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Biomolecular Characterization of Human Glioblastoma Cells in Primary Cultures: Differentiating and Antiangiogenic Effects of Natural and Synthetic PPARγ Agonists

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Gliomas are the most commonly diagnosed malignant brain primary tumors. Prognosis of patients with high-grade gliomas is poor and scarcely affected by radiotherapy and chemotherapy. Several studies have reported antiproliferative and/or differentiating activities of some lipophylic molecules on glioblastoma cells. Some of these activities in cell signaling are mediated by a class of transcriptional factors referred to as peroxisome proliferator-activated receptors (PPARs). PPARγ has been identified in transformed neural cells of human origin and it has been demonstrated that PPARγ agonists decrease cell proliferation, stimulate apoptosis and induce morphological changes and expression of markers typical of a more differentiated phenotype in glioblastoma and astrocytoma cell lines. These findings arise from studies mainly performed on long-term cultured transformed cell lines. Such experimental models do not exactly reproduce the in vivo environment since long-term culture often results in the accumulation of further molecular alterations in the cells. To be as close as possible to the in vivo condition, in the present work we investigated the effects of PPARγ natural and synthetic ligands on the biomolecular features of primary cultures of human glioblastoma cells derived from surgical specimens. We provide evidence that PPARγ agonists may interfere with glioblastoma growth and malignancy and might be taken in account as novel antitumoral drugs.

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Gliomas are the most commonly diagnosed malignant brain primary tumors in humans (Holland, 2001). These highly aggressive tumors are characterized by high proliferative rate and invasiveness, as well as secretion of proteins leading to T-cell immunosuppression. Persistent invasion of malignant glioma tumor cells into the adjacent normal brain parenchyma hinders complete surgical resection, therefore radiation and chemotherapy are required (Stupp et al., 2005). Nevertheless, prognosis of patients with high-grade gliomas is poor and poorly improved by radiotherapy and chemotherapy (Moynihan and Grossman, 1994) and several gliomas eventually become drug-resistant. A number of mechanisms may contribute to cellular drug resistance, including reduced intracellular drug concentrations, rapid drug inactivation, and increased rate of DNA repair. Inhibition of apoptosis may also contribute to drug resistance since chemotherapeutic agents mostly act by inducing apoptosis. The finding that brain tumors either deficient in the p53 tumor suppressor gene or over-expressing the antiapoptotic protein Bcl-2 escape apoptosis and are resistant to radiotherapy and chemotherapy (Rohini et al., 2006; Stegh et al., 2007) indicates that tumor-specific genetic lesions confer a survival advantage to tumor cells. Malignant progression of gliomas involves accumulation of genetic alterations inactivating p53, p16, RB, and PTEN tumor suppressor genes and/or activating oncogenes including the epidermal growth factor receptor (EGFR), CDK4, CDK6, and MDM2 genes (Schiffer, 1998; Ushio et al., 2003).

Recently, several studies have reported the in vitro and in vivo antiproliferative and/or differentiating activities of some lipophylic molecules on glioblastoma cells (Driever et al., 1999; Costa et al., 2001; Engelhard et al., 2001). It is known that some of the activities of lipophylic molecules in cell signaling are mediated by a class of transcriptional factors referred to as peroxisome proliferator-activated receptors (PPARs). At first, PPARs were identified as critical controllers of lipid metabolism, mainly regulating the expression of peroxisomal enzymes that catalyze fatty acid oxidation. Presently, it is well known their wider role in controlling important cell functions and processes such as energy homeostasis, cell proliferation and death, differentiation, inflammation, diabetes, atherosclerosis and cancer (Kersten and Wahli, 2000). PPARs belong to the nuclear receptor superfamily for thyroid, steroid hormones, vitamin D, and retinoic acid (Green, 1995). Upon ligand activation, they form heterodimers with the receptor for 9-cis retinoic acid (RXR) and bind to specific DNA sequences, named peroxisome proliferator responsive elements (PPREs), therefore triggering the transcription of target genes (Kliewer et al., 1994). So far, three PPAR isotypes have been identified,

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namely α, β/δ and γ, differing in tissue distribution, developmental expression, ligands and target genes (Forman et al., 1995). PPAR isotypes can both share ligands or act in a specific ligand-dependent manner. Many functions of PPARs can be extrapolated on the basis of their target genes which mainly belong to the pathways of lipid transport and metabolism. PPARγ has been demonstrated to inhibit inflammatory response in vivo. By antagonizing STAT, AP-1, and NFkB transcription factors activities, it downregulates inducible nitric oxide synthase (NOS II) and proinflammatory cytokines expression in macrophages (Colville-Nash et al., 1998; Ricote et al., 1998; Petrova et al., 1999) astrocytoma cells (Pérez-Ortiz et al., 2007), microglial cells (Jiang et al., 1998), cerebellar granule cells (Heneka et al., 1999). NO may enhance vasodilatation and promote neovascularization, thereby improving tumor growth (Heneka et al., 2000; Yang et al., 2002; Hara and Okayasu, 2004). Expression of NOS II in malignant gliomas has been extensively documented (Pérez-Ortiz et al., 2007). Recently, a role for PPARγ in cancer has been stressed. PPARγ has been identified in transformed neural cells of human origin and it has been demonstrated that PPARγ natural and synthetic agonists decrease cell proliferation, stimulate apoptosis and induce morphological changes and expression of markers typical of a more differentiated phenotype in glioblastoma and astrocytoma cell lines (Chattopadhyay et al., 2000; Morosetti et al., 2004; Cimini et al., 2005; Spagnolo et al., 2007). Moreover, PPARγ downregulates VEGF and, as a consequence, inhibits angiogenesis, primary tumor growth and metastasis formation (Xin et al., 1999; Panigrahy et al., 2002).

Noteworthy, all the above findings arise from studies mainly performed on long-term cultured transformed cell lines. Such experimental models do not reproduce the in vivo environment since long-term culture often results in the accumulation of further molecular alterations in the cells. To be as close as possible to the in vivo condition, in the present work we investigated the effects of PPARγ natural (CLA) and synthetic (GW347845) ligands on the biomolecular features of human glioblastoma cells in primary cultures derived from surgical specimens. We provide evidence that PPARγ agonists may interfere with glioblastoma growth and malignancy and thus be considered as potential antitumoral drugs.

Materials and Methods

Reagents

Conjugated linoleic acid (CLA) was generously provided by Natural Lipids Ltd AS (Hovdebygda, Norway); Triton X-100, Nonidet P40, sodium dodecylsulfate, Tween-20, L-glutamine, phalloidin-TRITC, anti-GFAP monoclonal antibody, FITC-labeled anti-rabbit IgG and anti-mouse IgG antibodies, PVDF were purchased from Sigma Chemical Co. (St. Louis, CO); ELISA nucleosome detection kit, BrdU incorporation kit and RNA-ase were from Roche Diagnostic (Indianapolis, IN); trypsin–EDTA solution, streptomycine–penicillin, Trizol reagent and Platinum Taq RNA plymerase, Trizol reagent were from Gibco Invitrogen GmbH (Paisley, UK); rabbit anti-caspase 3, anti-caspase 9, anti-bcl-2, anti-p21, anti-CDK4, anti-Cyclin D1, anti-PTEN from St. Cruz Biotechnology (Santa Cruz, CA); RPMI-1640, fetal bovine serum (FBS) were from Euroclone Ltd (Erba, MI, Italy); Vectashield was from Vector (Burlingame, CA); ECL kit was from Amersham Life Sciences (Little Chalfont, Buckinghamshire, UK); micro BCA protein detection kit from Pierce (Rockford, IL). All other chemicals were of the highest analytical grade.

The PPAR γ agonist and antagonist, GW347845 and GW9662 respectively, were generous gifts of Dr. Timothy M. Willson, GlaxoSmithKline, Discovery Research (Research Triangle Park, NC).

Cell cultures

Patients, harboring cerebral lesions resembling grade IV astrocytomas (GBL), have been subjected to gross total removal (apparently radical exeresis) of the tumor by microsurgical aggression of the lesion from the margins, with the aid of Image Guided Surgery and, in selected cases, of the Photo Dynamic Diagnoses (PDD).

A part of the surgical tissue corresponding to a certainly tumoral portion (preferably a portion corresponding to the main contrast enhanced part on preoperative MRI), assessed intraoperatively with the use of neuronavigation and by extemporary histological examination has been used by to establish autologous glioblastoma cell lines.

All patients were submitted, within 48 h from surgery, to CT scan or MRI with contrast enhancement to evaluate the extent of the resection of the tumor.

Informed consensus has been provided by the patients prior to participation in this study, in accordance with L'Aquila University and S. Salvatore Hospital Bioethical Committee approval. Human glioblastoma surgical specimens were extensively washed in DMEM (Gibco Invitrogen GmbH), mechanically dissected and incubated at 37°C under continuous shaking in DMEM containing 0.125% trypsin and 0.125% EDTA (10 ml/g of tissue) to obtain a single cell suspension. After 15 min the incubation medium was diluted 1:2 with fresh DMEM to block trypsin and EDTA action. Cell suspension was centrifuged at 1,200 rpm and 4°C for 10 min. Cell pellet was dissolved in DMEM containing 5% G-5 supplement (Gibco Invitrogen GmbH) and single cell suspension was plated and incubated at 37°C and 5% CO_2. After 24 h medium was replaced with fresh G-5 containing DMEM and maintained for further 3 days. Medium was then replaced with 10% FBS (Gibco Invitrogen GmbH) DMEM. Cells were allowed to reach 70% confluence prior to be frozen in 10% FBS DMEM containing 7.5% DMSO. In the present study data obtained from cell cultures derived from five different glioblastoma specimens (five different patients) are presented.

For treatments with conjugated linoleic acid (CLA) and PPARγ ligand and antagonist, FBS was replaced with charcoal (5 mg/ml) stripped serum (overnight at 4°C) in order to eliminate endogenous lipid components.

Treatments

CLA was prepared from a solution containing 1 mM CLA, 1 mM NaOH, 10 mg/ml defatted BSA in serum-free medium. The solution was incubated for 10 min at 37°C and sonicated (5 cycles of 5 sec), incubated at 50°C for 10 min and used at the indicated final concentrations. The PPARγ ligand, GW347845, was dissolved in 0.01% DMSO and used at the final concentration of 1 μM. The PPARγ antagonist GW9662 was used at 1 μM (final concentration) and administered 30 min before CLA or GW347845 treatment. Controls received the vehicle alone (0.001% DMSO for GW347845 and GW9662, delipidated serum for CLA).

Cell growth, proliferation and death assays

Cell growth curves were obtained by counting Trypan blue excluding cells, both in control and treated cultures, at different time points. Cell proliferation was measured by BrdU incorporation assay according to manufacturer's directions.

For apoptosis detection, cells were seeded in 96-well plates at a density of 3×10^4 cells/cm^2 in delipidated serum. Control and treated cells were analyzed for apoptosis using an ELISA nucleosome detection kit. Absorbances at 405 and 490 nm were recorded according to manufacturer's directions.

Protein assay

Proteins were assayed by the micro-BCA kit. Briefly, this assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total

protein. This method combined the reduction of Cu + 2 to Cu + 1 by protein in alkaline medium (the biuret reaction) with the high sensitive and selective colorimetric detection of the cuprous cation (Cu + 1) using a reagent containing BCA. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This complex exhibits a strong absorbance at 562 nm.

Immunoblotting

For Western blotting, cells treated for 4 or 72 h (depending on the parameter to be studied: 4 h for transcription factors and cell cycle proteins; 72 h for undifferentiation or differentiation markers) were washed in ice-cold PBS and harvested in ice-cold RIPA buffer (phosphate buffer saline pH 7.4 containing 0.5% sodium deoxycolate, 1% Nonidet P-40, 0.1% sodium dodecylsulfate, 5 mM EDTA, 100 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM PMSF, 2 mM ortovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin). Cell lysates were centrifuged and the supernatants were assayed for protein content. Proteins (40 μg) from whole cell lysates were electrophoresed through a 10% SDS–polyacrylamide gel under reducing conditions. Proteins were transferred onto PVDF membrane sheets and nonspecific binding sites were blocked by overnight incubation at 4°C in 20 mM TRIS, 55 mM NaCl and 0.1% Tween-20 pH 7.4 (TST) containing 5% nonfat dry milk (blocking solution). Membranes were then incubated for 1 h at RT with the following rabbit primary antibodies: anti-caspase 3 and anti-caspase 9 (1:200), anti-CDk4 (1:200), anti-cyclin D1 (1:200), anti-p21, anti-PTEN, anti-GFAP (1:500), anti-N-cadherin (1:500), anti-bcl-2 (1:500) dissolved in the blocking solution. After extensive washings with TST, the membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:2,000). Immunoreactive bands were visualized by chemiluminescence (ECL).

RT-PCR analysis

Total cellular RNA was extracted by Trizol Reagent according to manufacturer's directions. The total RNA concentration was determined spectrophotometrically in RNAase-free water. Reverse transcription (RT) was performed using Gene Specific Realtime TR-PCR kit and polymerase chain reaction (PCR) amplification of specific target RNA was performed using Platinum Taq DNA polymerase following the manufacturer's directions. For RT-PCR of human p-TEN primers Fw5′-GGACGAACTGGTGTAATGATATG-3′; Rev5′-TCTACTGTTTTTGTGAAGTACAGC-3′; were used to amplify a 671 bp fragment. Human β-actin: 5′-TGA CGG GGT CAC CCA CAC TGTGCC CAT CTA3′ (upstream) and 5′-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG3′ (downstream) with an amplification product of 661 bp. Thermal cycling was performed by 35 cycles at 94°C for 1 min, 57.5°C for 1 min and 72°C for 3 min, followed by a final extension at 72°C for 10 min. For RT-PCR of human NOSII primers Fw5′-TAGAGGAACATCTGGCCAGG-3; Rev5′-TGGCAGGGTCCCCTCTGATG-3′ were used to amplify a 372 bp fragment. Thermal cycling was performed by 35 cycles at 95°C for 1 min, 61°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. For RT-PCR of human VEGF isoforms primers: Fw5′-GAGTGTGTGCCCACTGAGGAGTCCAAC3′; Rev5′CTCCTGCCCGGC TACCCGCCTCGGCTT-3′ were used to amplify 311 bp (VEGF 165) and 145 bp (VEGF 121) fragments. Thermal cycling was performed by three cycles at 93°C for 30 sec, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

PCR products were electrophoretically separated on 1.5% agarose gel and identified by ethidium bromide staining. To normalize differences in total RNA concentration among samples, bands were normalized against β-actin. Semiquantitative analysis was performed by TOTAL LAB V.I.II. System (Phoretix, Newcastle, UK).

Immunofluorescence

Cells grown on coverslips (3×10^4 cells/cm^2) and treated for 48 h were fixed for 10 min at RT in 4% paraformaldehyde in PBS and permeabilized in PBS containing 0.1% Triton X-100 for 10 min at RT. Cells were then incubated with N-cadherin, CD133, nestin, A2B5, β-catenin, fibronectin, GFAP (1:100 in PBS containing 3% BSA), overnight at 4°C. After extensive washings with PBS, cells were incubated with fluorescein-labeled anti-rabbit IgG secondary antibodies (1:100 in PBS containing 3% BSA) for 30 min at RT. Actin staining was performed incubating fixed cells with phalloidin-TRITC (1:100 in PBS containing 3% BSA) for 30 min at RT. After extensive washings with PBS, cells were mounted with Vectashield mounting medium and photographed at fluorescence microscope (AXIOPHOT, Zeiss, Oberkochen, Germany).

Nitrates/nitrite assay

Cell culture media were collected and centrifuged. Nitrite concentrations were evaluated on supernatants by the Griess method. Briefly, supernatants were treated (1:1) with Griess reagent (1% sulfanilamide, 2.5% H_3PO_4, 0.02% *N*-1-naphthyl-ethylendiamine dissolved in H_2O). The concentration of nitrite is determined by ELISA reader at 550 nm against a standard curve.

Cell adhesion, migration, and invasiveness assay

Adhesion: control and 48 h-treated cells were seeded onto plastic, Matrigel (25 μg/ml) or gelatin (0.1% v/v) coated 96-multiwell (2×10^4 cells/well) in serum free medium and incubated at 37°C, for 15, 30, and 60 min. Cells were then washed twice with PBS and fixed with ice cold methanol for 10 min. After washings, cells were allowed to air dry for 5 min and stained with 20 μl/well 0.5% (w/v) crystal violet for 15 min at RT. After washings, cells were washed with methanol for 15 min, until no more staining was released. Absorbance at 595 nm of methanol is directly proportional to adhesion grade.

Migration and invasiveness: migration and invasiveness assays were performed on Boyden chambers containing PVPF8 filters coated by gelatin 0.1% (v/v) or Matrigel (25 μg/ml), respectively. Control and 48 h-treated cells were detached, washed twice with PBS and resuspended in serum free medium at 7×10^5 cells/ml. Cell suspension (600 μl) was seeded in the upper chamber and allow to migrate for 6 h. After removing the nonmigrated cells on the upper face of the filter, the migrated ones on the lower face of the filter were stained with Deef-Quick, mounted on slides and counted under light microscope. For each conditions three filters were prepared and for each filter five fields were counted.

Statistics

Statistical significance of paired samples was analyzed by Student's *t*-test. $^{**}P < 0.01$; $^{***}P < 0.001$. For RT-PCR analysis, samples were processed by SPSS software and analyzed by ANOVA test, followed by Scheffe's "post hoc test" analysis. $^{**}P < 0.005$; $^{***}P < 0.001$.

Results

Immunofluorescence

Actin staining in treated cells revealed that changes in microfilament organization accompany morphological modifications (Fig. 1A–C). For further characterization, glioblastoma cells were also immunostained to evaluate differentiation markers prior to and after 72 h treatments. Untreated cells were strongly immunopositive for nestin, a marker of undifferentiated neuroepithelial cells (Fig. 1D). Following CLA or PPARγ agonist treatment, the fluorescence intensity for nestin strongly decreased (Fig. 1E,F, respectively).

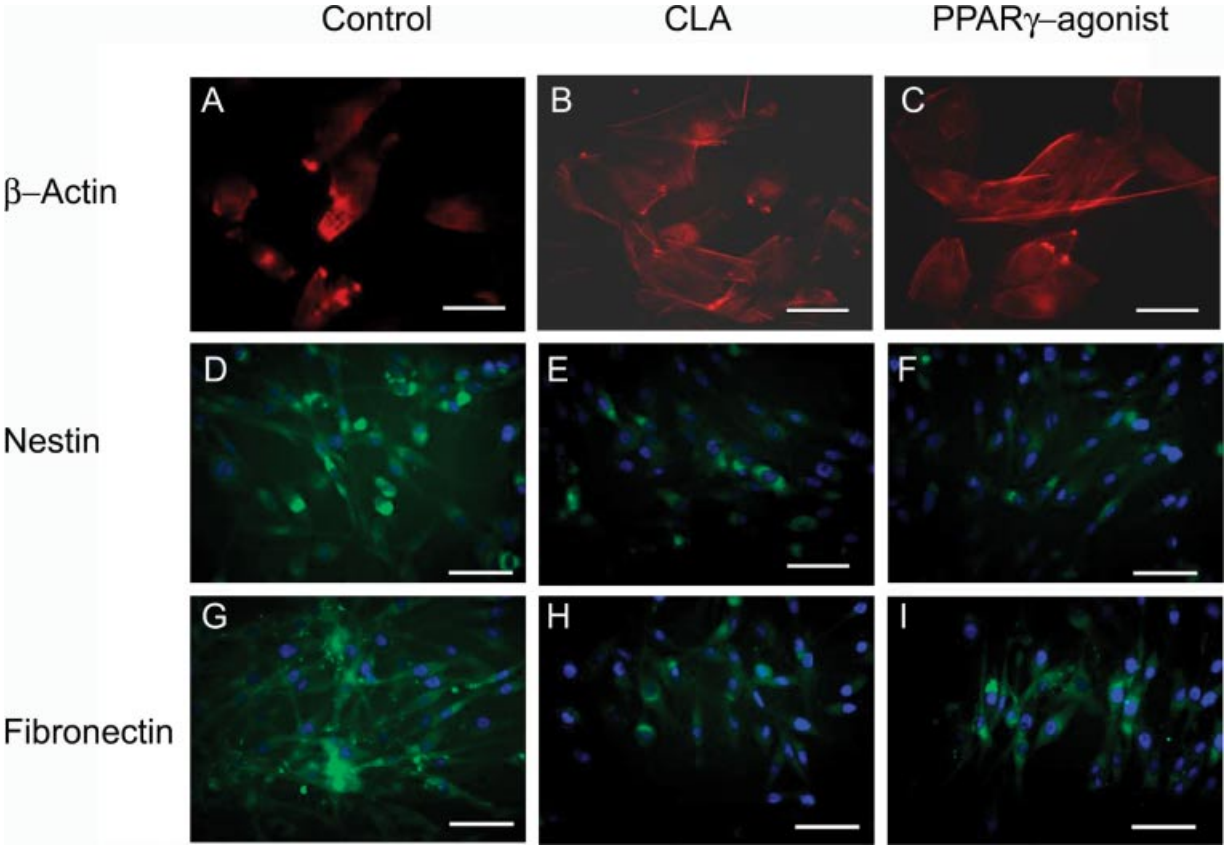

Fig. 1. Actin staining in control (A), CLA and PPARγ agonist treated cells (B,C, respectively) shows reorganization of microfilament network in treated cells. Nestin immunofluorescence of control (D) or CLA (E) or PPARγ agonist (F) treated cells shows a strong decrease of the protein upon both treatments. Untreated cells appear immunopositive for fibronectin (G). Upon CLA or PPARγ agonist treatments, intra- and extracellular fibronectin fluorescence intensity decreases (H,I, respectively). Bar = 125 μm.

Untreated cells appeared strongly immunopositive for fibronectin, which was also detected in the extracellular matrix (Fig. 1G). Both intra- and extracellular fibronectin immunofluorescence intensity was decreased after CLA or PPARγ agonist treatments (Fig. 1H,I, respectively). In untreated primary cultures, several neurospheres immunopositive for the neural stem cell marker CD133 were observed (Fig. 2A). Following CLA or PPARγ agonist treatments, cells were still CD133-immunopositive, though not organized in neurospheres anymore (Fig. 2B,C, respectively). Some untreated cells in primary cultures showed positivity for A2B5, a marker of glial restricted progenitors (Fig. 2D). Both treatments slightly increased the number of A2B5 immunopositive cells (Fig. 2E,F). β-catenin antibody marked few cells in untreated cultures (Fig. 2G), whereas CLA or PPARγ agonist treatments highly increased the number of immunopositive cells and the protein appeared to be localized to the cytoplasm, including cell processes (Fig. 2H,I, respectively). In untreated cultures, weak N-cadherin immunoreactivity was present in few cells (Fig. 3A); treatments remarkably enhanced fluorescence intensity as well as the number of positive cells (Fig. 3B,C). Few GFAP-immunoreactive cells were observed in control cultures (Fig. 3D), whereas CLA or PPARγ agonist treatments increased the number of immunopositive cells and induced strong modifications in cell morphology (Fig. 3E,F, respectively). Parallel Western blot analyses for N-cadherin and GFAP confirmed the significant increase of both proteins in treated cells (Fig. 3).

Cell proliferation and death

We had previously shown that 25 μM CLA was able to modulate growth in a human glioblastoma cell line without exerting toxic effects (Cimini et al., 2005). Preliminary experiments indicated 50 μM as the optimal concentration for CLA treatment of glioblastoma cells in primary culture (not shown). CLA strongly decreased glioblastoma cell growth rate. Similarly to CLA, though to a lesser extent, the PPARγ agonist GW347845 induced a reduction of cell growth, especially dramatic after 72 h of treatment (Fig. 4A). The specific PPARγ antagonist completely abolished the effects of the two compounds. BrdU incorporation shows decreased cell proliferation after CLA and GW347845 treatments, particularly at 48–72 h, though with different time courses (Fig. 4B). The specific antagonist completely reverted these effects. The same Figure (Fig. 4C) shows the apoptotic rate of untreated and treated glioblastoma cells, evaluated as nucleosome concentration. CLA increased nucleosome concentration, showing maximum activity at 24 h, while GW347845 effect was apparent at 72 h. The PPARγ antagonist completely abolished these effects. Western blot analysis shows a reduction of the caspase 3 inactive form and bcl-2 and an increase of caspase 9 levels upon both treatments, thus

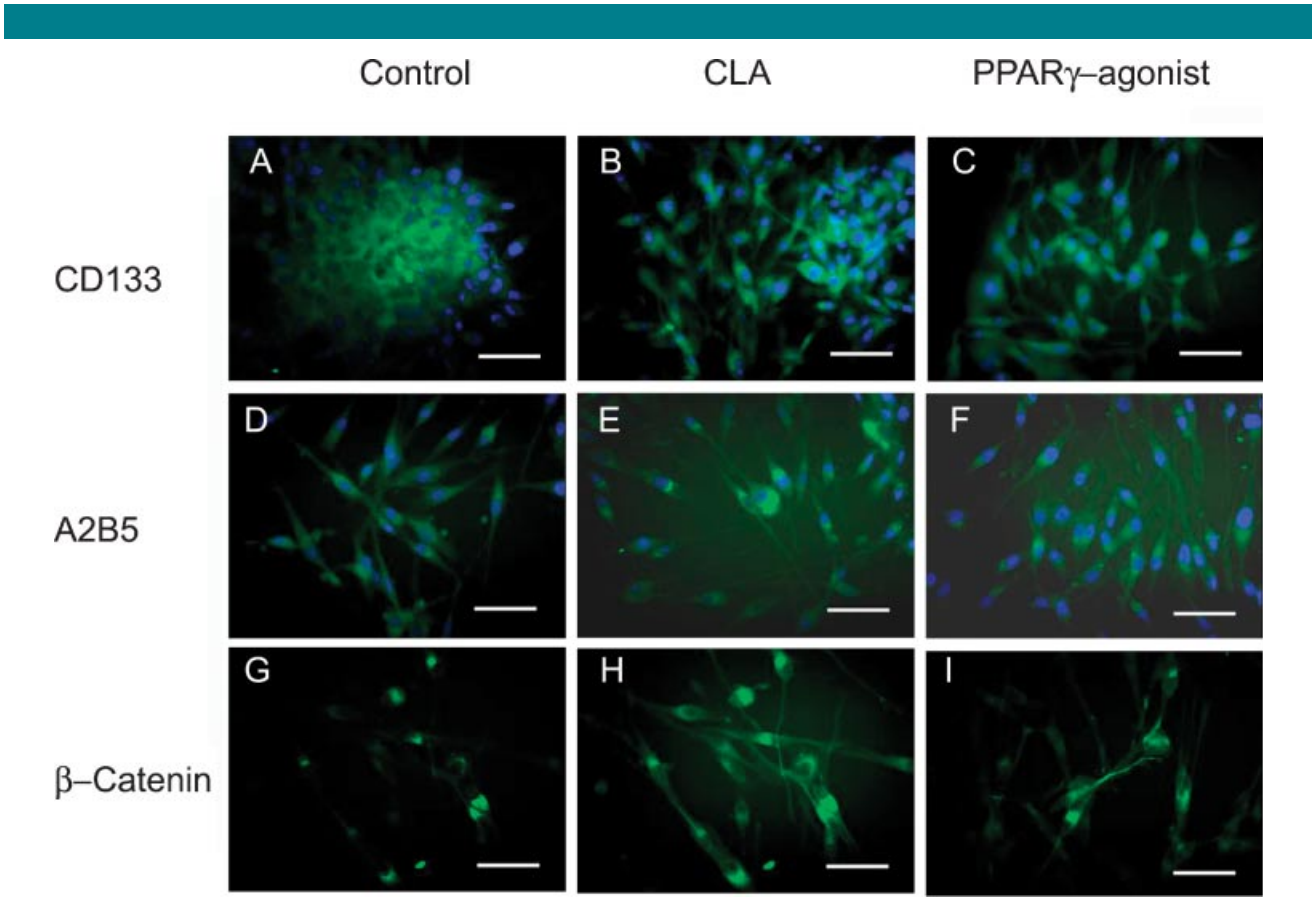

Fig. 2. CD133 immunostaining in control (A) and CLA or PPARγ agonist (B,C, respectively) is shown. Several untreated cells appear organized in neurospheres which are no more observed upon CLA or PPARγ agonist treatments. A2B5 immunostaining shows some positive cells in untreated conditions (D). Both treatments slightly increased the number of A2B5 immunopositive cells (E,F). β-catenin staining marks few cells in untreated cultures (G), whereas CLA or PPARγ agonist treatments highly increased the number of immunopositive cells and the protein appears localized to the cytoplasm and cellular elongations (H,I, respectively). Bar = 125 μm.

confirming the induction of apoptosis by CLA and the PPARγ agonist (Fig. 4D).

Cell cycle control

Expression levels of the cell cycle positive regulators such as cyclin D1 and CDK4 cell, and of the cell cycle negative regulators such as p21 and PTEN, were investigated in treated and untreated primary culture glioblastoma cells. CLA and the PPARγ agonist decreased cyclin D1 and CDK4 protein levels, while greatly increasing p21 protein levels and PTEN mRNA and protein (Fig. 5).

Cell adhesion, migration and invasiveness

CLA and the PPARγ agonist dramatically altered glioblastoma cell substrate interactions, by enhancing adhesion capability. Noteworthy, both treatments inhibited in vitro cell motility through gelatin and invasiveness through basement membrane matrix (Matrigel) (Fig. 6).

NOSII and VEGF expression

Proinflammatory cytokines and LPS treatment induced NOS II expression in primary cultures glioblastoma cells, as revealed by RT-PCR. CLA, but not the PPARγ agonist, reverted such biological effect. Consistently, the nitrate/nitrite levels were down modulated by CLA, but were not modified upon PPARγ agonist treatment (Fig. 6). VEGF 165 and VEGF 121 isoform expression in primary culture glioblastoma cells was decreased by both treatments, CLA being more effective. The effect was reverted by PPARγ antagonist (Fig. 6).

Discussion

Present knowledge about gliomas biology is mostly based on studies performed on long-term cultured cell lines. Since in time such cultures undergo an extensive clonal selection, results obtained using this model might substantially differ from data on biopsy specimens. To overcome this problem, in the present work we used primary cultures of human glioblastoma cells, freshly derived from surgical specimens, as an experimental model closer to the in vivo condition. Our cells contained, besides neurosphere-forming and CD133-expressing cells, adherent nestin-positive cells and also cells weakly positive for β-catenin, A2B5, N-cadherin and GFAP. The finding of a heterogeneous population of cells at different degrees of differentiation might partially account for the frequent failure of radio- and chemotherapy protocols followed for glioblastomas.

It is well known that the response of tumor cells to antiproliferative treatments is strongly dependent on their differentiation degree. PPARγ not only plays a crucial role in apoptosis but also in differentiation of a variety of cell types, including malignant cells (since induction of differentiation through PPARγ activation has been observed in several malignant cells; Tontonoz et al., 1997; Mueller et al., 1998; McCarthy, 2000). It has been reported that several effects of CLA on both normal and malignant cells are mediated by the

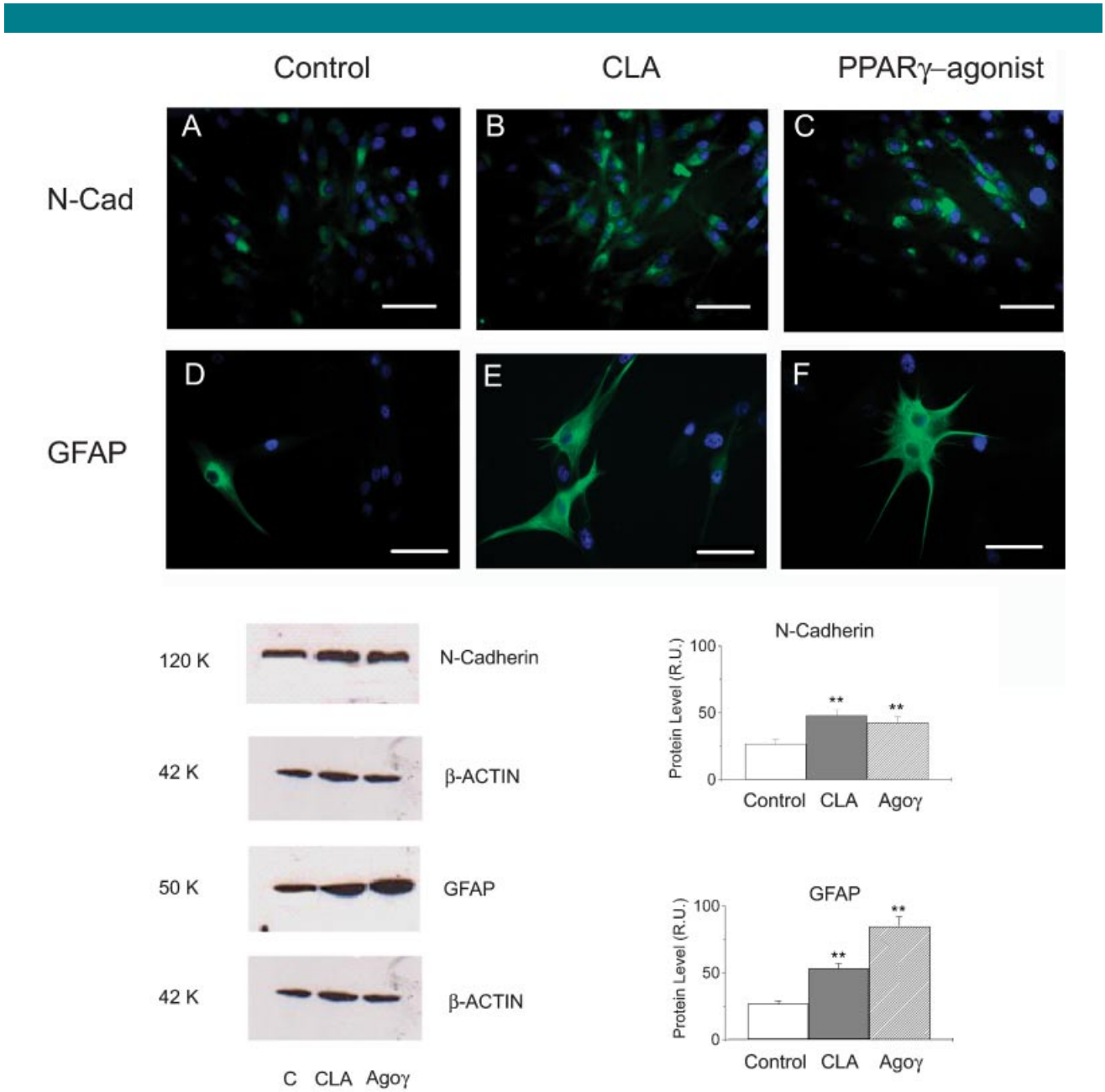

Fig. 3. In untreated cultures N-cadherin appears weakly present (A); treatments clearly increase fluorescence intensity as well as the number of positive cells (B,C), Bar = 125 μm. GFAP immunostaining shows few immunopositive cells in controls (D), whereas CLA (E) or PPARγ agonist (F) treatments increase the number of immunopositive cells and induce strong modification of cell morphology, Bar = 75 μm. Western blot analyses for N-cadherin and GFAP confirm the strong increase of both proteins in treated cells. Data are mean ± SE of five experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

induction and activation of PPARs (Moya-Camarena et al., 1999a,b). In a previous article we demonstrated the expression of PPARs in a human glioblastoma cell line (ADF) and showed that the growth inhibitory effects of CLA were mediated by PPARγ activation (Cimini et al., 2005).

In this article, we investigated whether PPARγ activation could influence differentiation of primary culture glioblastoma cells. Herein we show that treatment with natural or synthetic ligands of PPARγ decreases the expression of undifferentiation markers such as CD133, nestin, fibronectin, while increasing the expression of differentiation markers such as A2B5, GFAP, β-catenin, N-cadherin.

In adult and pediatric brain tumors, a small population of cancer stem cells has been recently identified (Singh et al., 2003). Although its biological function is not fully understood, CD133 is currently employed as a useful marker for the identification and isolation of brain cancer stem cells (Singh et al., 2004; Liu et al., 2006). CD133-positive cancer stem cells are highly resistant to temozolomide, carboplatin, VP16 and Taxol (Liu et al., 2006). CD133-positive cells isolated from human glioblastoma may initiate tumors (Strojnik et al., 2007), thus representing potential therapeutical targets. In our model, we observed cells organized in neurospheres and positive for CD133/nestin, that is, stem-like cells. Upon both treatments,

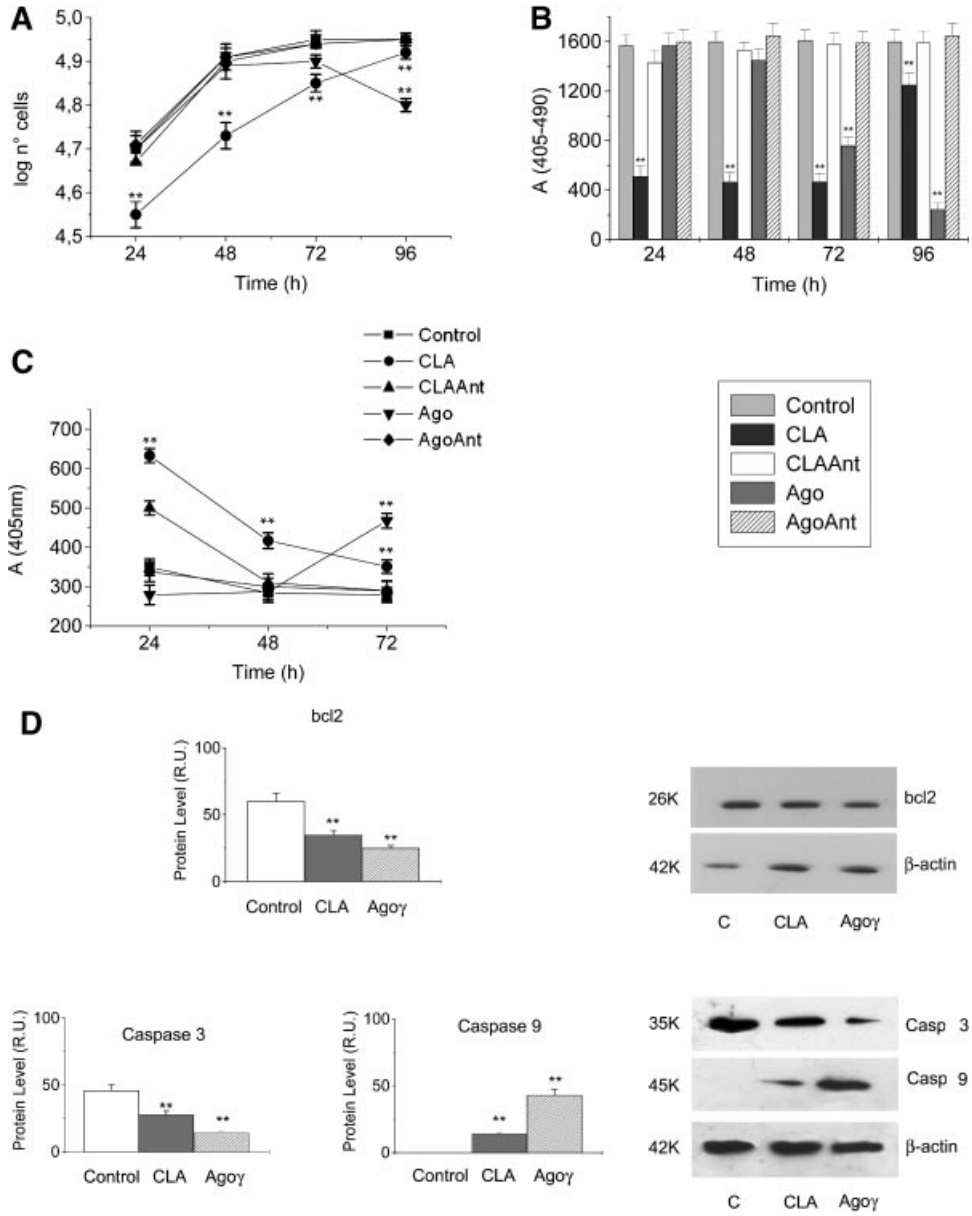

Fig. 4. Growth curves in control and treated cells show that CLA strongly decreases cell growth rate. Similarly to CLA, even if to a lesser extent, PPARγ agonist induced a reduction of cell growth after 72 h of treatment. A: The specific PPARγ antagonist abolishes these effects. B: Cell proliferation analysis of glioblastoma cells upon treatments, as evaluated by BrdU incorporation. Both CLA and PPARγ agonist decrease BrdU incorporation but with different time courses. C: The apoptotic rate in control and treated cell evaluated as nucleosome concentration. CLA increased nucleosome concentration, showing maximal activity at 24 h, while PPARγ agonist effect was evident at 72 h. Western blot analysis shows a reduction of the caspase 3 inactive form and bcl-2 and an increase of caspase 9 levels upon both treatments. Data are mean ± SE of five experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

CD133 expression decreased, cells did not form neurospheres but adhered to the substrate, suggesting that treatments induced towards a more differentiated phenotype.

The intermediate filament protein nestin has been detected in gliomas/glioblastomas showing expression levels correlating with the malignancy degree of brain tumors (Strojnik et al., 2007). In our experimental conditions, the strong downregulation of nestin upon both treatments clearly suggests the induction of a differentiation pathway.

Both intracytoplasmic fibronectin expression and the strial fibrillary pattern characteristic of the extracellular matrix fibronectin have been shown in human glioma cell lines (Enam et al., 1998). Moreover, strong interstitial fibronectin staining has been found in vivo, similar to that observed for human glioma cell lines grown in nude mice. We show that glioblastoma cells in primary culture produced and secreted fibronectin and that both treatments are able to decrease its production, thus suggesting that this event may be related to the decrease of invasiveness observed upon treatments.

We show that, upon treatments, the number of A2B5 positive glioblastoma cells in primary cultures increases, concomitant with a decrease in their invasiveness. This result is consistent with the reported dose-dependent inhibition of invasiveness by A2B5 expression in different cell lines derived

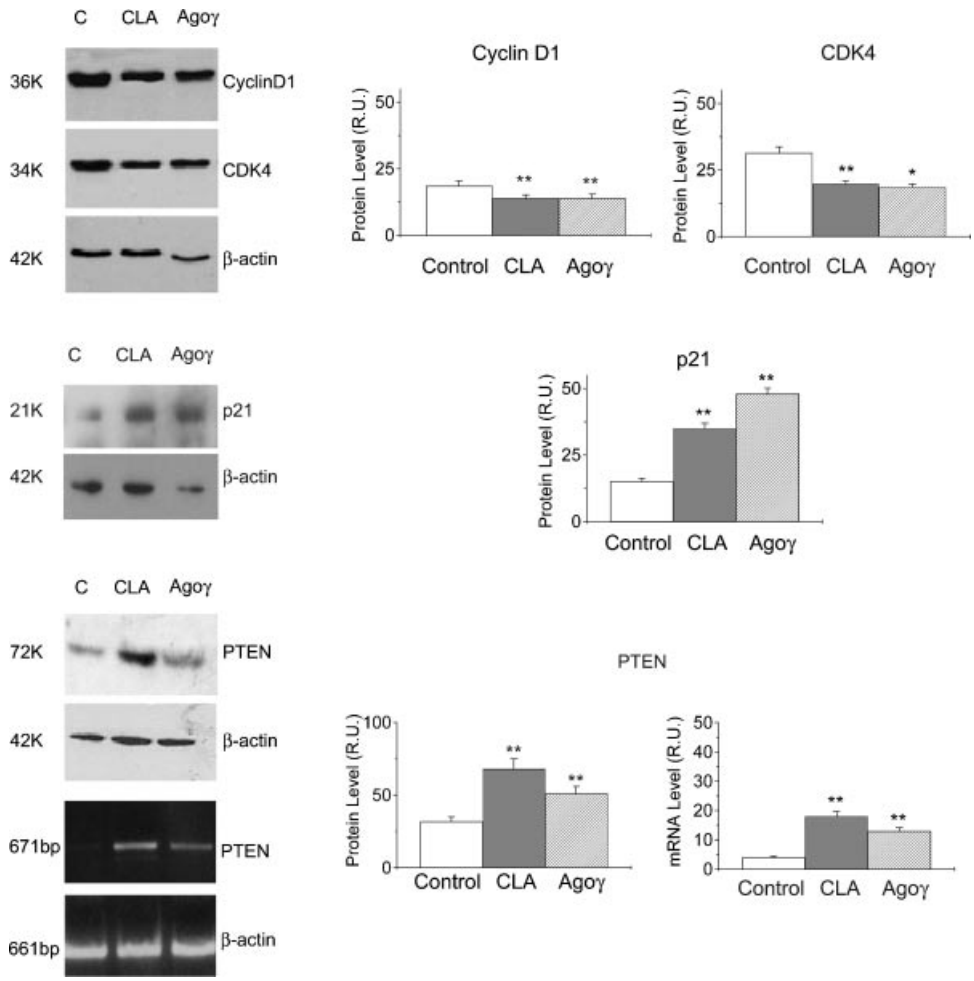

Fig. 5. Western blot and densitometric analyses of cyclin D1, CDK4, and p21 in control and treated cells. CLA and the PPARγ agonist decrease Cyclin D1 and CDK4 protein expression levels, whereas greatly increased p21 protein. Data are mean ± SE of five experiments. $^{**}P < 0.01$; $^{***}P < 0.001$. RT-PCR and Western blot analyses for PTEN show a strong increase after both treatments. Data are mean ± SE of five experiments. $^{**}P < 0.005$; $^{***}P < 0.001$.

from glial tumors of different histological grades (Merzak et al., 1994). It has been demonstrated that invasive behavior of glioblastoma cell lines is also associated with altered organization of the cadherin–catenin adhesion system (Perego et al., 2002).

In our experimental conditions, few glioblastoma cells were positive for β-catenin. Following treatments, the protein increased and appeared mainly localized at the cytoplasm and membrane levels, thus suggesting a reorganization of the junction/cytoskeletal interactions, also supported by the observation of more organized microfilament components.

Over the last few years, reports have described the involvement of cadherins in tumor invasion and metastasis. N-cadherin levels are important in the malignant behavior of gliomas, and may serve as a prognostic indicators for patients with high-grade gliomas (Asano et al., 1997, 2004). Our data indicate low N-cadherin levels in untreated glioblastoma cells, whereas a significant increase of N-cadherin is observed upon both treatments, correlating with the inhibition of in vitro migration and invasiveness.

CLA and PPARγ agonist suppress proliferation and induce apoptosis in primary cultures of glioblastoma cells. Consistently with growth inhibition, both ligands downregulate cyclin D1 and CDk4 protein levels, while inducing the transcription of the tumor suppressor gene PTEN. Recently, a critical role of PTEN lipid phosphatase activity in stabilizing the E-cadherin junctional complexes and reversing invasiveness has been reported (Kotelevets et al., 2001). Moreover, a direct implication of PTEN in stabilizing adherent junctions and suppressing invasiveness has been reported (Kotelevets et al., 2005). Our findings show that PPARγ activators stimulate PTEN transcription as well as inhibit invasiveness of glioblastoma cells, and are consistent with the presence of PPREs in the PTEN promoter (Patel et al., 2001) and with the involvement of PTEN in controlling migration and invasiveness.

Besides being utilized for glioma histological characterization, astroglial marker GFAP is also recognized as an indicator of glioma differentiation since its expression increases upon several anticancer drug treatments (Chen et al., 2006). In our model, we observed a significant increase of GFAP protein levels as well as the acquirement a more differentiated phenotype upon treatment with PPARγ agonists, indicating that activated-PPARγ induces differentiation of glioblastoma cells.

Taken together, our results strongly indicate that the PPARγ natural and synthetic ligands exert differentiation effects on these cells both by promoting the expression of differentiation markers, the increase of apoptosis and by decreasing proteins related to cell cycle progression, migration and invasiveness.

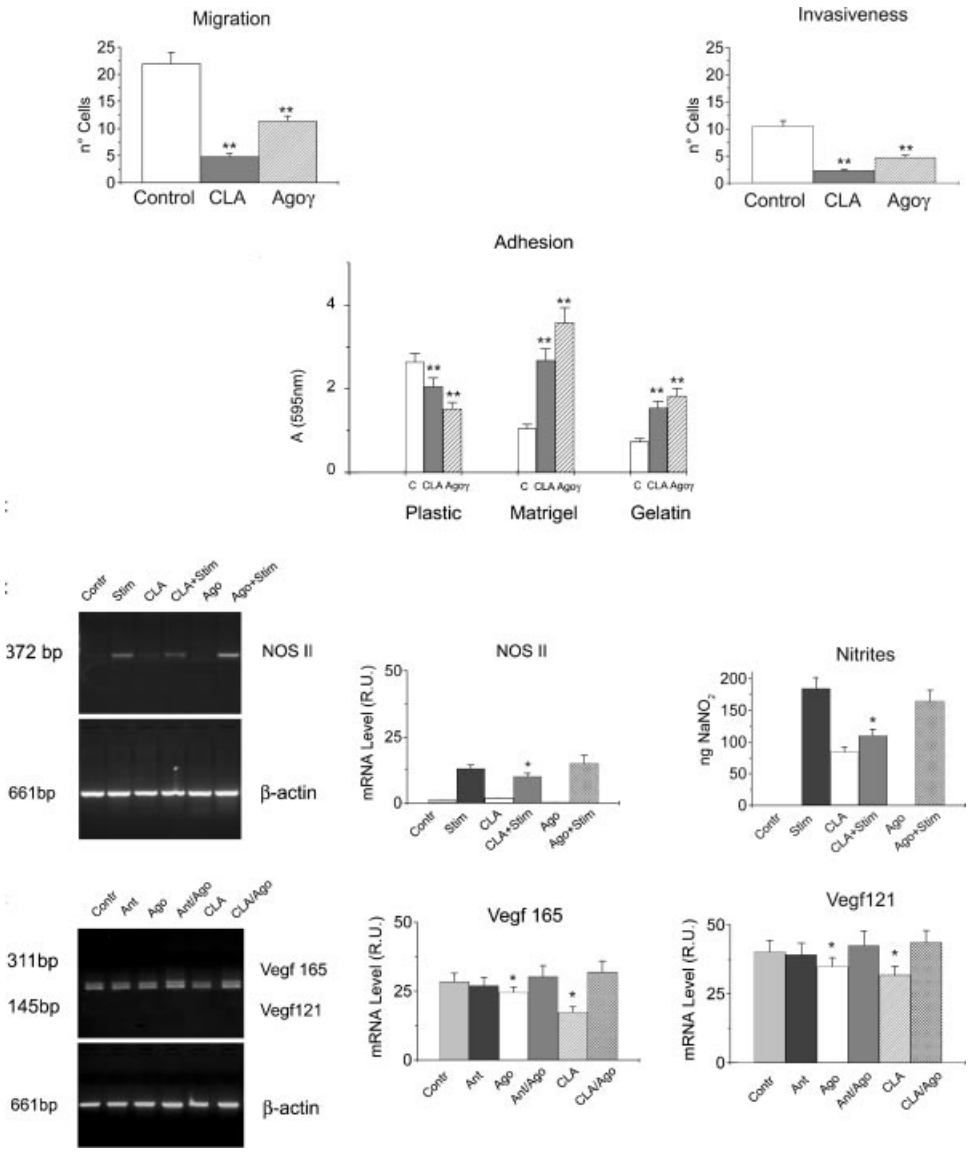

Fig. 6. Cell adhesion, migration and invasiveness in control and treated cells. CLA and the PPARγ agonist affect cell substrate interactions enhancing adhesion. Both treatments inhibited cell motility and invasiveness. Data are mean ± SE of five experiments. $^{**}P < 0.01$; $^{***}P < 0.001$. RT-PCR analysis of control and treated cells show that proinflammatory cytokines and LPS treatment induced NOS II expression in primary cultures glioblastoma cells. CLA, but not the PPARγ agonist, reverted such biological effect. The nitrate/nitrite levels were down modulated by CLA, but were not modified upon PPARγ agonist treatment. RT-PCR analysis for VEGF 165 and 121 isoforms shows a decrease by both treatments, being CLA more effective. The effect was reverted by PPARγ antagonist. Data are mean ± SE of five experiments. $^{**}P < 0.005$; $^{***}P < 0.001$.

Gliomas express NOS II in vivo; in our experimental conditions, we show that glioblastoma cells in primary culture do not express NOS II, strongly suggesting that the in vivo environment is crucial for NOS II expression. Upon proinflammatory challenge, glioblastoma cells express NOS II. CLA co-treatment limits the effect, whereas the synthetic PPARγ agonist does not. Conceivably, NOS II is not likely to be modulated by PPARγ, and CLA may affect its expression through different pathways.

In other experimental models, it has been demonstrated, that PPARs—particularly the γ isotype inhibit primary tumor growth and metastasis by inhibiting angiogenesis, through VEGF downregulation (Xin et al., 1999; Panigrahy et al., 2002). In agreement, both CLA and PPARγ agonist treatments led to a significant decrease of the VEGF isoforms, thus indicating that even in glioblastoma PPARγ is able to inhibit the angiogenetic pathways.

In conclusion, in this article we focused on the antineoplastic effects of PPARγ agonists in an in vitro model system more closely resembling the human glioblastoma natural environment, than cell lines. Our data clearly indicate that PPARγ natural and synthetic ligands promote a combination of growth inhibitory, pro-apoptotic, antiangiogenetic, and cell differentiating effects on glioblastoma cells, suggesting that PPARγ agonists might be considered as novel therapeutic drugs for patients affected with malignant gliomas. Specifically, CLA, a natural occurring fatty acid, which is known to cross the blood brain barrier (Spector, 1988), appears a suitable candidate as chemopreventive agent to be used in the near future, as it would not require clinical trials, being already used on humans. On the

other hand, the γ agonist GW347845, once experimented and validated on man, may represent a potentially useful adjuvant in glioblastoma therapy and in the prevention of recurrencies.

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