AACAATGTGTAATGAGTAC-3′, 5′-GGTTATCAGTGTAATGAAG-3′. In each sequence, the first nucleotide sequence begins from numbers 284, 589, 843, 1002, respectively, of IE1 cDNA starting from A in the initiation codon ATG as number 1.

The oligonucleotides were desalted and deprotected according to the manufacturer's instructions. For siRNA expression, U373MG-IE1 cells were transfected using Oligofectamine™ (Invitrogen, Carlsbad, CA) as described (Elbashir et al., 2001). Briefly, siRNAs [1.2 nmol (60 μl)] were mixed with 60 μl of Opti-MEM (Gibco-BRL, Carlsbad, CA) and added to diluted Oligofectamine; the mixture was placed onto a 100-mm dish of U373MG-IE1. Total RNA or cell lysates were prepared 3 days after transfection and used for real-time quantitative RT-PCR and Western blot analysis.

Astrocyte Culture

Primary human astrocyte cultures were prepared from embryonic human brain of 15 weeks gestation, as described previously (Kim, 1985; Nagai et al., 2001). Briefly, brains were dissected into small blocks and incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin and 40μg/ml DNase I for 30 min at 37°C. Enzyme-treated tissues were dissociated into single cells by gentle pipetting and dissociated cells were suspended in DMEM supplemented with 5% horse serum, 5 mg/ml glucose, and 20μg/ml gentamicin, and then plated in T75 flasks at a density of 10^6 cells/ml. After 2–3 weeks in vitro, microglia floating in the medium were detracted, neurons and oligodendrocytes were removed by vigorous shaking and then surface-adherent astrocytes were isolated by a brief incubation in PBS containing 0.1% trypsin and 1 mM EDTA and plated in new flasks. Confluent astrocyte cultures were subcultured every 2 weeks. In the present study, human astrocyte culture of third passage was used and the purity of astrocytes was better than 99% as determined by GFAP immunocytochemistry. The use of human tissue samples was approved by the ethics committee of the University of British Columbia.

Astrocyte Transfection

Purified plasmids, pEQ273 (containing IE1 genomic sequence) and pEQ336 (empty vector) (Biegalke and Geballe, 1991) were mixed with Lipofectamine PLUS reagent (Life Technologies) in serum-free and antibiotic-free media for 15 min at room temperature. Lipofectamine was diluted with the same media and then combined with the DNA mixture for 15 min at room temperature. Astrocytes were seeded on the glass cover slips in 6-well plates and used the next day at confluency of 70%. The preexisting media were removed, and 800 μl of antibiotic-free medium were then added followed by incubating with the transfection mixture at 4 μg of DNA per well for 3 h at 37°C. At the end of the incubation, 2 ml of 10% FBS-containing DMEM was added to the well, and then incubated for additional 8 days.

RESULTS

Construction of Cell Lines Constitutively Expressing IE1

To construct U373MG cells stably expressing IE1, a retroviral vector expressing IE1, LNC-gIE1, was constructed by inserting the genomic DNA sequence of IE1 into plasmid LNC (Fig. 1A). Cell-free retroviral vectors were prepared by the three-plasmid transfection method (Kim et al., 1999) and used to transduce U373MG cells followed by G418 selections. Both drug-resistant populations and subcloned cell lines were established. As a control, the same retroviral vector lacking the IE1 genomic DNA sequence, LNC-X, was also used. The presence and expression of integrated IE1 cDNA were tested by measuring DNA, RNA, and protein specific for IE1. In all assays, the same results were obtained from both drug-

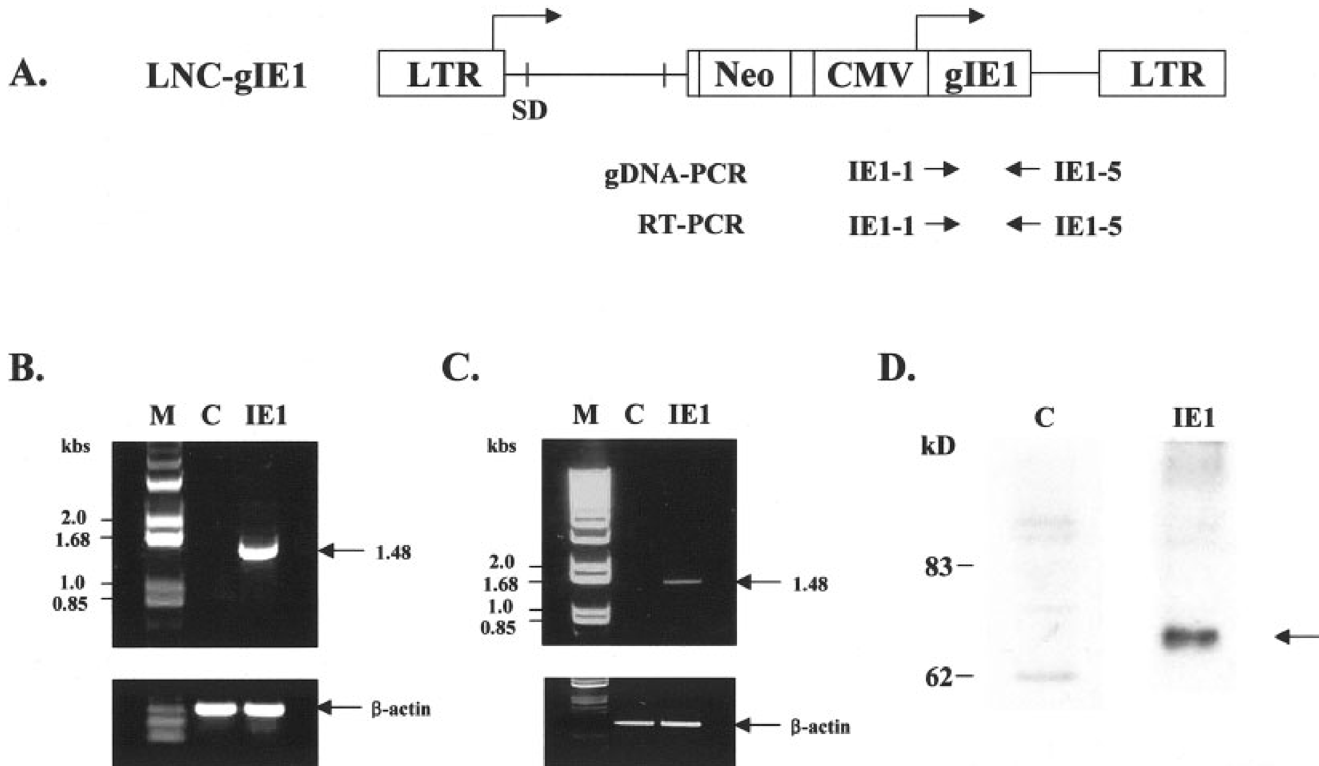

Fig. 1. Construction of U373MG stably expressing IE1. **A:** LNC-gIE1, an MLV (murine leukemia virus)-based retroviral vector expressing IE1. LNC-gIE1 was derived from LNC-X by inserting the genomic DNA sequence of IE1 to the latter (Kim et al., 1999). Neomycin resistant gene (Neo) is expressed from the long terminal repeat (LTR), while the IE1 gene is driven from the human cytomegalovirus MIE promoter (CMV), respectively. The backbone of the vector was previously described in detail (Miller et al., 1993). The positions of oligonucleotide primers used in polymerase chain reaction (PCR) are indicated as an arrow. **B:** Amplification of integrated IE1 cDNA by genomic DNA PCR. The approximate size of the amplified DNA band would be 1.48 kb. β-Actin was used as a control. **C:** Detection of IE1 mRNA by RT-PCR using primers specific for LNC-IE1. As an internal control, human β-actin RNA was also amplified. **D:** Western blot analysis using antibody specific for IE1. M, size marker; C, control cells containing LNC-X lacking IE1 cDNA sequence; IE1, U373MG stably expressing IE1.

resistant populations and subcloned lines. Therefore, only the results obtained from the subcloned cells are presented in this report. Cells expressing IE1 and containing the control vector lacking IE1 cDNA sequence are collectively called U373MG-IE1 and U373MG-X, respectively.

The presence of IE1 cDNA was confirmed by detecting the integrated IE1 cDNA of 1.48 kb from total DNA samples by genomic DNA PCR using the oligonucleotide primer set specific for IE1 (Fig. 1A,B). Expression of IE1 mRNA in U373MG cells was confirmed by amplifying RNAs with RT-PCR, using the same primer set (Fig. 1A,C). The amplified products from both genomic DNA PCR and RT-PCR were cloned into a pGEM-T Easy vector (Promega, Madison, WI) and their oligonucleotide sequences were determined. There was no mutation in the IE1 sequence that might result in any change in amino acid sequence. Finally, expression of the IE1 protein was confirmed by observing an ~72-kDa band by Western blotting, using a specific antibody (Fig. 1D). In control cells containing LNC-X, no specific band was detected in any of these assays.

To confirm that the IE1 protein expressed in U373MG-IE1 cells was indeed functional, we tested whether IE1 could upregulate gene expression from the major immediate-early (MIE) promoter. Both U373MG-IE1 and U373MG-X cells were transfected with a reporter plasmid, pCN-CAT, which drives expression of the CAT gene from the HCMV MIE promoter (Lee et al., 2000), and the CAT assay performed (Fig. 2). The level of CAT activities was higher in U373MG-IE1 cells by approximately 7-fold than in control cells lacking the IE1 protein, suggesting that the U373MG-IE1 cells produced functionally competent IE1 protein.

Effect of IE1 on Cellular Gene Expression

It was recently reported that a high fraction of glial tumor samples contained HCMV and expressed several viral proteins, one of which was IE1 (Cobbs et al., 2002). Because U373MG originated from glial tumor cells, we thought that U373MG-IE1 cells might be a good model to study effects of IE1 on the expression of a variety of human genes using DNA chips. Total RNA was prepared from U373MG-IE1 and U373MG-X cells. Fluorescence-labeled cDNAs were generated from each RNA sample and hybridized to a microarray harboring approximately 1,000 human cDNAs. To minimize experimental errors inherent to the microarray analysis, two separate cDNA microarrays were used in two independent experiments.

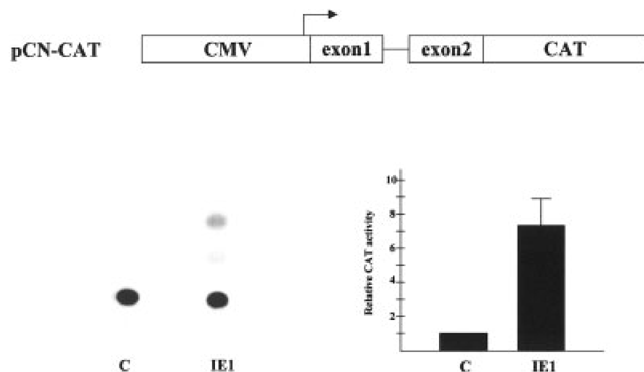

Fig. 2. Activation of the major immediate-early promoter of HCMV by IE1 in U373-IE1 cells. The schematic diagram of the reporter plasmid, pCN-CAT, is shown above. In this construct, not only the promoter, but also the entire untranslated, transcribed regions are present. CAT assay confirming the expression of functional IE1 in U373MG-X (C) and U373MG-IE1 (IE1) cells. Respective cells were transfected with pCN-CAT. Two days later, cells were harvested and the levels of CAT activities measured. All experiments were performed more than three times, and one representative result is shown.

The change in the level of expression of respective genes was calculated by comparing the difference in the fluorescence intensity obtained from RNA samples of U373MG-IE1 with that from those of U373MG-X. We found that among 1,050 genes, expression of 61 genes was downregulated more than 1.5-fold in IE1-cell lines, while that of 16 genes was upregulated more than 1.5-fold. When the difference was more than 3-fold and the signal intensity was considerably higher than that of background, the change in the level of gene expression was considered to be significant, and the list of such genes is shown in Table 1. Among the listed genes, we chose to work further on three genes because of their previously known biological roles in tumorigenesis. They are glial fibrillary acidic protein (GFAP), thrombospondin-1 (TSP-1), and p53.

Downregulation of GFAP by IE1 protein

GFAP is an intermediate filament protein specific for mature cells of the astroglial lineage and it has a molecular weight of ~50 kDa (Rutka et al., 1997). It has been reported that GFAP is progressively lost with an increase in astrocytic malignancies (Deck et al., 1978; Eng and Rubinstein, 1978; Jacque et al., 1979; de Armond et al., 1980; Duffy, 1982; Duffy et al., 1982; Rutka et al., 1997). In our cDNA microarray analysis, the level of GFAP mRNA was decreased reproducibly by 15–fold in U373MG-IE1 cells, as compared with that in control cells (Fig. 3A). This decrease in the RNA level was confirmed by using two different methods: real-time quantitative RT-PCR and Northern blot analysis. In the former assay, the change in the level of GFAP mRNA was approximately 12-fold (Fig. 3B). The GFAP locus is known to produce five different RNA isoforms resulting from alternative splicing. The expression level of different isoforms varies depending on cell types. For example, GFAPα is known to be the dominant isoform in the central nervous system. In Northern blot analysis of U373MG cells, two isoforms, known as GFAPα and ε, had sizes of 2.9 kb and 1.8 kb, respectively (Nielsen et al., 2002). Levels of both mRNAs were decreased in U373MG-IE1 cells by approximately 15- to 20-fold, as determined by phosphoimage analysis (Fig. 3C).

TABLE 1. mRNAs of Cellular Genes Modulated 3-fold or Greater by the IE1 Protein

Genbank accession numbers	Genes	Fold
S40719	Glial fibrillary acidic protein	−14.7
X14787	Thrombospondin-1	−6.7
NM_000546	Tumor protein p53	−3.0
J04177	Collagen, type XI, αa1	−12.4
J05016	Protein disulfide isomerase-related protein	−4.3
X02419	Plasminogen activator, urokinase	−3.5
M59371	EphA 2	−4.6
AL162086	Villin 2	−7.4
X13482	Small nuclear ribonucleoprotein polypeptide A′	−3.6
D87930	Myosin phosphatase, target subunit 1	−7.1
X80692	Mitogen-activated protein kinase 6	−3.5
M90657	Transmembrane 4 superfamily member 1	−3.7
M86752	Stress-induced-phosphoprotein 1	−4.6
S45630	Crystallin, αB	+6.2

We also examined whether the change in the GFAP mRNA level indeed resulted in the change in the protein level by two-color immunocytochemistry using antibodies to IE1 and GFAP (Fig. 3D). The dramatic decrease of GFAP protein was evident in U373MG-IE1 cells. These results clearly indicated that IE1 could significantly downregulate expression of GFAP at the protein as well as mRNA levels. It is interesting to note that the morphology of cells was significantly changed in IE1-expressing cells. When IE1 was expressed, cells became smaller and had shorter stellate processes than control cells (Fig. 3D). It remains to be seen what causes such morphological changes.

Downregulation of TSP-1 by IE1 protein

TSP-1 is an extracellular matrix glycoprotein whose molecular weight is 170–180 kDa (Adams, 1997; Chen et al., 2000; de Fraipont et al., 2001). This protein has been shown to inhibit the formation of new blood vessels by binding to the membrane receptor CD36 (Bornstein, 1995; Roberts, 1996; Tuszynski and Nicosia, 1996). The cDNA microarray data showed that TSP-1 was downregulated approximately 7-fold by IE1 (Fig. 4A). This result was confirmed by not only real-time quantitative RT-PCR (Fig. 4B) but also by the fact that Northern blot analysis generated approximately a 4-fold difference between U373MG-IE1 and U373MG-X cells (Fig. 4C). It has to be noted that the mRNA level of TSP-1 in Northern blot analysis was fluctuating from 1.5- to 4-fold depending on the culture condition. To test whether the protein level of TSP-1 was also changed, a well-established fluorescence-activated cell sorting (FACS) analysis was performed using a mouse monoclonal antibody that specifically detects TSP-1. As

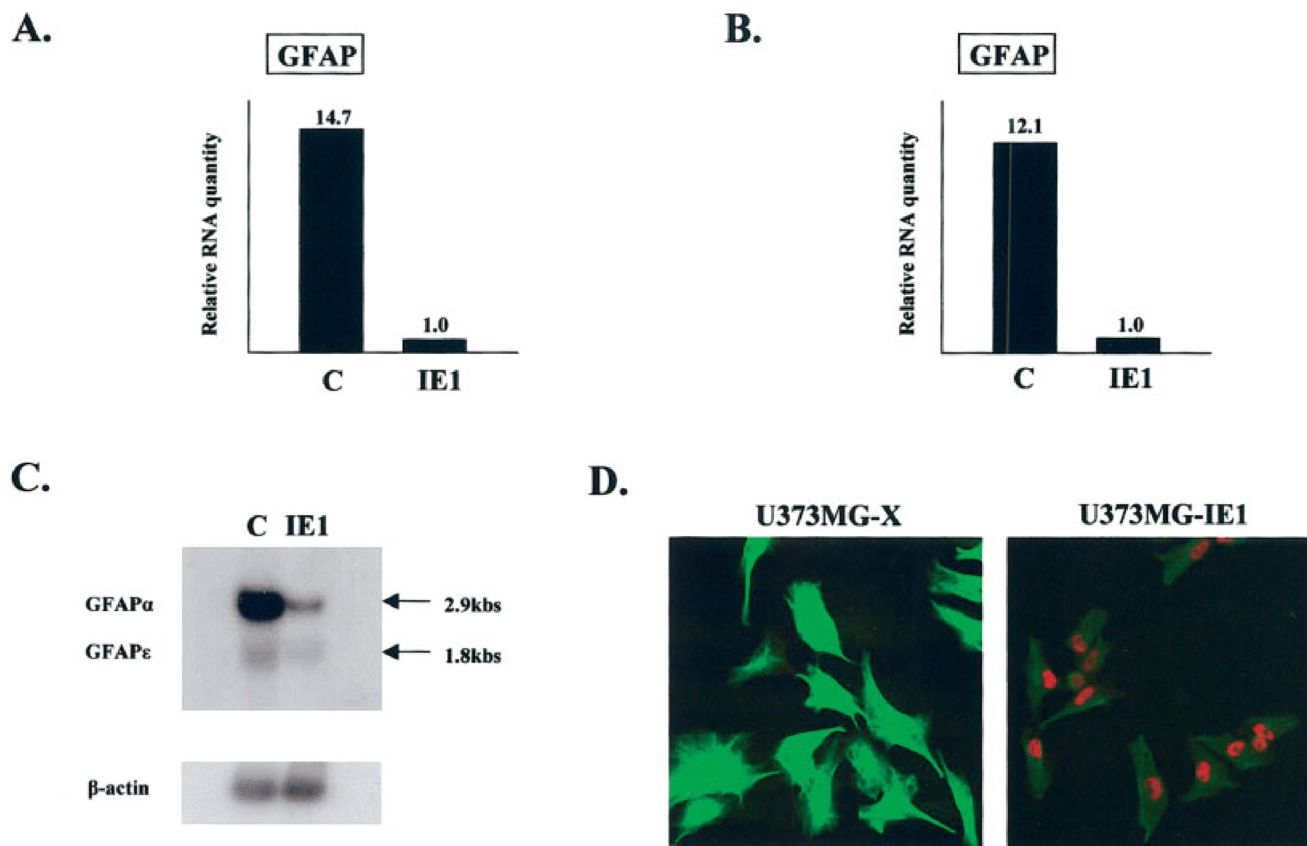

Fig. 3. Decrease in level of GFAP in U373MG-IE1 cells. cDNA microarray data of GFAP (**A**) normalized with β-actin level (**B**) Real-time quantitative RT-PCR analysis of GFAP mRNA. β-Actin was used as a control. **C:** Northern blot analysis of GFAP mRNA. **D:** Immunocytochemical analysis of GFAP in U373MG-IE1 (IE1) and U373MG-X (C) cells. GFAP is shown as green, while IE1 is indicated as red.

shown in Figure 4D, the level of the TSP-1 protein, as measured by fluorescence intensity and cell number of positive cells, was significantly decreased in U373MG-IE1 cells, as compared with that in U373MG-X cells. These results demonstrated that IE1 could downregulate the expression of TSP-1 at both mRNA and protein levels.

Downregulation of p53 by IE1 protein

p53 is a well-known tumor suppressor gene. It is a potent transcriptional regulator of cell growth whose induction leads to either cell-cycle arrest or apoptosis (Levine, 1997; Prives and Hall, 1999). The loss of p53 function or downregulation of p53 expression is well-known to have a strong correlation with cell transformation and tumorigenesis (Finlay et al., 1989; Hollstein et al., 1991; Levine et al., 1991; Levine, 1997; Prives and Hall, 1999). In our chip data, the mRNA level of p53 was about 3-fold lower in U373MG-IE1 cells than in control cells (Fig. 5A). A similar magnitude of decrease in the level of mRNA was also observed by using real-time quantitative RT-PCR and Northern blot analysis (Fig. 5B,C). The change in the level of p53 protein was investigated by FACS assay using a mouse monoclonal antibody that specifically detects p53. As shown in Figure 5D, the level of p53 was appreciably decreased in U373MG-IE1 cells as compared with control cells. These results confirmed that IE1 could downregulate the expression of p53 at both mRNA and protein levels of p53.

Effects of Transient Expression of IE1 Protein

To confirm that downregulation of GFAP in U373MG-IE1 was not due to artifacts that might have resulted from the use of cell lines stably expressing the IE1 protein, we examined the effects of transient expression of IE1 protein. To transiently express IE1 protein in U373MG, a new retroviral vector, MT5-cIE1, was constructed that could produce relatively high viral titer (Fig. 6A). The cDNA sequence of IE1 was inserted into retroviral vector MT5 (Lee et al., 2004). Cell-free retroviral vectors were prepared by the three-plasmid transfection method and used to transduce U373MG cells. The expression of IE1 was confirmed by transducing U373MG cells with MT5-cIE1 and performing Western blot analysis using a specific antibody (Fig. 6B). At 48 h after transduction, total RNA was prepared and subjected to real-time quantitative RT-PCR using a pair of oligonucleotides primers specific for GFAP. As in the

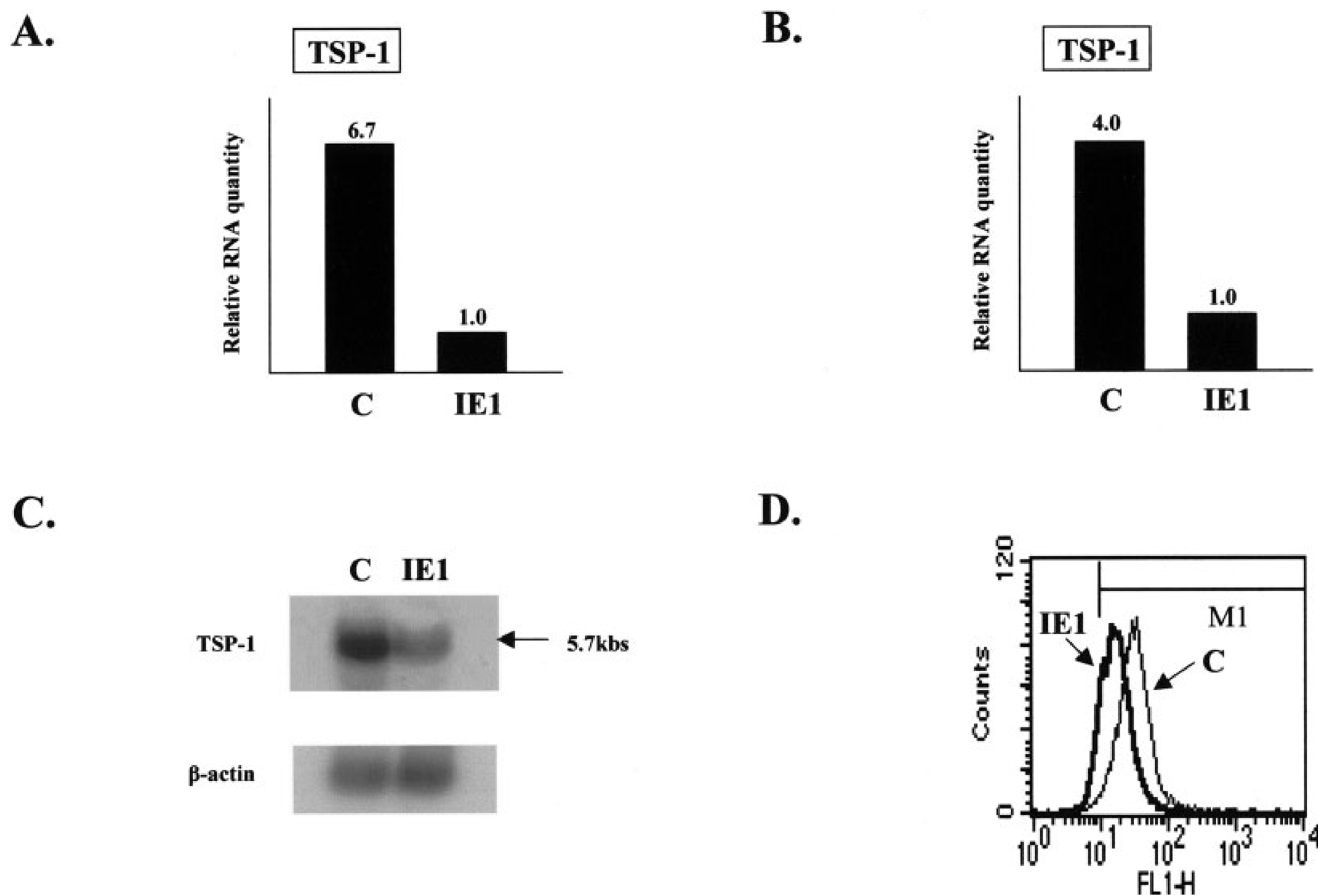

Fig. 4. Decrease in level of TSP-1 in U373MG-IE1 cells. cDNA microarray data of TSP-1 (**A**) normalized with β-actin level (**B**) Real-time quantitative RT-PCR analysis of TSP-1 mRNA. β-Actin was used as a control. **C:** Northern blot analysis of TSP-1 mRNA. **D:** Representative FACS assay of TSP-1 in U373MG-IE1 and U373MG-X cells. TSP-1-specific intensity in U373MG-IE1 (thick line) is visibly shifted to the left, in comparison with that in U373MG:LNC-X (thin line).

case of stable cell line, the level of GFAP was decreased by almost 8.7-fold (Fig. 6C). Because the transduction efficiency was approximately 40%, the magnitude of change in the GFAP RNA looked lesser in this transient assay. These data confirmed the effects on GFAP observed using stable cell line.

Effects of Inhibition of IE1 Expression by RNA Interference

Recently, RNA interference (RNAi) has become a powerful tool to silence gene expression in mammalian cells. To confirm that the downregulation of GFAP in U373-IE1 is truly the result of IE1 expression, four regions in the IE1 coding sequence were selected to make siRNA duplexes. U373-IE1 cells were transfected with four siRNA duplexes, and 3 days post-transfection, whole cell lysates were subjected to Western blot analysis using an antibody specific for IE1. Among four RNA duplexes, siIE1-4 inhibited IE1 expression most effectively (Fig. 7A). The same experiment was performed, this time specifically using siIE1-4, except that total RNA was prepared from cells transfected with siRNA and the level of GFAP RNA was measured by real-time quantitative RT-PCR. When IE1 expression was inhibited in U373-IE1 cells by siIE1-4, the level of GFAP RNA was increased up to 45% of the original level in U373MG, by almost 4-fold higher than that in untreated U373-IE1 cells (Fig. 7B). These results reinforced the observation that the GFAP RNA was decreased by the presence of IE1 in U373MG cells.

Effects of IE1 in Various Human Glioma Cell Lines and Primary Human Astrocytes

To confirm that our observations were not restricted to U373MG, we also studied the effects of IE1 in various human glioma cell lines using Northern blot analysis. Cell lines used in this study include U251MG, U343MG, and A172. These cells were transduced with same retroviral vector expressing IE1 as used to construct IE1-expressing U373MG cells and the RNA level of respective genes was measured by Northern blot analysis. The results are shown in Figure 8A. The RNA expression

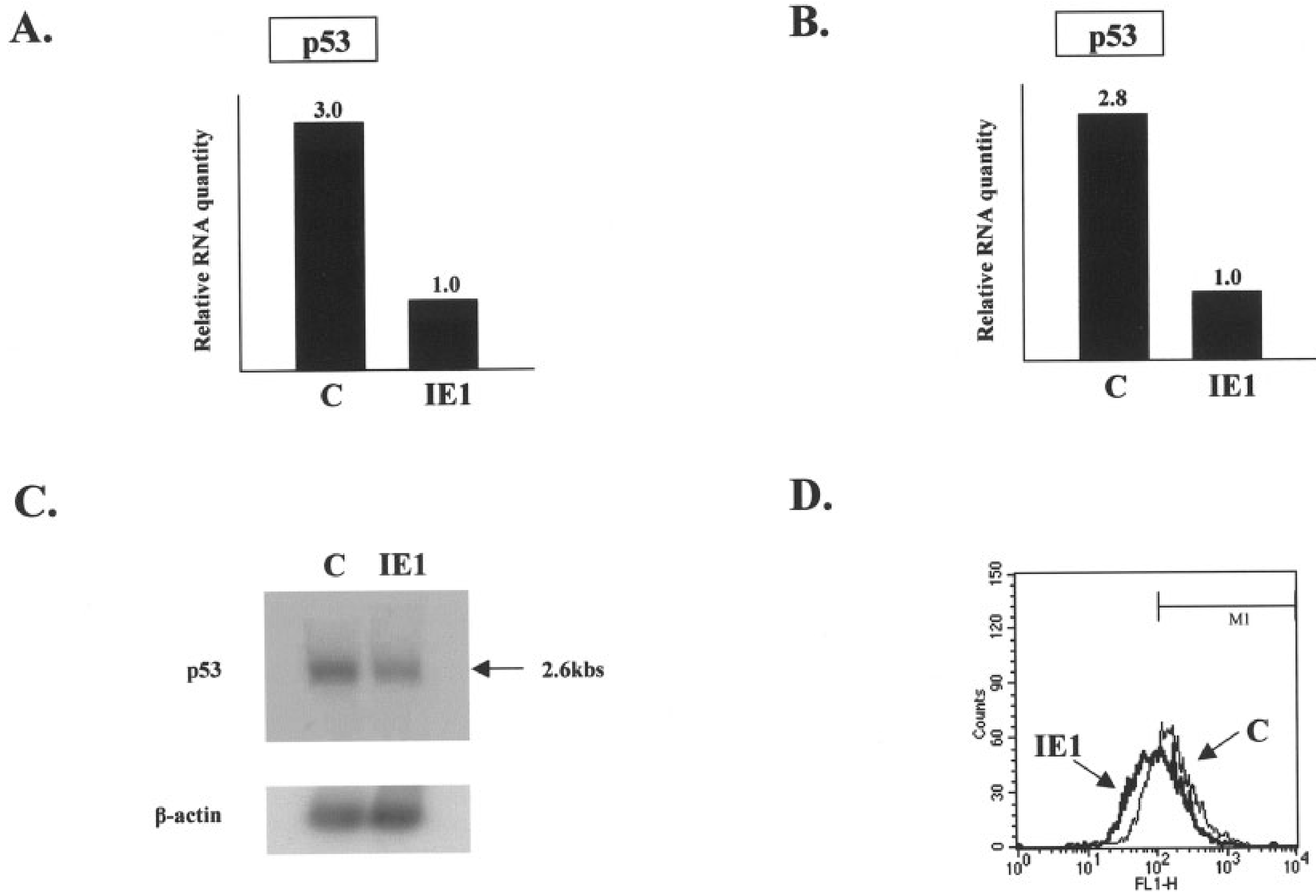

Fig. 5. Decrease in level of p53 in U373MG-IE1 cells. cDNA microarray data (**A**) of p53 normalized with β-actin level (**B**) Real-time quantitative RT-PCR analysis of p53 mRNA. β-Actin was used as a control. **C:** Northern blot analysis of p53 mRNA. **D:** Representative FACS assay of p53 in U373MG-IE1 and U373MG-X cells. p53-specific intensity in U373MG-IE1 (thick line) is shifted to the left, in comparison with that in U373MG:LNC-X (thin line).

pattern of GFAPα and GFAPε in these cell lines was different from that in U373MG. In U373MG, the RNA level of GFAPα was much higher than that of GFAPε. However, it was opposite in U251MG and U343MG, while GFAPα RNA was not detectable in A172. In both U251MG and U343MG, the level of GFAPα RNA was significantly decreased, from a low but visible band to an undetectable level. The amount of GFAPε RNA was also lowered in these three cell lines, but to a lesser extent than that of GFAPα RNA. It is not yet clear how IE1 exerted differential effects on these two isoforms of GFAP. Whatever the reason, the level of GFAPα RNA was decreased by more than 10-fold as determined by phosphoimage analysis, consistent with the result obtained from using U373MG.

Primary human astrocytes were also tested. Astrocytes were prepared from the fetal brain and transfected with an IE1-expressing vector, pEQ273, together with a control plasmid lacking the IE1 sequence, pEQ336. Eight days later, cells were analyzed by two-color immunocytochemistry using antibodies specific for IE1 and GFAP. Transfection efficiency was less than 5%, as determined by the number of IE1-positive cells. In control cells, GFAP was intensely visible (Fig. 8B); however, the fluorescence intensity of GFAP became considerably lower than in IE1-expressing cells (Fig. 8C). These results confirmed that IE1 could downregulate the level of GFAP in not only established glioma cell lines but also in primary astrocytes.

DISCUSSION

In this study, we showed that the expression of GFAP, TSP-1, and p53 was significantly downregulated by HCMV IE1 protein in glioblastoma cell line U373MG. Together with recent data showing the possible involvement of HCMV in malignant glioma (Cobbs et al., 2002), our results strongly implicate IE1 in the pathogenesis of glial tumors. U373MG cells expressing IE1 may provide an interesting model for study of the viral gene in glial tumors. The pathogenesis of glial tumors is likely to involve persistent infection allowing expression of only a limited number of viral genes, rather than productive infection, which may lead to cell death directly or indirectly by immune responses (Ogura et al., 1986; Poland

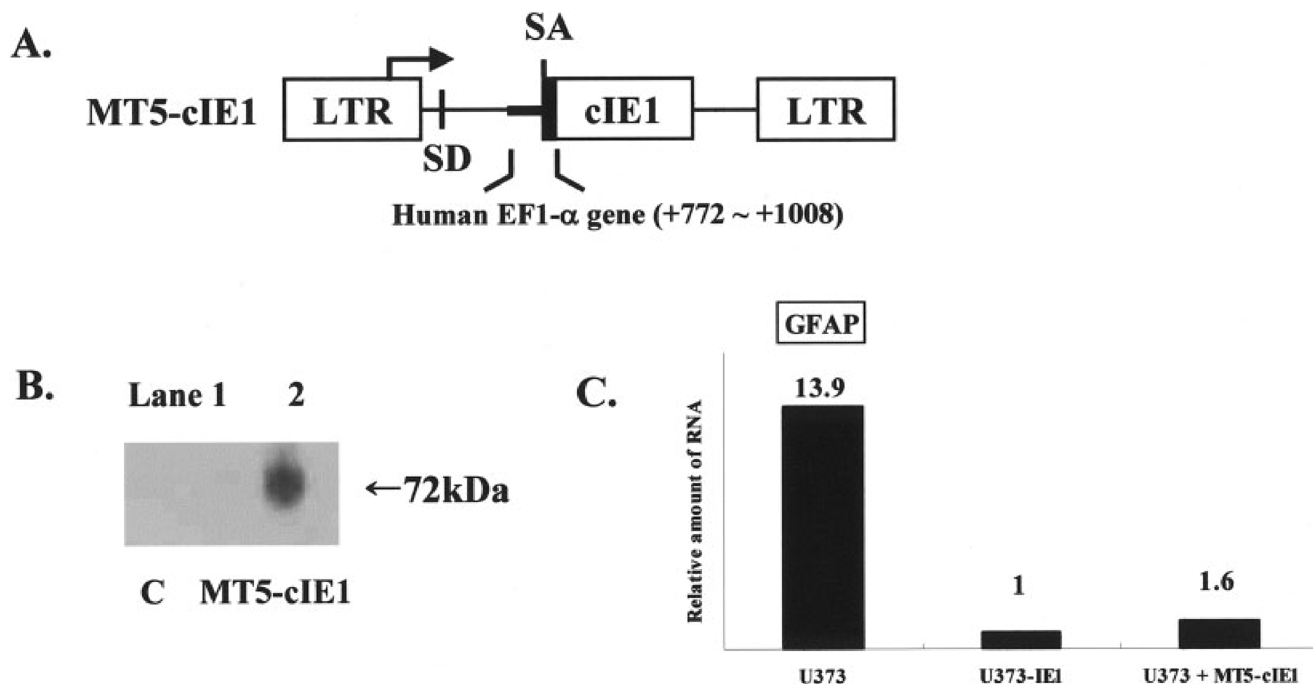

Fig. 6. Transient expression of IE protein in U373MG. **A:** MT5-cIE1, an MLV (murine leukemia virus)-based retroviral vector expressing IE1. MT5-cIE1 was derived from MT5 by inserting the cDNA sequence of IE1 to the vector. **B:** Detection of IE1 protein by Western blot analysis using antibody specific for IE1. **Lane 1**, U373MG as negative control; **lane 2**, U373MG transduced with MT5-cIE1. **C:** Detection of GFAP mRNA level in U373MG cells transiently expressing IE1 by real-time quantitative RT-PCR analysis. β-Actin RNA was used as a control.

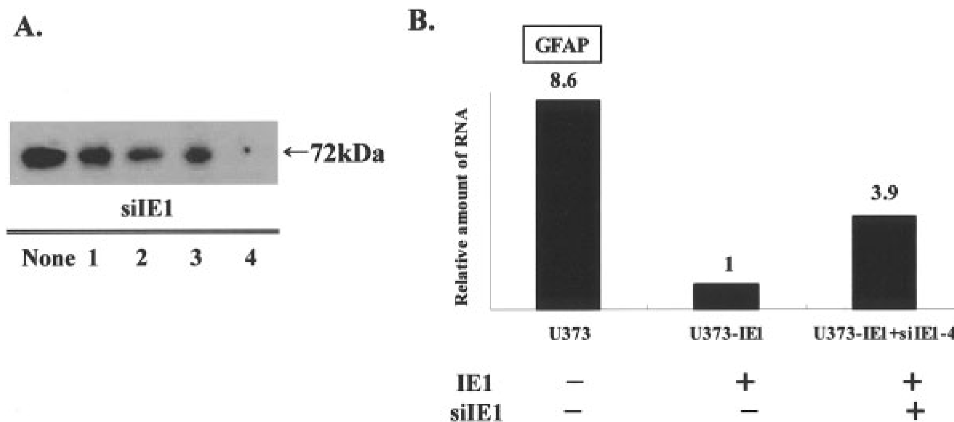

Fig. 7. Inhibition of IE1 expression by RNA interference. **A:** Detection of IE1 protein by Western blot analysis using antibody specific for IE1. Numbers indicate the names of siRNAs against IE1. **B:** Detection of GFAP mRNA level in U373-IE1 cells transfected with siRNA against IE1 by real-time quantitative RT-PCR. β-Actin RNA was used as a control.

et al., 1990; Wolff et al., 1994). U373MG-IE1 cells used in this study may simulate such an in vivo situation.

Our findings are somewhat surprising in that the expression of genes affected by IE1 was expected to be upregulated rather than downregulated, because IE1 has been known to be a transactivator of various cellular and viral promoters (Stenberg and Stinski, 1985; Tevethia et al., 1987; Davis et al., 1987; Cherrington and Mocarski, 1989; Sambucetti et al., 1989; Stenberg et al., 1989; Iwamoto et al., 1990; Hagemeier et al., 1992; Monick et al., 1992; Michelson et al., 1994; Margolis et al., 1995; Yurochko et al., 1995; Kim et al., 1999; Murayama et al., 2000). However, most affected genes harbored in the cDNA chip were downregulated by the IE1 protein. One possible explanation is that the effect of IE1 on the transiently transfected reporter plasmid employed in most of previous studies might be fundamentally different from that on the naturally existing human gene. For example, in transient transfection assays, the promoter under investigation is present in many copies and in an extrachromosomal form. It might better represent the actual situation when the gene to be tested exists in the chromo-

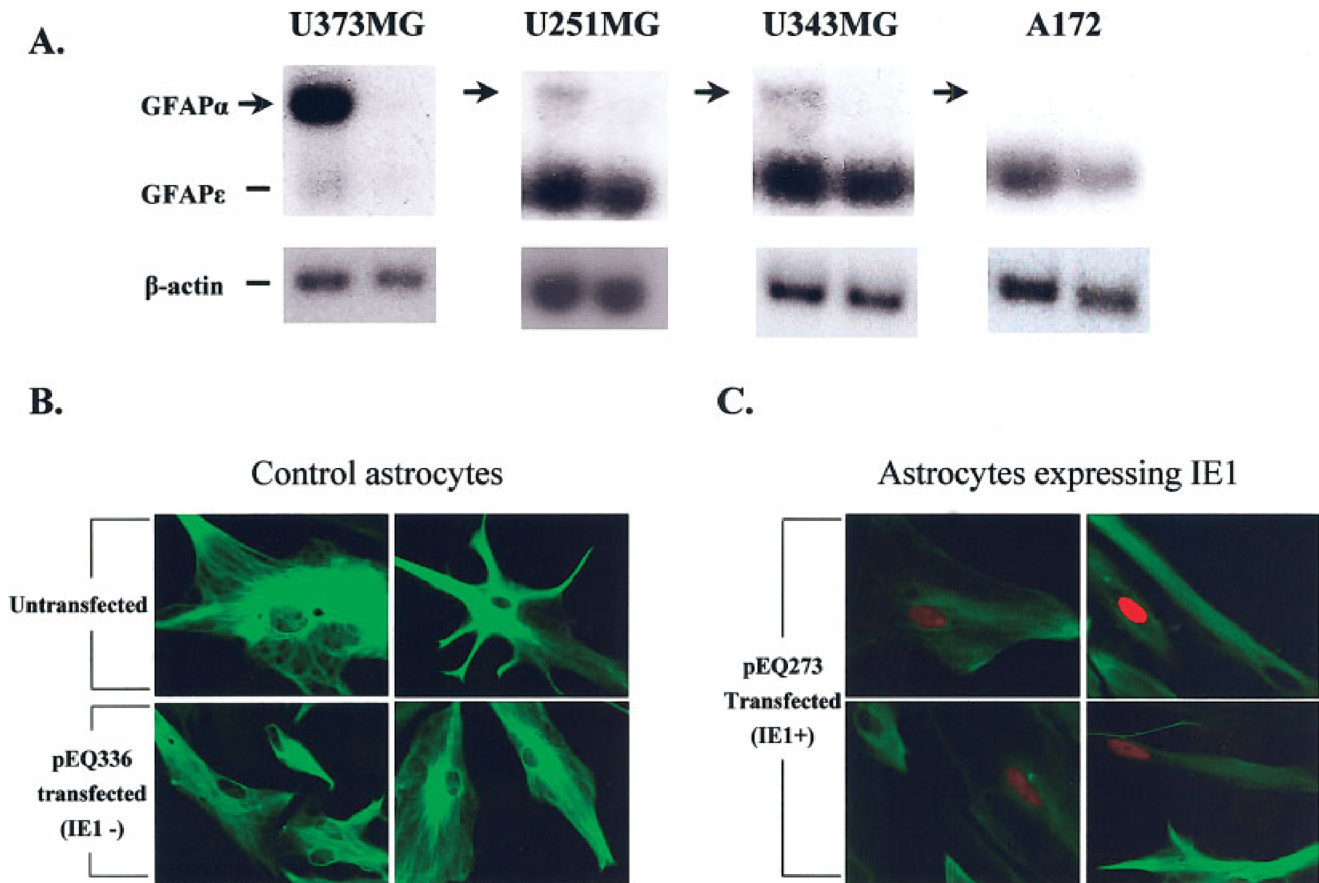

Fig. 8. Decrease in the level of GFAP in various human glioma cell lines and primary human astrocytes. **A:** Northern blot analysis of GFAP RNA in U251MG, U343MG, A172. Immunocytochemical analysis was performed for GFAP and IE1 in untransfected (**B**, top), mock-transfected (**B**, bottom) and IE1-transfected human astrocytes (**C**). GFAP is shown as green, while IE1 is indicated as red.

some. Further experiments are needed to resolve the possible differences between transient and stable systems.

In U373MG cells, GFAP was dramatically downregulated by IE1 protein both at mRNA and protein levels. This finding was confirmed in primary human astrocytes as well as other glioma cell lines. The molecular mechanism underlying the effects of IE1 on GFAPα RNA currently remains to be unraveled. Our preliminary data from nuclear run-on assay indicated that the downregulation might occur at the transcriptional, rather than the posttranscriptional, level (data not shown). GFAP is the most widely used marker of astroglial cells, and its protein level appears to correlate inversely with the malignancy of gliomas (Deck et al., 1978; Eng and Rubinstein, 1978; Jacque et al., 1979; de Armond et al., 1980; Duffy, 1982; Duffy et al., 1982; Rutka et al., 1997). Consistent with these observations, high-level expression of the exogenously added GFAP gene in glioma cells was reported to reduce cell motility, invasiveness and proliferation (Rutka and Smith, 1993; Rutka et al., 1994, 1997; Murphy et al., 1998; Toda et al., 1999; Elobeid et al., 2000). Indeed, the transition from a GFAP-positive to a GFAP-negative astrocyte was often interpreted to represent transformation to a blastic, less mature, more aggressive state. For example, glioblastoma multiforme is the most common and most malignant form of glioma characterized by rapid growth and high invasiveness, and has often been associated with low-level expression of GFAP. In contrast, normal astrocytes and low-grade astrocytomas were usually associated with high-level expression of GFAP. All of theses data indicate that GFAP might play some roles in the tumorigenesis of glial tumors. However, it should be noted that GFAP-negative mice did not necessarily lead to an increase in tumor incidence (Wilhelmsson et al., 2003), suggesting that GFAP might be one of various factors influencing the status of the glioma.

IE1 protein also decreased the expression of TSP-1. Our result is consistent with previous reports showing that HCMV infection of U373MG cells decreased expression of TSP-1 at both mRNA and protein levels (Cinatl et al., 1999, 2000; Margraf et al., 2001). When IE gene expression was inhibited using a specific antisense oligonucleotide during HCMV infection, the magnitude of TSP-1 downregulation by HCMV was significantly decreased (Cinatl et al., 1999). Our data suggest that IE1 has the potential to downregulate the expression of TSP-1. Various studies have correlated the loss of TSP-1

in cultured cells and tumors with the switch to an angiogenic phenotype. Therefore, IE1 protein might contribute to angiogenesis in the pathology of malignant glioma.

p53, a well-known tumor suppressor gene, was also downregulated by IE1 in U373MG, although to a lesser extent than two other cellular proteins. Since U373MG has a mutant p53 genotype, the downregulation of p53 gene by IE1 might not be biologically important in this cell lines. Therefore, it is a clearly next step to test if the downregulation of p53 mRNA by IE1 is independent of p53 genotype. If such is the case, this observation might extend to the general situation where wild type p53 gene exists. In addition, this result suggests that IE1 might be one of the rare viral gene products, such as HBV X protein and HTLV-1 Tax protein, which can directly suppress p53 gene transcription, although the magnitude of repression is smaller (Lee et al., 2000).

siRNA duplexes can act as powerful target-specific catalysts for destruction of specific RNA sequence through the evolutionarily conserved mechanism known as RNA interference (RNAi). Currently, RNAi is applied to therapeutic intervention in viral infections targeting genes essential for the viral life cycle including HIV-1 (Jacque et al., 2002) influenza virus (Ge et al., 2003), and hepatitis C virus (Randall et al., 2003), and also in tumorigenesis targeting specific oncogenes (Scherr et al., 2003). Our data showed that RNAi could effectively inhibit the biological functions of IE1, leading to the recovery of the GFAP RNA quantity to one-half the original level. If such an inverse in the level of GFAP indeed negatively influence the tumorigenesis as shown in the cell culture system (Rutka and Smith, 1993; Rutka et al., 1994, 1997; Murphy et al., 1998; Toda et al., 1999; Elobeid et al., 2000), RNA interference against IE1 might be useful for the treatment of glial tumors.

To our knowledge, this study is the first to demonstrate that IE1 might be involved in the pathogenesis of malignant glioma by modulating key gene that is already known to be involved in tumorigenesis. Considering the high consistency rate in data obtained from various sources and well-known roles of cellular proteins, further investigation into the exact mechanism for the IE1-mediated gene regulation and the possible involvement of other HCMV genes is warranted.

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