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Inhibition of cell growth by EGR-1 in human primary cultures from malignant glioma

Antonella Calogero*[1,2], Vincenza Lombari[2], Giorgia De Gregorio[2], Antonio Porcellini[1,2], Severine Ucci[1], Antonietta Arcella[2], Riccardo Caruso[2,3], Franco Maria Gagliardi[2,3], Alberto Gulino[1], Gaetano Lanzetta[2,4], Luigi Frati[1,2], Dan Mercola[5,6] and Giuseppe Ragona[1,2]

Address: [1]Department of Experimental Medicine and Pathology, University of Rome "La Sapienza", Rome, 00161 Italy, [2]IRCCS Neuromed, Pozzilli, 86077 Italy, [3]Department of Neurological Sciences, University of Rome "La Sapienza", Rome, 00161 Italy, [4]INI, Istituto Neurotraumatologico Italiano, Grottaferrata, 00046 Italy, [5]Sidney Kimmel Cancer Center, San Diego, California 92121, USA and [6]The Cancer Center, University of California at San Diego, La Jolla, California 92093, USA

Email: Antonella Calogero* - antonella.calogero@uniroma1.it; Vincenza Lombari - lombari@neuromed.it; Giorgia De Gregorio - antonella.calogero@uniroma1.it; Antonio Porcellini - antonio.porcellini@uniroma1.it; Severine Ucci - antonella.calogero@uniroma1.it; Antonietta Arcella - arcella@neuromed.it; Riccardo Caruso - caruso@neuromed.it; Franco Maria Gagliardi - gagliardi@neuromed.it; Alberto Gulino - alberto.gulino@uniroma1.it; Gaetano Lanzetta - antonella.calogero@uniroma1.it; Luigi Frati - luigi.frati@uniroma1.it; Dan Mercola - danmercola@skcc.org; Giuseppe Ragona - giuseppe.ragona@uniroma1.it

* Corresponding author

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Abstract

Background: The aim of this work was to investigate in vitro the putative role of EGR-1 in the growth of glioma cells. EGR-1 expression was examined during the early passages in vitro of 17 primary cell lines grown from 3 grade III and from 14 grade IV malignant astrocytoma explants. The explanted tumors were genetically characterized at the *p53*, *MDM2* and *INK4a/ARF* loci, and fibronectin expression and growth characteristics were examined. A recombinant adenovirus overexpressing *EGR-1* was tested in the primary cell lines.

Results: Low levels of EGR-1 protein were found in all primary cultures examined, with lower values present in grade IV tumors and in cultures carrying wild-type copies of *p53* gene. The levels of EGR-1 protein were significantly correlated to the amount of intracellular fibronectin, but only in tumors carrying wild-type copies of the *p53* gene (R = 0,78, p = 0.0082). Duplication time, plating efficiency, colony formation in agarose, and contact inhibition were also altered in the *p53* mutated tumor cultures compared to those carrying wild-type p53. Growth arrest was achieved in both types of tumor within 1–2 weeks following infection with a recombinant adenovirus overexpressing EGR-1 but not with the control adenovirus.

Conclusions: Suppression of EGR-1 is a common event in gliomas and in most cases this is achieved through down-regulation of gene expression. Expression of EGR-1 by recombinant adenovirus infection almost completely abolishes the growth of tumor cells in vitro, regardless of the mutational status of the *p53* gene.

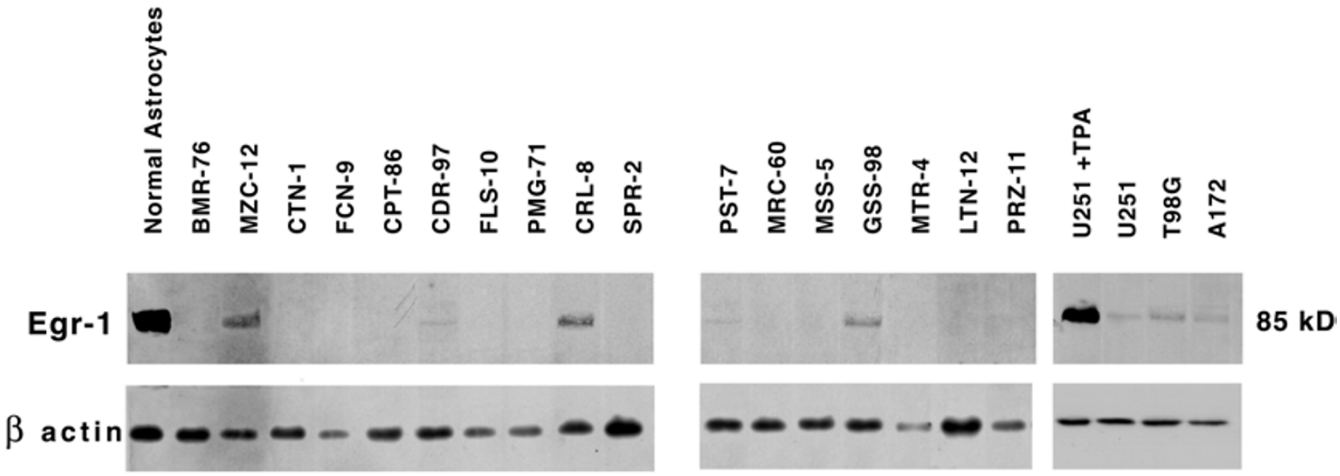

Figure 1
Western blot of EGR-1 expression in glioma-derived primary cell cultures Western blot of EGR-1 expression in 17 primary cell cultures derived from tumors resected from patients affected by grade III astrocytoma or glioblastoma multiforme (grade IV). Controls include normal adult astrocytes, the established glioblastoma cell lines U251, T98G and A172, and the U251 cell line after stimulation with PMA. EGR-1 is clearly detected in normal astrocytes, in PMA stimulated U251 cells, and at lower levels in a few tumor primary cultures and in all three established cell lines. EGR-1 was detected by enhanced chemiluminescence after film exposure of 10 min.

Background

EGR-1 encodes a nuclear phosphoprotein that binds to DNA and regulates transcription through a GC-rich consensus sequence [1-4]. *EGR-1* is involved in the regulation of cell responses to a wide array of stimuli such as mitogens, growth factors and stress stimuli [5-7]. Recent studies have shown that EGR-1 expression is altered in several types of neoplasia, compared to normal tissue [1,8,9]. Gene deletion or *EGR-1* mutations have been reported in sporadic hematological malignancies [10]. EGR-1 expression has been found to be either decreased or undetectable in human breast cancer tissue and small cell lung carcinoma [11,12]. *EGR-1* is altered in a different manner in prostate cancer, where higher levels of EGR-1 expression are found correlated to more advanced stages of malignancy [13]. Later studies confirmed in two independent mouse models that EGR-1 up-regulates tumor progression [14,15]. From these various studies it is clear that EGR-1 is involved in regulation of cell proliferation.

In order to test whether *Egr-1* is implicated in the pathogenesis of astrocytic tumors, we recently examined the expression of *EGR-1* RNA and protein in fresh glioma samples [16]. Compared to human normal brain tissue, EGR-1 expression was strongly reduced in these tumors, particularly in cases with normal *p53* alleles, suggesting a role as a tumor suppressor gene. To explore this finding further, we have extended our study to a new series of 17 primary cell cultures established in our laboratory from anaplastic astrocytoma and glioblastoma multiforme. During the first passages in vitro, we not only evaluated EGR-1 expression but also examined the primary cultures for proliferative activity and expression of fibronectin, an important factor in the organization of extracellular matrix [17]. Fibronectin facilitates cell adhesion and migration [18] and has been shown to be positively regulated by EGR-1 in the human glioblastoma cell line U251 [19]. In addition, the primary cultures were genotypically characterized for mutations in the *p53*, *MDM2* and *p16/INK4a/ARF* genes [20]. Finally, the effect of EGR-1 on proliferative activity of primary cells carrying wild type or mutant *p53* genes was investigated by infection with recombinant adenovirus engineered to overexpress EGR-1.

Results

EGR-1 expression in glioma cells cultured in vitro

EGR-1 protein expression in the 17 primary cultures was examined using Western blot analysis. In CRL-8, GSS-98 and MZC-12 an 85 kD band corresponding to full-lenght EGR-1 was clearly visible (Fig. 1). In the remaining primary cultures, basal expression of EGR-1 was either

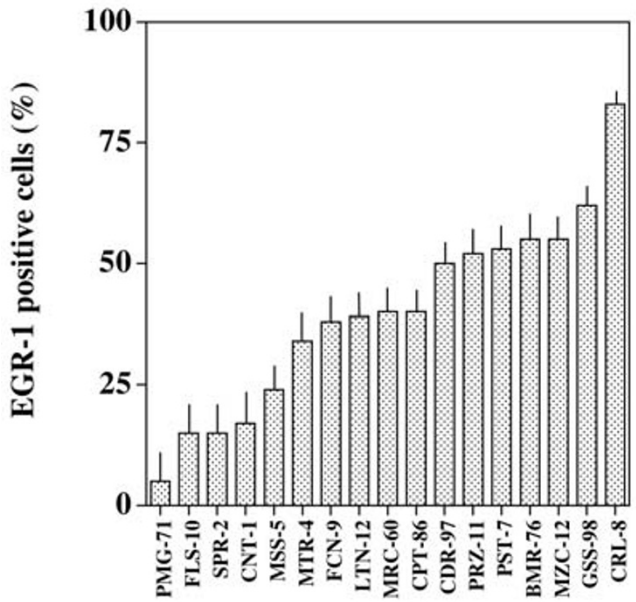

Figure 2
Quantitative evaluation of EGR-1 expression Quantitative evaluation of EGR-1 expression in 17 glioma primary cell lines. Data are given as percent of labelled cells measured by immunocytochemistry.

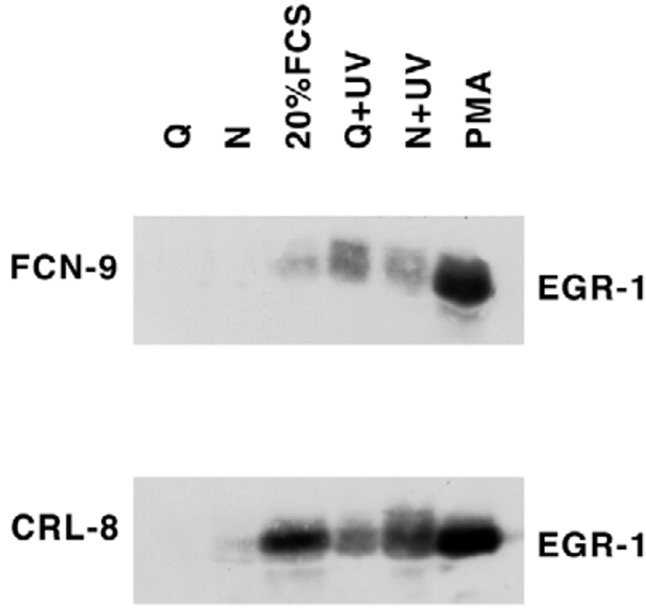

Figure 3
Stimulation of EGR-1 expression by the endogenous gene Western blot of EGR-1 in CRL-8 and FCN-9, established from glioblastoma multiforme and astrocytoma grade III, respectively. EGR-1 protein synthesis was up-regulated by the endogenous gene in the two primary cell cultures after being exposed to different types of external stimuli, suggesting that EGR-1 expression was regulated at the level of transcription. The characteristics of the two cell lines are described in Table 1 (see Additional file 1). Q, cells made quiescent in serum depleted medium for 24–48 hrs; N, cells growing at log phase in medium with 10% serum (control cells); 20% FCS, quiescent cells stimulated with serum enriched medium for 24–48 hrs; Q + UV, quiescent cells stimulated by UV irradiation (40 J/m^2); N + UV, exponentially growing cells stimulated by UV irradiation. PMA, quiescent cells stimulated by PMA (100 ng/ml). A typical experiment is shown.

undetectable or faintly present. EGR-1 protein was detected in normal astrocytes and in the U251 cell line stimulated with PMA [19], serving as positive control; The basal expression of EGR-1 in the U251 and two additional glioblastoma cell lines (T98G, and A172) was also examined and found moderately present in all the three established glioblastoma cell lines. Densitometric measurements indicated that EGR-1 protein levels in the three positive tumor cultures were at least 20-fold lower than the level found in the unstimulated normal astrocytes, supporting the hypothesis that EGR-1 expression is downregulated in malignant tumors.

EGR-1 protein expression in tumor primary cultures was also examined by immunocytochemistry. Weakly positive cells were present in all primary cultures. As shown in Fig. 2, the fraction of immunolabeled cells ranged from 5 to 83%, with a mean ± s.e value of 39.8 ± 4.9. The three primary cultures with the highest frequency of EGR-1-positive cells by immunocytochemistry (CRL-8, 83%; GSS-98, 62%; and MZC-12, 55%) were also the three cultures that showed clear expression of EGR-1 protein by Western blot analysis.

The finding that all tumor primary cultures showed EGR-1 protein expression by immunocytochemistry led us to reexamine the Western blot analysis by increasing the exposure time from approximately 10 minutes to over 30 minutes. With increased exposure, EGR-1 protein was detected as a weak signal in all tumor primary cultures previously found negative. The EGR-1-specific bands in the primary cultures were quantified following densitometric scanning and normalization (see Table 1, in Additional file 1). Western blot data and immunocytochemistry data correlate significantly ($R = 0.703$, $p = 0.0084$). In addition, Northern blot analysis of EGR-1 expression confirmed the results seen with Western blot analysis (data not shown). The results of these analyses suggest that *EGR-1* gene expression is strongly down-

regulated in primary cultures derived from malignant glioma.

EGR-1 expression in glioma can be up-regulated in vitro by PMA and UV stimulation

The reduced levels of EGR-1 in glioma primary cultures could be due to gene inactivation or mutation. To determine whether EGR-1 expression could be up-regulated in these cells in response to external stimuli, we treated quiescent tumor cells with PMA (100 ng/ml), UV irradiation (40 J/m^2) for 2 hours, or serum. These stimuli are known to induce both mRNA and protein synthesis of EGR-1 [4,21]. EGR-1 protein expression was differentially induced by all these stimuli. The protein migrated with an apparent size of 85 kDa, very similar to full-length normal EGR-1. Our data suggest that EGR-1 gene transcription and transcription processing remain intact after the oncogenic process, but that steady-state expression is selectively reduced. Results of a typical experiment performed with primary cultures FCN-9 and CRL-8 are shown in Fig. 3.

Mutational profile and differentiation markers

The 17 primary cell lines were analyzed for mutations in exons 5–9 of the p53 gene, for MDM2 gene amplification, and for loss of heterozygosity at the INK4A-ARF locus (Table 1, see Additional file 1). p53 gene mutations were present in three cases. CRL-8 and MZC-12 had homozygous mutations leading to amino acid substitutions at codon 216 of exon 6 (Val -> Met) and at codon 248 of exon 7 (Arg -> Glu), respectively. In the third case, FCN-9, a mutation occurred at the AG donor (AG to AA) located at the 3' splice site of exon 6. In order to determine whether these mutations affected protein stabilization, we performed immunofluorescence staining for the p53 protein. In FCN-9 cells, accumulation of p53 protein was seen in only a small fraction (10%) of cells. By contrast, p53-positive cells were detected at a >70% frequency in CRL-8 and MZC-12 cells (Table 1, see Additional file 1). We concluded that EGR-1 was expressed at significantly ($p = 0.026$) higher levels in the p53-mutated primary cultures (370.6 integrated O.D. ± 167.2) compared to primary cultures with wild-type p53 (133.3 integrated O.D. ± 18.9).

Southern analysis indicated that none of the primary cell lines carried amplified copies of MDM2 gene (data not shown). In all cell lines but one, indirect immunofluorescence detected low levels of MDM2 protein, compatible with the presence of normal copy number of the MDM2 gene. The exception was the primary cell line BMR-76, which was negative for MDM2 protein (Table 1, see Additional file 1). Homozygous deletions at the INK4A-ARF locus were found in FLS-10 and GSS-98.

The differentiation status of tumor cells was assessed by examining glial fibrillary acidic protein (GFAP) and vimentin expression, which were demonstrated in 6 and 11 tumor primary cultures, respectively (Table 1, see Additional file 1). Neither MDM2 levels nor the presence of GFAP or vimentin correlated with EGR-1 expression.

Glioblastoma (grade IV) tumors carrying wild-type p53 have less EGR-1 than do anaplastic astrocytoma (grade III) tumors

Primary cultures from glioblastoma multiforme carrying normal copies of p53 gene had both a lower percentage of EGR-1 positive cells (32.2% ± 4.8) and lower levels of EGR-1 protein (116.2 integrated O.D. ± 16.9) than did grade III astrocytoma-derived cultures (57.5% ± 4.5 and 335.5 integrated O.D. ± 84.5, respectively). The difference between the two tumor types with respect to EGR-1 protein levels was highly significant ($p = 0.0009$).

Fibronectin expression and EGR-1 expression are strongly correlated in tumors carrying wild-type copies of p53 gene

Fibronectin has an important role in organizing the extracellular matrix and facilitates cell adhesion, migration and tumor metastasis [18], and recent work has shown that EGR-1 can positively regulate expression of the fibronectin gene in vitro [19]. Since the low levels of EGR-1 protein found in our primary cultures may suggest that EGR-1 is functionally inactivated, we examined fibronectin expression in these cells to determine whether fibronectin protein levels were similarly decreased. To rule out the possibility that fibronectin expression might arise from contamination of the primary cultures by mesenchyme cells or other tumor supportive cells, we first tested for cell contamination by performing extensive immunocytochemical reactions with S-100 protein and neurofilament proteins, cytokeratins, basic myelin protein and neuron-specific-enolase. Results showed that our primary cultures consisted almost entirely of tumor cells (data not shown) and supported the conclusion that cell contamination was negligible during the short time the cells were under observation

We examined the intracellular accumulation of fibronectin by Western blot analysis in 11 randomly selected cultures from the panel of the 17 tumor primary cultures and detected fibronectin in all tumors tested. The normalized values of integrated O.D.^{3} varied from less than 200 to more than 1600 and increased linearly with the amount of EGR-1 in the tumor primary cultures homozygous for wild type *p53* ($R = 0.78$, $p = 0.0082$). In the primary cell lines with mutated *p53* genes, only low levels of fibronectin were found, despite a wide range in EGR-1 levels.

Growth assays

To determine whether primary cell cultures with different levels of EGR-1 have different growth properties in vitro, we tested the tumor cells cultured between the second and third passage for duplication time, plating efficiency, ability to grow as colonies in soft agarose, and sensitivity to contact inhibition (Table 1, see Additional file 1). The duplication time ranged from 36 hours to over 10 days (mean ± s.e. = 82.3 hours ± 13.8; median duplication time = 69 hrs). Plating efficiency, measured as the percentage of cells that proliferate as surface-attached colonies when plated at low density, ranged from 11% to 80% (mean ± s.e. = 29.2% ± 4.8; median plating efficiency = 30%) among primary cultures that formed colonies. Two of the tumor primary cultures tested, CDR-97 and GSS-98, did not form colonies. Lowering the serum concentration from 10% to 2.5% reduced or abolished plating efficiency: only six cultures, including the three with *p53* mutations, proliferated under this condition (mean ± s.e. = 2.1% ± 1.9). Eleven of 17 tumor primary cultures formed colonies in soft agarose, the percentage of cells growing as colonies ranging from 0.01% to 6%. Finally, growth inhibition by cell-to-cell contact was evaluated by culturing the 17 tumor primary cultures at high cell density. Only cell cultures with p53 mutations continued to grow after cell confluence, giving rise to typical transformation-associated growth foci.

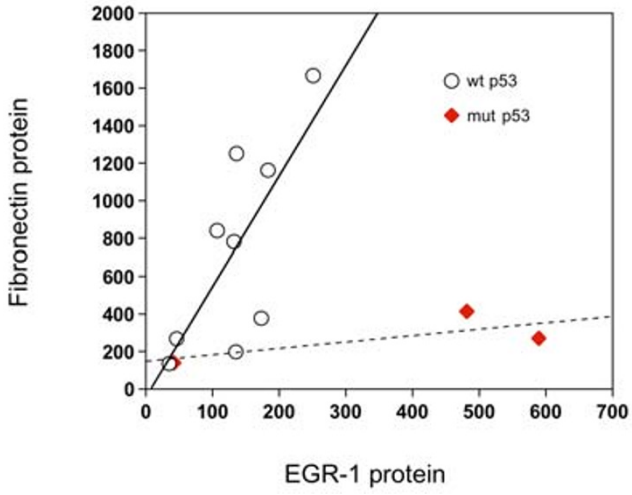

Figure 4

Expression of fibronectin in primary cell lines carrying wild-type or mutant p53 Both fibronectin and EGR-1 are expressed as units of integrated O.D. measured by densitometric analysis of Western blot. Fibronectin protein increases linearly with the amount of EGR-1 in tumors homozygous for wild-type p53 (wt p53) (R = 0.78, p = 0.0082). Tumors with mutated copies of p53 (mut p53) have only low levels of fibronectin.

In summary, compared to the 14 primary cultures homozygous for wild-type *p53*, the three tumor primary cultures with p53 mutations not only contain significantly higher levels of EGR-1 protein (see previous section "Mutational profile and differentiation markers"), but also have shorter duplication times (compared to the median duplication time of 69 hrs), higher mean plating efficiency at low serum concentration (9.33% vs. 0.61%, p = 0.0017) and higher mean agarose cloning ability (4.03% vs. 0.65%, p = 0.005). Within the group of cultures homozygous for wild-type p53, none of the growth parameters correlated with EGR-1 expression, and no differences in the levels of EGR-1 were found between any of the cell lines homozygous for wild-type *p53*, regardless of growth characteristics.

Functional EGR-1 introduced via recombinant Adenovirus infection drastically reduces cell growth in vitro

In view of the above results, we finally investigated the effect of EGR-1 on cell growth after reconstitution of functional EGR-1 levels via recombinant Adenovirus infection. A replication deficient Adenovirus vector harbouring EGR-1 cDNA (AdGFP-EGR-1) was used to deliver EGR-1 into five different primary cell cultures (MZC-12, LTN-12, CRL-8, FCN-9 and BRT-5, this last not included in Table 1, see Additional file 1), and into the established glioma cell lines U251 and U87MG for comparison. The AdGFP adenovirus vector carrying the GFP gene alone was used as a control.

Within 24 hours after viral infection EGR-1 could be detected in cells infected with the adenovirus constructs carrying the EGR-1 transgene, but not in cells infected with the control virus. EGR-1 reached maximal expression after approximately two days and remained detectable for as long as 12 days, which was the maximum time investigated in time-course protein expression experiments. Fig. 5 shows EGR-1 expression in three representative cell lines following infection with the control and EGR-1-containing constructs. Cell growth experiments from repeated infections were highly reproducible, yielding comparable results among the different cell lines. Growth arrest of the AdGFP-EGR-1 infected cells was clearly manifest from day 5 post-infection. A four- to six-fold reduction of proliferation was usually achieved between days 7 to 12 by the AdGFP-EGR-1 infected cells compared to cells infected with control vector. Interestingly, cell growth inhibition by EGR-1 occurred in both primary cultures (regardless of p53 status) and established cell lines. Differences in the growth magnitude between CRL-8 and LTN-12 can be largely explained by the much longer duplication time of LTN-12 (see Table 1, in Additional file 1). U251 cells were examined only up to seven days post-infection, as control cells reached full confluence thereafter.

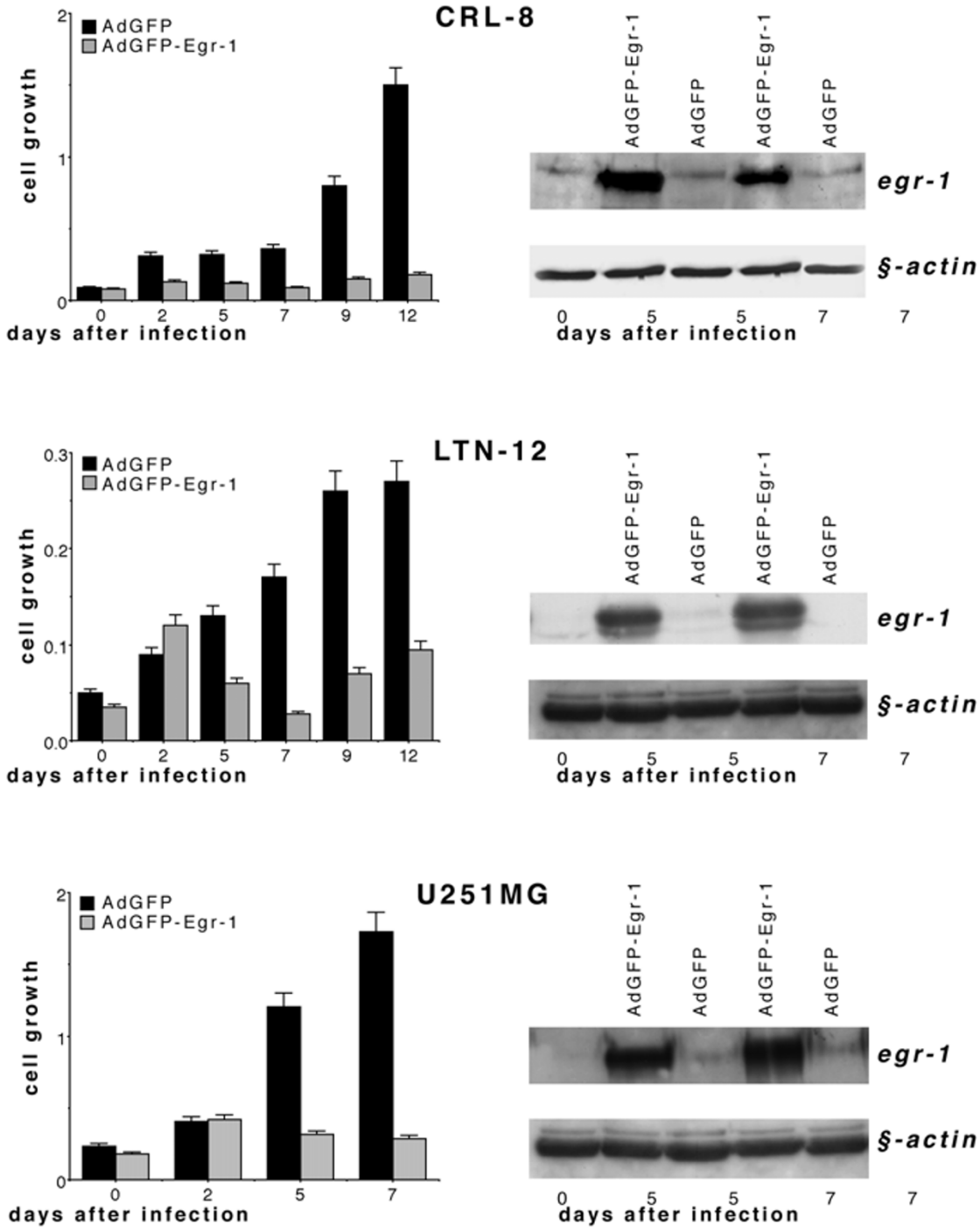

Figure 5

Exogenous EGR-1 induces growth arrest in glioma primary cell lines and in U251 cells. Left side: growth curves of CRL-8 (upper), LTN-12 (middle), and U251 (lower) following infection at day 0 with either a recombinant adenovirus expressing an *EGR-1* transgene (AdGFP-EGR-1), or control vector carrying the GFP gene insert (AdGFP). Cell growth is given by absorbance units of cell lysates pre-treated with sulphorhodamine B. Cell viability exceeded 90% in control vector-infected cell lines. Duplication times are 23 hrs for U251, 66 hrs for CRL-8, and 100 hrs for LTN-12, allowing for differences between cell lines for cell growth (see LTN-12 versus CRL-8 and U251) or for the length of the reported observation period (see U251 versus CRL-8 and LTN-12). Right side: immunoblot analysis of EGR-1 and β-actin protein extracted from CRL-8 (upper), LTN-12 (middle), and U251 (lower) at days 0, 5 and 7 following infection at day 0 with either AdGFP-EGR-1 or control vector.

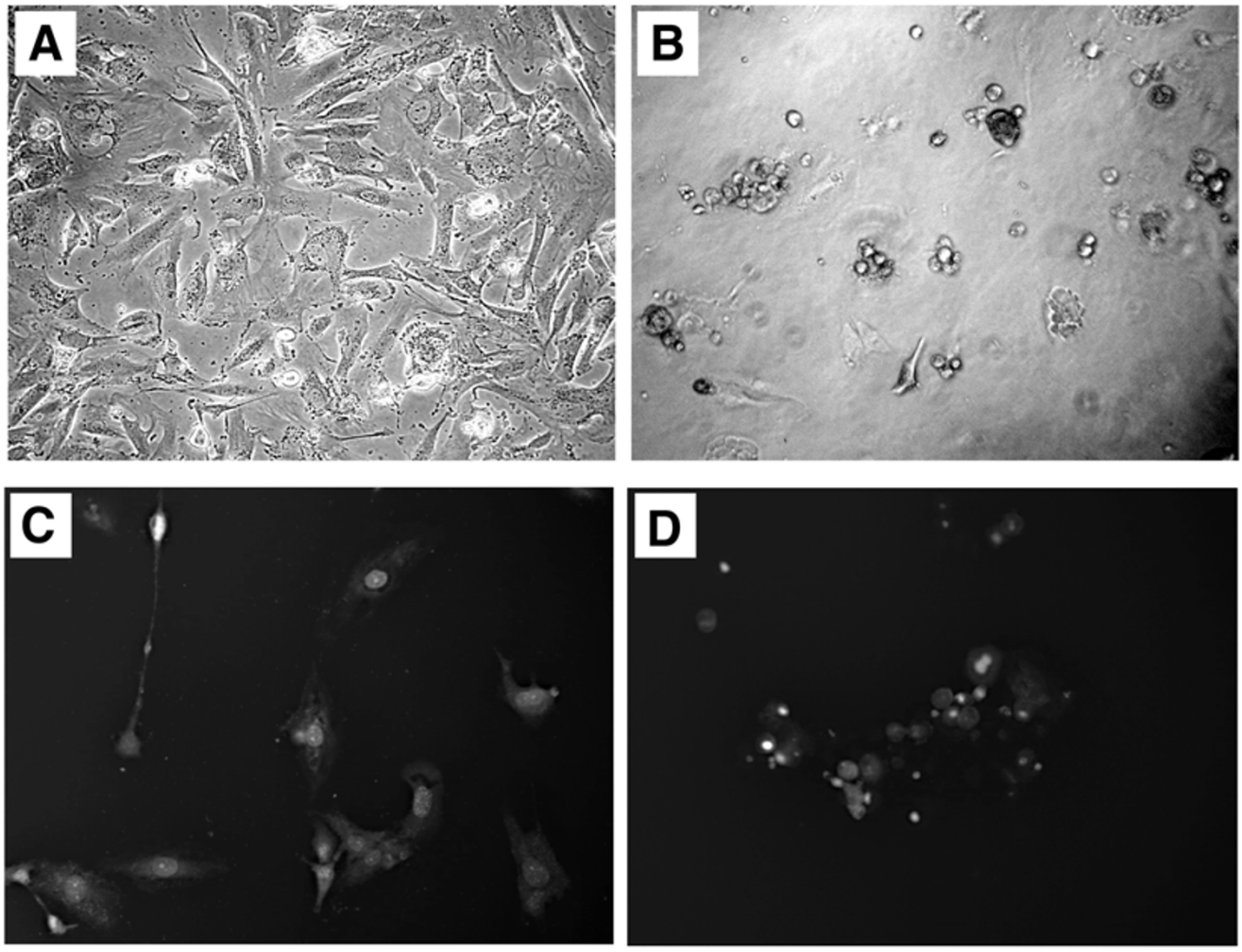

Figure 6
Morphology of cells treated with recombinant Adenovirus Morphology of LTN-12 at seven days after infection with either a recombinant adenovirus expressing an *EGR-1* transgene (B, D), or control vector carrying the GFP gene insert (A, C). Upper panel: phase contrast magnification. Lower panel: cell nuclei were stained with Hoechst dye.

For more than 70% of the growth-arrested cells expressing exogenous EGR-1, growth inhibition was eventually followed by drastic changes in morphology. These changes consisted of reduction in cell volume, cell rounding, and eventual detachment of the cells from the bottom of the culture dish (Fig. 6). Similar changes were observed in fewer than 10% of cells infected with control virus.

Discussion

EGR-1 is an important factor in regulating cell growth. In tumor cells two alternative patterns of EGR-1 expression have been recognized. In prostate cancer, EGR-1 levels are elevated in tumor cells [13]. By contrast, EGR-1 expression is often low or absent in breast and lung tumors [11,12], suggesting that EGR-1 may have tumor suppressive functions in some cancer types.

In the present study we investigated EGR-1 expression in a group of 17 newly established primary cultures of anaplastic astrocytoma and glioblastoma multiforme and showed that EGR-1 is down-regulated in these tumors compared to normal astrocytes. Basal levels of EGR-1 expression in normal astrocytes have been reported also by other investigators [22-24]. Our data thus support the hypothesis that EGR-1 may have tumor suppressive functions, as for breast and lung tissues. Among the tumor cultures with wild-type p53, those derived from glioblastoma multiforme had significantly ($p = 0.0009$) lower

EGR-1 expression than did tumor cultures derived from grade III astrocytoma. In addition, cultures with wild-type p53 had significantly (p = 0.026) lower EGR-1 expression than cultures with mutant *p53*. All together, these findings are entirely consistent with our previous observations concerning EGR-1 RNA expression in fresh biopsies from glioma [16], and support the hypothesis that primary cultures provide a reliable model for examining the role of the *EGR-1* gene in the maintenance and progression of the glioma phenotype. In fact, permanent cell lines may offer a less realistic model of the tumor of origin. Their inherent instability at the genomic level and the elevated number of duplications they have gone through during their lifespan in vitro result in accumulation of a series of genetic alterations, leading to the expansion of highly transformed, clonally selected subpopulations [25,26]. This is further exemplified by the finding that the *p16* gene is deleted in most established cell lines, irrespective of the *p53* gene status, whereas in our tumor primary cultures mutations in the *p53* and *p16/INK4a/ARF* genes are carried in a mutually exclusive pattern, as in fresh biopsies [16,27,28].

Another issue we investigated was whether EGR-1 preserves its role as a regulator of cell proliferation in the in vitro explanted tumors, where it is expressed in lower levels compared to normal tissue. We investigated several cell growth parameters including duplication time, contact inhibition, plating efficiency and agarose clonability, and we attempted to find a correlation between these parameters and either the levels of EGR-1 protein and/or the presence of genetic mutations. We found a correlation between aberrant cell growth and *p53* mutations. Compared to the tumor cultures homozygous for wild-type *p53*, the primary cultures with mutated copies of the *p53* gene show loss of contact inhibition, have lower duplication times, have significantly (p = 0.0017) higher plating activity at low serum concentration, and significantly (p = 0.005) higher ability to form colonies in soft agarose. Interestingly, these cells also expressed higher levels of EGR-1 thus pointing to a link between the occurrence of *p53* mutations and greater EGR-1 expression. Primary cultures carrying wild type *p53* have lower levels of EGR-1, and there is no correlation between EGR-1 expression and growth properties in these cases. On the basis of this evidence we hypothesize that inactivation of either p53 (through gene mutations) or EGR-1 (via down-regulated expression) is an important step leading to unrestricted proliferation of tumor cells. Glial tumor cells carrying mutant copies of p53 are more aggressive, irrespective of EGR-1 expression, than cells carrying wild type copies of p53, in which EGR-1 expression is almost undetectable.

We examined the intracellular expression of fibronectin in the tumor primary cultures and observed two distinct patterns of fibronectin expression. In tumor primary cultures carrying wild type copies of *p53*, fibronectin accumulated in amounts proportional to the levels of EGR-1 protein. In tumor primary cultures with *p53* mutations, however, only small amounts of fibronectin were seen, with no correspondence to EGR-1 levels. Our results provide further data in support for the experimental observation that EGR-1 directly transactivates the fibronectin gene in the U251 cell line [19] and suggest that EGR-1 may have retained this control only in tumors carrying wild type copies of *p53*. Whether regulation of fibronectin is part of a wider control EGR-1 exerts on the glioma phenotype, other than growth inhibition, remains to be investigated. Fibronectin expression is also inversely correlated with the degree of histological malignancy, cell transformation and dedifferentiation [29,30]. Together, these observations support our findings of a direct involvement of EGR-1 in the negative regulation and control of glial malignancies.

We used a recombinant adenovirus to introduce a functional *EGR-1* cDNA into glioma primary cell lines with either normal or mutated copies of *p53* gene. The exogenous expression of EGR-1 resulted in growth arrest and eventual cell death, even in the presence of mutated copies of *p53*. Mutant p53 may hinder the suppressor activity of EGR-1, perhaps by direct protein-protein interaction. Liu et al [31] have recently shown that EGR-1 and P53 form molecular complexes in vitro. Although these findings have not been further confirmed, they allow speculation that binding of mutant p53 protein might inhibit functional EGR-1 protein. This scenario would be consistent with our results showing that differences in fibronectin expression are supported by differences in EGR-1 levels only in tumor cultures carrying wild-type p53, but at variance with our finding that the exogenously added EGR-1 arrested the growth of primary cultures irrespective of p53 status. One explanation may be that the high levels of EGR-1 protein expressed by the transgene saturates the inhibitory effects of the dominant mutant *p53* gene.

In summary, the simplest interpretation of our observations is to attribute the failure of EGR-1 protein to inhibit glioma cell proliferation to downregulation of the EGR-1 gene in the tumor cells. Our data bear strong implications for *EGR-1* as a future candidate in gene therapy of tumors.

Methods

Glioma primary cell cultures and cell lines

Primary cell lines were established from 17 astrocytic neoplasms obtained from patients undergoing surgery. Tumors were classified according to the WHO classification system as anaplastic astrocytoma (W.H.O. grade III; 3 tumors) and glioblastoma multiforme (W.H.O. grade IV; 14 tumors) [32]. Written informed consent for research

use of tumor tissue was obtained from each patient prior to surgery, according to a protocol approved by the institutional ethics committee.

Tumor specimens were immediately transported to the laboratory, finely minced to single cell suspension and cultured in complete medium (Dulbecco's modified Eagle medium containing 10% fetal calf serum and 2% glutamine) into 100 cm^2 tissue culture plastic dishes (Falcon, Becton Dickinson) until passage 2. Cells were then collected and aliquots were cryopreserved in liquid nitrogen. One aliquot of cells was kept in culture and grown to confluence. Cells used in these experiments were subcultured for no more than three additional passages. If additional cells were needed, another aliquot was thawed and cultured. The human glioblastoma cell lines U251, T98G and A172 (obtained from the American Type Culture Collection, Rockville, MD) were cultured in complete medium.

RNA extraction and Northern Analysis

Total RNA was extracted from tumor primary cultures using the Ultraspec(tm) RNA isolation system (Biotecx Laboratories, Houston, TX, USA). Northern blots were performed as described [16], with 20 μg of RNA used in each lane. A 1.6 Kb Bgl II fragment from pCMV-EGR-1 plasmid was the probe for *EGR-1* [9].

Western Blot Analysis

EGR-1 is easely detected in whole cell extracts [11]. Proteins from cultured cells were extracted in TBS 1% Triton lysis buffer (150 mM NaCl; 50 mM Tris-HCl pH 8; 5 mM EDTA; 1 mM NaF; 1 mM $Na_4P_2O_7$; 1.5 mM KH_2PO_4; 0.4 mM Na_3PO_4) and incubated on ice for 20 min. After samples were centrifuged briefly at 12,000 g, protein concentration was determined using the BioRad protein assay reagent (Bio-Rad, Hercules, CA, USA). One hundred micrograms of protein were added to an equal volume of 2 × sample buffer (125 mM Tris-HCl pH 6.8; 4% SDS; 10% glycerol; 0.006% bromophenol blue; 2% mercaptoethanol), boiled for 5 min, separated on 7% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell, Germany). After transfer, Ponceau staining was used for confirmation of equal protein loading and for signal normalization.

The membranes were incubated for 1 h at room temperature with PBS containing 5% non-fat dry milk (Bio-Rad) and then incubated overnight at 4°C in PBS containing 2.5% non-fat dry milk and 1 μg/ml of specific antibody, either rabbit polyclonal anti-EGR-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal anti-human fibronectin (Sigma, St. Louis, MO, USA). The membranes were washed twice with PBS and re-incubated for 1 h with anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase (Amersham Italia Srl, Milano, Italy). Signals were detected using enhanced chemiluminescence (ECL detection system; Amersham Italia Srl) according to the manufacturer's instructions.

Immunofluorescence and immunocytochemistry

Antibodies used were as follows: the rabbit polyclonal anti-EGR-1 also used for Western blots (Santa Cruz Biotechnology), a mouse monoclonal antibody against GFAP (Sigma), and mouse monoclonal antibodies against p53, MDM2 and vimentin (Pharmingen Corporation, San Diego, CA, USA). For immunofluorescence, primary cells were fixed in 4% fresh paraformaldehyde for 5 min and then permeabilized with 0.1% Triton-X100/PBS for 5 min. Anti-rabbit or anti-mouse fluorescein-conjugated secondary antibody (Sigma) was used at a 1:100 dilution.

Immunocytochemistry was performed using avidin-biotin-peroxidase (ABC Universal kit, Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol. Endogenous peroxidase was inhibited by incubation with freshly prepared 3% hydrogen peroxide for 10 min. Slides were then washed in PBS buffer (0.05 M Tris-HCl pH 6.7, 0.3 M NaCl, and 0.1% Tween 20), and cells fixed on the slide were treated with 1.5% blocking serum (Santa Cruz Biotechnology) in PBS for 20 min to reduce non-specific antibody binding. Cells were incubated overnight with primary antibody, washed, and further processed with the ABC Universal Quick kit (Vector Laboratories,), according to the manufacturer's instructions. Peroxidase activity was visualized after 6 min incubation with freshly prepared 3,3'-diaminobenzidine substrate solution. Cells were rinsed in water, counterstained with hematoxylin, dehydrated, and mounted. All the values for immunochemistry are expressed as percentage of positive cells stained.

Fluorescence at the single cell level was captured with a Spot II CCD camera (Diagnostic Instruments Inc, Sterling Heights, MI, USA) mounted on a Axiophot II microscope (Zeiss, Germany) equipped for epifluorescence. The fluorescence intensity was determined by semi-quantitative densitometric analysis using the NIH Image software (see below).

Densitometric Analysis

Densitometric analysis of Western blots was performed on a Macintosh G3/233 computer using the public domain NIH Image software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Total protein content of each sample after loading was estimated by averaging the intensity values of the single major bands of protein detected by Ponceau staining of filter blots. The

EGR-1 and fibronectin chemiluminescence signals were normalized with respect to actin content of each sample.

Evaluation of MDM2 content in cells stained by immunofluorescence was performed by semi-quantitative densitometric analysis of the intensity of fluorescence at the single cell level.

DNA Sequencing of p53 Exons 5–9

For analysis of *p53* mutations, previously described primer sets were used for PCR amplification from high molecular weight DNA of three DNA fragments corresponding to p53 exons 5 and 6, exon 7, and exons 8 and 9, respectively [33]. Direct sequencing of the specific DNA fragments was performed using a Big Dye terminator DNA sequencing kit with the ABI PRISM 377 DNA Sequencer (PE Applied Biosystems Inc., Foster City, CA, USA), according to the manufacturer's instructions. Results were analyzed using the ABI sequencing analysis software.

Analysis of allele dosage for MDM2 and loss of heterozygosity for p16/ARF

Southern blotting was used for monitoring *MDM2* gene amplification and loss of heterozygosity at the p16INK4a/ARF locus, using procedures and probes as previously reported [16].

Growth assays

Duplication time

cells were seeded at 2×10^3 per well in 96-well plates in complete medium. Every two days the growth was measured by crystal violet assay. The cells were stained and the dye was released by citrate buffer for quantification in an ELISA reader. Since cells vary in size between different primary cultures, the relationship between cell number and absorbance was first calculated for each primary culture. The duplication time was calculated by measuring the distance on the time axis during the log phase of cell growth.

Plating efficiency

clonogenicity on plastic substrate was tested by seeding 200 cells per well in 6-well plates. Cells were cultured in complete medium containing either 10% or 2.5% FCS and fed every two days. After three weeks, the cells were washed in PBS, fixed in 70% ethanol and stained with crystal violet for colony counting.

Soft agar colony growth

2×10^3 cells were seeded in 0.35% NuSieve low melting agarose (FMC Bioproducts, Rockland, ME, USA) into 35-mm dishes previously lined with 0.7% agarose medium. After three weeks the colonies were counted under the inverted microscope.

Construction of recombinant Adenoviruses

Recombinant adenoviruses were generated following the method described by He et al. [34]. Briefly, a 1.7-kb Ava II fragment containing the *EGR-1* coding sequence was subcloned from plasmid pRSVEgr-1-2.1 [35] into the multiple cloning site of the shuttle vector pAdCMV-GFP containing the coding sequence of green fluorescent protein (GFP) under the control of CMV promoter. The resulting plasmid was linearized by digestion with PmeI and co-transfected with the adenoviral backbone plasmid pAdEasy-1 (Stratagene, San Diego, CA, USA) into E. coli BJ5183 cells, generating recombinant plasmid pAdCMV-GFP-EGR-1. The recombinant adenovirus AdGFP-EGR-1 was obtained by transfecting pAdCMV-GFP-EGR-1 into adenovirus packaging 293 cells. A recombinant AdGFP construct without an insert was also generated and used as a control. The recombinant adenoviruses were collected at seven days post-infection. All adenovirus constructs were amplified and titrated in 293 cells.

For transgene expression the U251 and glioma primary cultures were grown to about 70% cell confluence and infected by adding the virus with serum-free medium. The virus concentration used for infection was chosen by determining the highest dilution at which optimal transgene expression could be obtained with low cell toxicity. At this concentration between 70% to 90% of cells were infected each time.

For the cell proliferation assay, cells were seeded at 24 hours after infection onto microtiter plate wells at the density of 1500 cells/well. After cell fixation in 50% TCA, the sulforhodamine B assay was performed to quantitate the viable cells [36].

Statistical analysis

The mean values of measurements of either protein expression made by densitometry or growth parameters were compared by two side Student's t test or non parametric Mann-Whitney U test. Statistical analysis and calculation of the regression coefficients were performed using StatView software (SAS Institute Inc., Cary, NC, USA).

List of abbreviations

The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; GFP, green fluorescent protein; GFAP, glial fibrillary acidic protein; O.D., optical density.

Additional material

Additional File 1

the Table describes tumor gene mutations, protein expression, and growth properties of 17 primary cell cultures established from malignant glioma.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1475-2867-4-1-S1.doc]

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