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Histone deacetylase inhibitor 4-phenylbutyrate modulates glial fibrillary acidic protein and connexin 43 expression, and enhances gap-junction communication, in human glioblastoma cells

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Abstract

Human glioblastoma cell cultures were established and the expression of glial fibrillary acidic protein (GFAP) and the gap-junction protein connexin 43 (Cx43) was confirmed by Western blot. Following treatment with 4-phenylbutyrate (4-PB), increased concentrations of non-phosphorylated GFAP were seen, while phosphorylated isoforms remained intact. Immunocytochemical staining of glioblastoma cells revealed an intracellular redistribution of GFAP. In addition to cytoplasmic immunostaining, GFAP immunoreactivity was also associated with the nucleus and/or the nuclear membrane. Phosphorylated and non-phosphorylated Cx43 proteins were increased 2- to 5-fold following 4-PB treatment, and were redistributed to areas of the cell surface, participating in cell-to-cell contacts. In addition, functional gap-junction coupling was amplified, as indicated by increased fluorescent dye transfer, and elevated levels of Cx43 protein were detected in parallel with enhanced gap-junction communication. Induced cell differentiation, with improved functional coupling of tumour cells, may be of importance for therapeutic strategies involving intercellular transport of low molecular-weight compounds.

Keywords: Connexin 43; Gap junction; GFAP; Malignant glioma; Immunocytochemistry; Phosphorylation; Primary culture; Western blot

1. Introduction

Intercellular communication is of paramount importance in the multicellular organism, allowing adaptation to an ever-changing environment by the co-ordinated responses of adjacent groups of cells to external stimuli. This type of co-ordination is mediated by gap junctions, permitting small water-soluble substances (<1000 Da) such as ions, second messengers and small metabolites to be exchanged between cells without secretion into the extracellular space. Thus gap junctions are to a large extent involved in the regulation of cell homeostasis, proliferation and differentiation [1].

Gap junctions are proteinaceous channels consisting of two semichannels, connexons, each belonging to one of the two plasma membranes of the cells participating in the contact. Each connexon consists of six multimeric protein subunits termed connexins. Connexins make up a protein family with at least 13 members [2], in which connexin 43 (Cx43) is the most ubiquitously expressed and is commonly found in nervous tissue and heart muscle [3]. The mechanism(s) of gap junction-mediated intercellular transfer are thought to be dependent on passive diffusion along a concentration gradient [4,5]. It is well known that gap junctions are involved in growth regulation and that the growth of many cells is inversely proportional to the extent of gap-junction communication (GJC). The proposed mechanism is the intercellular transfer of low molecular-weight growth regulators [6].

Aside from cellular mechanisms that control gross changes in the quantities of gap junctions and connexins, post-translational modification by phosphory-

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lation of Cx43 affects the functional status of the gap junction by changing its conformation. Phosphorylation of the COOH-terminal domain of Cx43 induces closure of the gap-junctional pore and disrupts channel permeability [7]. Compounds promoting Cx43 phosphorylation include oncogenes, growth factors and tumour promoters. Reduced expression of Cx43 has been demonstrated in numerous cancer cells [3]. Accumulated evidence indicates that connexins may function as tumour-suppressor proteins [1].

Previously, we demonstrated the presence of the gap-junction protein Cx43 in primary human glioblastoma cells in primary cultures and its distribution on the plasma membrane in conjunction with neighbouring cells [8]. We reported the ability of the glioblastoma cells to make new cell-to-cell contacts and their mediation of gap junction-dependent, fluorescent intercellular dye transfer. We also proved that GJC mediates the bystander effect of herpes simplex virus thymidine kinase (HSV*tk*) gene therapy in an *in vitro* model of glioblastoma cells [8].

The intermediate-filament protein, glial fibrillary acidic protein GFAP, is a cell type-specific marker for astroglial cells in the central nervous system. Like other intermediate-filament proteins, GFAP comprises an aminoterminal head domain, a central rod domain and a carboxyterminal head domain [9]. The assembly and disassembly of GFAP are regulated by its phosphorylation status. Protein phosphorylation affects the dynamic equilibrium of GFAP in the cell and breaks down the delicate cytoskeletal network [10]. Interestingly, increased concentrations of non-phosphorylated GFAP have been shown to suppress the growth of glial tumours [11].

Neoplastic transformation, characterised by inappropriate cell proliferation and/or altered patterns of cell death, does not necessarily destroy the cell's potential to differentiate and/or go into apoptosis under appropriate environmental conditions. One of the most effective agents to induce these processes are histone deacetylase (HDAC) inhibitors [12]. Histone acetylation is associated with the activation of gene transcription, while the opposite effect is mediated by HDAC [13]. *In vitro,* HDAC inhibitors cause cell-cycle arrest in the G1 and/or G2 phase, and apoptosis and/or differentiation in cultured, transformed cells. Several HDAC inhibitors can inhibit tumour growth in animal models with little toxicity [14–16]. However, the inhibition of HDAC is normally not sufficient to cause death in non-tumour cells. This selectivity for tumour cells makes these compounds particularly attractive for oncological therapy, and 4-phenylbutyrate (4-PB) is one of few HDAC inhibitors to have been tested on cancer patients in clinical trials [17].

Here we report on previously unknown effects of 4-PB on human malignant glioma cells, including the modulation of GFAP phosphorylation and of the expression and intracellular distribution of the gap-junction protein Cx43, with enhanced GJC.

2. Materials and methods

2.1. Tissue processing and primary culture

Tumour tissue specimens were obtained from patients during open surgical resection of glioblastoma multiforme (GBM; WHO grade IV) according to perioperative diagnosis on cryostat sections. Tissues were recovered with the permission of the ethics committee of Karolinska Institute and with the consent of the patient.

The three GBM cultures, hGBM-1, hGBM-5 and hGBM-14, used in this study were isolated and characterised as previously described [8]. Glioblastoma cells were cultured in DMEM/F12 culture medium supplemented with 10% fetal calf serum (Gibco) and penicillin/streptomycin (100 units/ml; Gibco).

2.2. Assessment of cell viability and cell proliferation by MTT assay

The proliferation of cultured glioma cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. MTT tetrazolium salt was dissolved in serum-free culture medium (0.5 mg/ml), added to the cells (150 μl/well) and incubated for 30 min at 37 °C. The formazan dye formed by viable cells was solubilised in 300 μl of isopropanol. Aliquots (100 μl) of the solutions were transferred to 96-well microplates. The absorbance at 570 nm (with reference at 650 nm) was measured with a microtitre-plate spectrophotometer (Anthos HT III). The results were expressed as percentage of viable cells compared to the control sample of untreated cells (100%).

2.3. Immunocytochemistry

Tumour cells cultured on laminin/poly-ornithine-coated glass chamber slides were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in phosphate buffer for 10 min. The cells were then washed in PBS three times and incubated with primary antibodies diluted in buffer (0.3% TX-100 (Sigma); albumin fraction V (Sigma); phosphate buffer, pH 7.0) overnight at 4 °C. Secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or Cy3 (Jackson Immunoresearch Laboratories) were applied for 1 h at room temperature, followed by rinsing with PBS. Bound antibodies were visualised by epifluorescence microscopy (Leica DMRB) and photomicrographs were taken with a Nikon F50 camera.

The following antibodies were used at specified dilutions: monoclonal anti-GFAP, 1:500 (DAKO), monoclonal anti-Cx43 (1:1000; Transduction Laboratories), and FITC (1:80)-conjugated secondary antibodies (Jackson Immunoresearch Laboratories).

2.4. Western blot

Primary glioblastoma cells were grown to 75% confluence and cultured for 48 h in the presence or absence of 4-PB (2, 5 and 10 mM), and then processed as previously described [8]. In brief, cell extracts containing equal amounts of protein (30 μg/lane for Cx43 detection and 60 μg/lane for GFAP detection) were loaded on to 10% polyacrylamide gels, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. A rabbit polyclonal antibody to Cx43, diluted 1:8000 (Sigma), or a mouse monoclonal antibody to GFAP, diluted 1:1000 (Pharmingen), were applied overnight at 4 °C. Detection was done with the enhanced chemiluminescence (ECL) kit (Amersham). The membranes were exposed to Hyperfilm-ECL (Amersham) for 1–5 min. For quantification, films were scanned and analysed with *Image Gauge* (version 3.12; Fuji Photo Film Corp.). Data were analysed using the program *Sigma Plot for Windows* (version 6; Jandel Corp.). All results are expressed as mean ± SEM for the indicated number of experiments.

As an internal control for whether equal amounts of protein had been loaded on to the gel, the nitrocellulose membranes were stained with Ponceau S solution (0.2% Ponceau S, 3% trichloroacetic acid, 3% sulphosalicylic acid) for 5 min. As the bands of protein became visible, the membranes were washed several times and the total amount of loaded protein was quantified by computerised densitometry.

2.5. Fluorescent dye transfer

Primary glioblastoma cells were cultured on 35-mm Petri dishes, labelled *in vitro* with 5 M calcein-AM (acetomethylic ester) and 10 M DiI (Molecular Probes) diluted in supplemented serum-free medium DMEM/F12, and incubated for 30 min at 37 °C. The cells were then washed once with the same medium, trypsinised and suspended in culture medium including 10% fetal bovine serum. The cells were then pelleted by centrifugation at 1600 rev/min for 1 min and resuspended in fresh culture medium. The procedure was repeated twice. Following the second resuspension, the labelled cells were plated on top of unlabelled cells of the same origin. These cells were either pretreated with 4-PB at a concentration of 5 mM or not treated, serving as a control. The labelled cells were then allowed to settle for 1 h, followed by scrutiny with epifluorescence microscopy every 30 min for up to 6 h. GJC were studied using an Olympus BX50WI microscope equipped with ultraviolet epifluorescence and red and green attenuating filters.

3. Results

3.1. Antiproliferative effects of 4-PB on glioma cells

The three cell cultures, hGBM-1, hGBM-5 and hGBM-14, were treated with 4-PB at various concentrations and assessed at various times using MTT test and phase-contrast microscopy. Fig. 1(A) shows the effect on cell proliferation in cultures treated with 2–20 mM 4-PB. The hGBM-1 culture showed significant sensitivity to 4-PB at 5 mM, while hGBM-5 and hGBM-14 were significantly affected at 10 mM 4-PB. We also performed longer treatments with a lower concentration of 4-PB (2 mM). Fig. 1(B–D) shows phase-contrast micrographs of hGBM-1 cells before treatment, at 1 day and at 10 days of treatment, respectively. Antiproliferative effects were seen after 24 h. In addition to 4-PB, other HDAC inhibitors were tested for their antiproliferative effects on these cell cultures. Splitomicin, trichostatin A, valproic acid and sodium butyrate were all shown to be less potent by MTT assay (Fig. 2).

3.2. Characterisation of cultured tumour cells

Tumour tissue specimens from patients undergoing surgical resection of GBM were used to establish long-

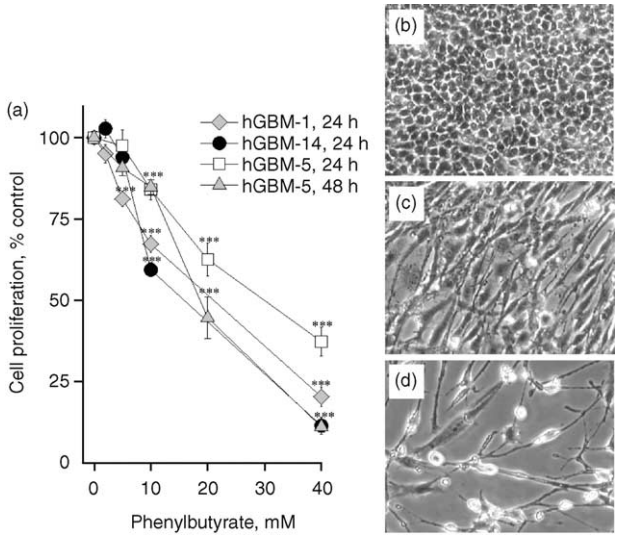

Fig. 1. Antiproliferative effects of 4-phenylbutyrate (4-PB) on glioblastoma cultures. (A) Cultures were treated with 4-PB at the concentrations and times indicated, after which they were analysed by MTT test. (B–D) Phase-contrast micrographs of a glioblastoma culture (hGBM-5) untreated (B), or treated for 24 h (C) or for 10 days (D) at a concentration of 5 mM 4-PB. Statistical significance between means was assessed by Student *t*-test for unpaired values. $***P < 0.001$ relative to control untreated cells ($n = 4$).

term primary cultures. The gross morphology of the tumour cells varied between cultures but was sufficiently consistent within each culture. Most cells were bipolar with short processes and identified as tumour cells of glial origin by their consistent expression of GFAP (Fig. 3A–C). Immunostaining for GFAP was repeated in culture to verify that the glial phenotype of the tumour cells had been maintained. The selected cultures, hGBM-1, hGBM-5 and hGBM-14, remained stable morphologically and in their expression of glial markers (including GFAP, nestin and S-100) over time in culture.

3.3. Effect of 4-PB on tumour cell morphology and GFAP

Semiconfluent glioblastoma cell cultures were incubated with 2 mM or 5 mM 4-PB in complete culture medium. Within the first 48 h of treatment, the cells gradually changed their shape to become more elongated with multiple fine cytoplasmic extensions (insets Fig. 3). This morphological transition was faster and more extensive in cultures exposed to 5 mM than 2 mM 4-PB (data not shown). When analysed for GFAP expression by immunocytochemistry, cells of all three cultures showed more intense immunostaining by epifluorescence after treatment with 4-PB. All three cultures also showed a novel subcellular distribution of GFAP immunoreactivity in the presence of 4-PB: in addition to the normal cytoplasmic pattern, GFAP was also associated with the nucleus and/or the nuclear membrane (Fig. 3D–F). The ratio of nuclear:cytoplasmic GFAP immunoreactivity varied between treated cultures.

Western blot of GFAP revealed two distinct bands with slightly different M_r (Fig. 4). These bands were interpreted as representing phosphorylated and non-phosphorylated isoforms of GFAP. 4-PB treatment specifically upregulated the non-phosphorylated isoform in two out of the three human glioblastoma cell cultures, hGBM-5 and hGBM-14 (Fig. 4), while the concentration of the phosphorylated isoform remained stable.

3.4. Effects of 4-PB on Cx43 protein and its subcellular redistribution

The gap-junction protein Cx43 was detected in all three primary cultures by immunocytochemistry and by Western blot using a commercially available, polyclonal, anti-Cx43 antibody. Immunocytochemistry demonstrated the presence of Cx43 in the plasma membrane as a patchy cell-surface fluorescence with a fine granular appearance (Fig. 5). Following treatment with 5 mM 4-PB for 48 h, cells showed a marked increase of

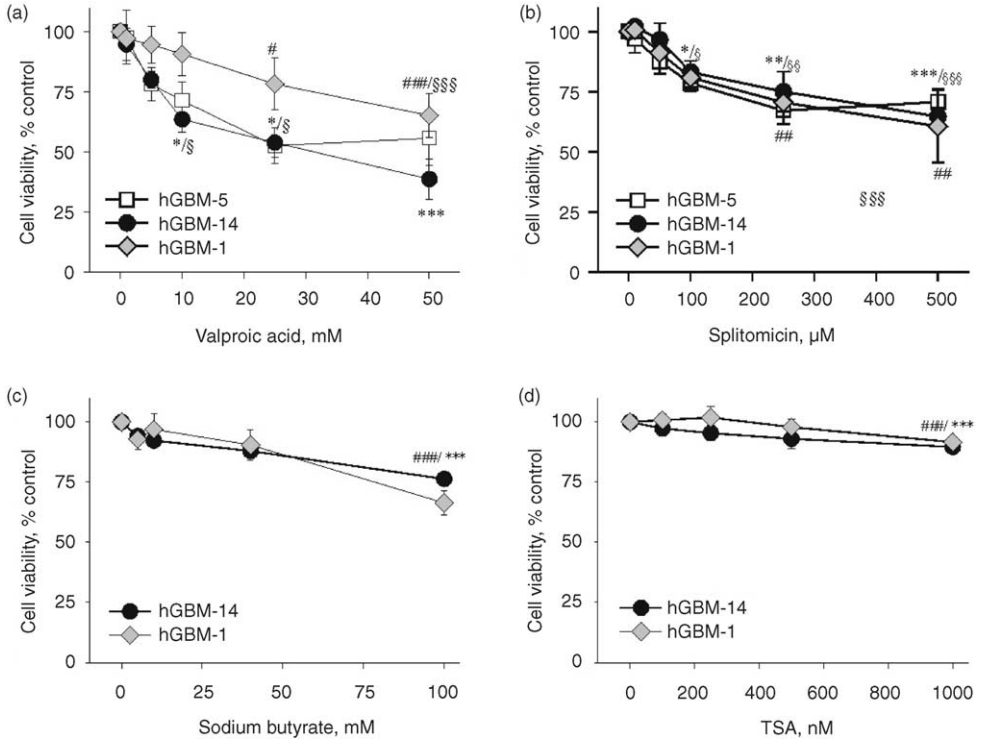

Fig. 2. Concentration-dependent effects of histone deacetylase inhibitors on cell viability shown by MTT test. Glioblastoma cells hGBM-1 (◆), hGBM-5 (□) and hGBM-14 (●) were cultured with valproic acid (A), splitomycin (B), sodium butyrate (C) and TSA (D) for 24 h. Data are expressed as a percentage of the untreated control cells±SEM, $n=4$. Statistical significance between means was assessed by Student t-test for unpaired values, $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$, relative to control untreated hGBM-14 cells; $^{\sharp}P<0.05$; $^{\sharp\sharp}P<0.01$, $^{\sharp\sharp\sharp}P<0.001$, relative to control untreated hGBM-1 cells and $^{\S}P<0.05$; $^{\S\S}P<0.01$, $^{\S\S\S}P<0.001$, relative to control untreated hGBM-5 cells.

Cx43 immunoreactivity at their surfaces. The density of Cx43 immunoreactivity varied between cells, but there was now a distinct lateralisation of Cx43 immunoreactivity favouring areas of intercellular contact and cellular processes (Fig. 5).

Western blot of whole-cell extracts detected three immunoreactive isoforms of Cx43. These isoforms differed slightly in M_r and migrated as smaller, non-phosphorylated and larger, phosphorylated Cx43 isoforms (Fig. 6). Following treatment with 4-PB (2-10 mM), the concentrations of both non-phosphorylated and phosphorylated isoforms increased (Fig. 6).

3.5. 4-PB effects on gap-junction communication

In order to study GJC in the glioma cultures, cells were labelled with two fluorescent dyes, DiI and calcein. Cells were trypsinised, washed and co-plated with unlabelled cells of the same origin cultured and adherent to a Petri dish. Within 1 h, preloaded cells had settled and attached to the subconfluent monolayer of unlabelled cells. Since calcein is a water-soluble acetomethylic ester that appears green after intracellular esterase cleavage, any green fluorescence in DiI-negative cells must have originated from preloaded donor cells, indicating functional contacts between labelled and unlabelled tumour cells. This dye transfer was mediated by gap junctions located on very thin and slender processes projecting from dye-loaded donor cells (Fig. 7A,B) [8]. When recipient cells were cultured in the presence of 5 mM 4-PB for 48 h preceding dye transfer, the intercellular spread of calcein was markedly more efficient than in untreated cells (Fig. 7).

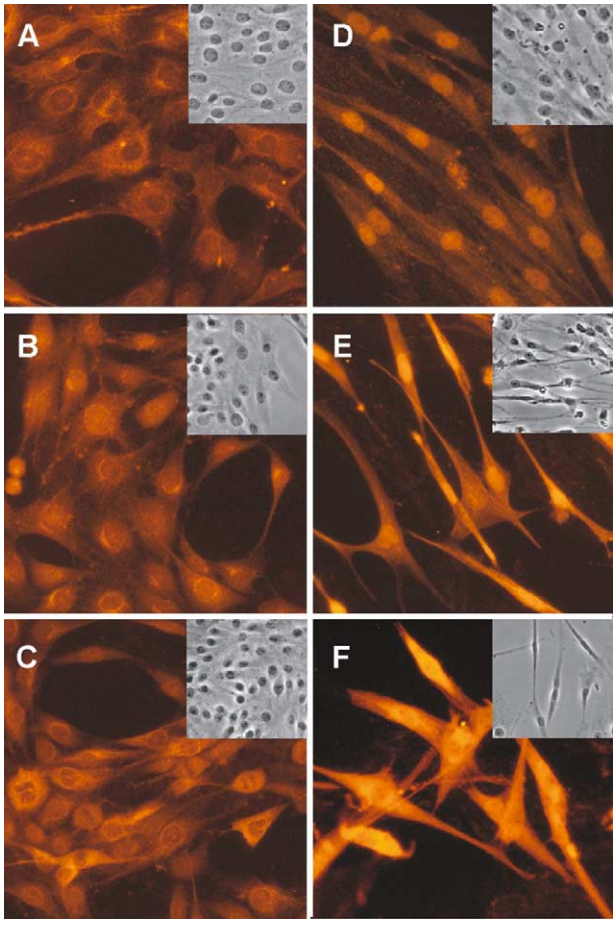

Fig. 3. Immunocytochemical analysis of glial fibrillary acidic protein (GFAP) expression in primary human glioblastoma cells. Glioblastoma cultures hGBM-1, hGBM-5 and hGBM-14 were immunostained for GFAP following treatment with 5 mM 4-PB for 48 h (D–F), compared to control cultures (A-C). Note the morphological differentiation of glioblastoma cells following 4-PB treatment as seen by GFAP fluorescence as well as in phase-contrast images (insets). GFAP immunodetection increased in parallel with the redistribution of GFAP to a nuclear/perinuclear pattern.

4. Discussion

We describe three previously unreported effects of 4-PB on primary human glioblastoma cells. First, non-phosphorylated GFAP was elevated in parallel with morphological differentiation of the cells. In addition to its usual cytoplasmic distribution, there was abundant GFAP immunoreactivity in the nucleus and in association with the nuclear membrane. Secondly, Cx43 expression increased and Cx43 immunoreactivity was redistributed to areas of cell-to-cell contact. Thirdly, intercellular transfer of fluorescent dye was enhanced, indicating improved gap junction-mediated intercellular communication.

The resistance of GBM to treatment is related to its cellular heterogeneity [18] and to the exceptional migratory nature of the tumour cells, which are able to diffusely infiltrate normal brain [19]. In parallel with preclinical research and clinical trials of various antineoplastic agents, it is important to explore new therapeutic paradigms, since only minor benefits of chemotherapy on overall survival have so far been reported [20].

HDAC inhibitors are potent regulators of gene expression through their effect on the acetylation of core histones; they thereby affect cell proliferation, differentiation and/or apoptosis, functions that are largely confined to transformed cells [21,22]. To evaluate the potential for differentiation and apoptosis in GBM cells, we chose to use the HDAC inhibitor 4-PB. We also tested other HDAC inhibitors for their antiproliferative effects, but 4-PB was the most potent. This compound can affect differentiation in transformed cells and has a well-documented pharmacokinetic profile [15], benign tolerability and promising antineoplastic effects [23,24]. Furthermore, 4-PB can be administered orally and is well absorbed via the gastrointestinal tract. Side-effects are reversible, mild to moderate, and without myelotoxicity.

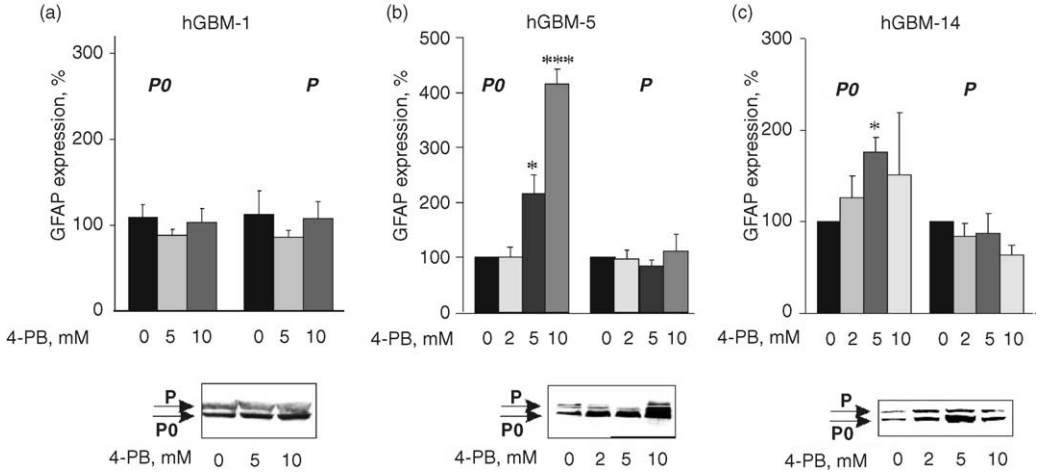

Fig. 4. Immunodetection of glial fibrillary acidic protein (GFAP) in human glioblastoma primary cultures by Western blot in the presence and absence of 4-phenylbutyrate (4-PB). Glioblastoma cells were recovered and processed for Western blot of GFAP expression in protein extracts (60 μg/lane). Anti-GFAP-positive proteins correspond to unphosphorylated (P0) and phosphorylated (P) species of GFAP. (A), (B) and (C) show the relative content of P0 and P forms of GFAP in the absence and in the presence of increasing concentrations of 4-PB in hGBM-1, hGBM-5 and hGBM-14, respectively. Note the increase of non-phosphorylated forms of GFAP in hGBM-5 and hGBM-14 when treated with 4-PB. Statistical significance between means was assessed by Student *t*-test for unpaired values. $*P < 0.05$ relative to control untreated cells ($n = 4$).

4-PB distributes to the cerebrospinal fluid after intravenous administration in non-human primates [25]. In malignant gliomas, the blood-brain barrier is defective at the site of the tumour, as visualised by iodine and gadolinium contrast enhancement in cranial computed tomograms and magnetic resonance images, respectively. This fact makes the penetration of 4-PB into the intracerebral compartment a realistic option in treating patients with hGBM. Since the 1980s substantial clinical experience with 4-PB has been acquired by treating children with urea-cycle disorders with large doses over extended periods of time [26].

In studying the effects of 4-PB on GBM cells, we made several novel observations about cell-cycle regulation and gene expression. While GFAP immunoreactivity is increased by 4-PB [27], we show that non-phosphorylated GFAP was specifically increased by 4-PB in two of our three GBM cultures. All three cultures also displayed intense GFAP immunoreactivity in or around the nucleus as well as in the cytoplasm.

4-PB arrests the cell cycle and causes significant morphological changes in glioblastoma cells, as reflected by the reorganisation of the intermediate filament GFAP. Intermediate-filament GFAP forms the cytoskeletal framework of glioblastoma cells and is also present in their nuclei as the major component of the nuclear lamina. We found that most glioblastoma cells exhibited GFAP immunofluorescence, albeit with variations in intensity. The diameter of the cell bodies was larger than the length of their processes. In contrast, the intensity of immunofluorescence in cells grown in the presence of 4-PB was much increased, as was the length of radially extending processes. Interestingly, GFAP immunoreactivity was redistributed within the glioblastoma cells following 4-PB treatment; immunostaining associated with the nucleus and nuclear membrane increased. The overall impression was an increased GFAP expression induced by 4-PB, which was confirmed by Western blot.

Reorganisation of the cytoskeleton is one of the distinct changes that occur during mitosis. The expression of intermediate-filament GFAP, known to be reorganised dramatically during mitosis, varies in a cell- and differentiation-dependent manner. *In vitro* the assembly state of purified GFAP is influenced by the ionic strength and pH of the medium, the presence of Mg^{2+} and Ca^{2+} [28], as well as by the phosphorylation status of the protein [9]. We detected both phosphorylated and non-phosphorylated isoforms of GFAP, and only the non-phosphorylated isoform was upregulated following 4-PB treatment.

Phosphorylation is particularly important in preparing the cytoskeleton for cell division. Site-specific phosphorylation of GFAP not only plays a fundamental part in mitosis but also regulates polymerisation and the dynamic equilibrium between the polymerised and depolymerised states. Assembly of GFAP is regulated by phosphorylation-dephosphorylation of the head domain by altering its charge. Six phosphorylation sites are phosphorylated by cyclic AMP-dependent protein kinase, Ca^{2+}/calmodulin-dependent kinase II, protein kinase C, the cdc-2 kinase and Rho kinase, which leads to disassembly of the filament structure *in vitro* [29–31]. Five of these sites are located in the N-terminal head domain (Thr^{7}, Ser^{8}, Ser^{13}, Ser^{17}, Ser^{34}) and one in the tail domain (Ser^{389}). The turnover of

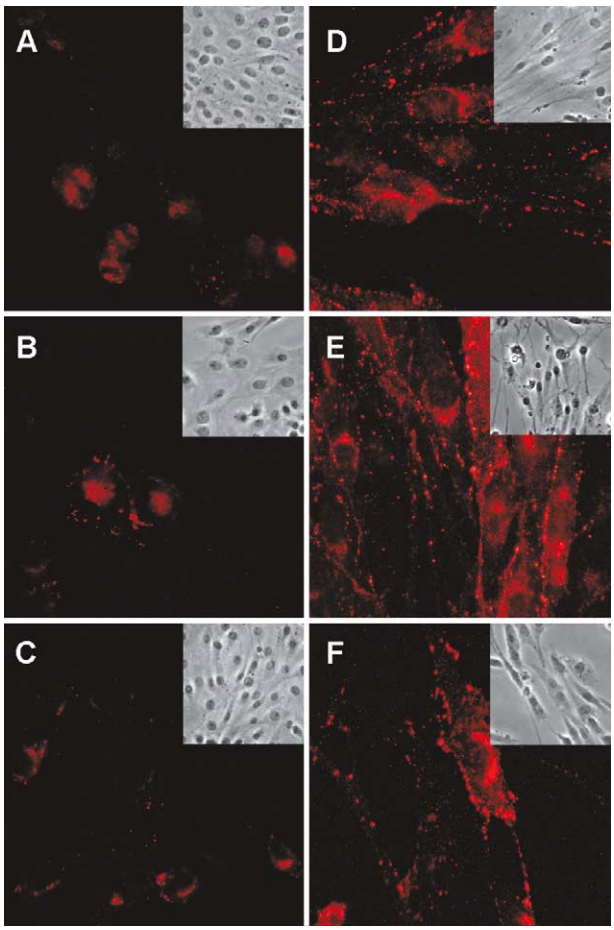

Fig. 5. Immunocytochemistry of connexin 43 (Cx43) and effects of 4-phenylbutyrate (4-PB) in human glioblastoma multiforme (GBM) cell cultures: Cx43 demonstrated in hGBM-1 (A), hGBM-5 (B) and hGBM-14 (C). Cells were allowed to grow in 4-PB for 48 h before fixation and immunostaining, which shows Cx43 expression and distribution on each of the three cultures hGBM-1 (D), hGBM-5 (E) and hGBM-14 (F). Note the marked increase of connexin 43 expression and redistribution to cytoplasmic processes shown in (D), (E) and (F).

protein phosphate in GFAP is very dynamic. While phosphorylation of residues is considered to occur on the intact filaments, the substrates of the dephosphorylation reaction are presumably disassembled subunits, such as dimers and tetramers [29].

The functional significance of the regulation of GFAP phosphorylation by 4-PB in glioblastoma cells may be associated with inhibition of cell-cycle progression and mitosis, and correlated with decreased proliferation of glioma cells. In our experiments, only the monomer was detected. Further investigation is required to understand the mechanism of this process and the effect of 4-PB on phosphorylation.

Major cell functions, including proliferation, differentiation, apoptosis and adaptive responses to cellular stress, are controlled by intra- and intercellular networks. These signals may either increase or diminish GJC, which is essential for maintaining homoeostatic balance through the modulation of cell growth and decay. Phenylbutyrate exerts prominent effects on cell proliferation and differentiation, which is why we investigated its effects on GJC and the gap-junction structural protein Cx43. We showed that 4-PB upregulates the expression of Cx43, which is reduced in high-grade human gliomas [1]. Upregulation of Cx43 potentially allows the restoration of GJC. Its restoration or upregulation is often associated with decreased proliferation, as was observed after treatment with 4-PB.

The function of the connexon as a regulator of intercellular communication is also modulated by phosphorylation, and we show that both the non-phosphorylated form of Cx43 (open gap junctions) as well as the phosphorylated form (closed gap junctions) were distinctly upregulated by 4-PB (Fig. 6). In order to respond to a mitogenic stimulus, i.e. a growth factor,

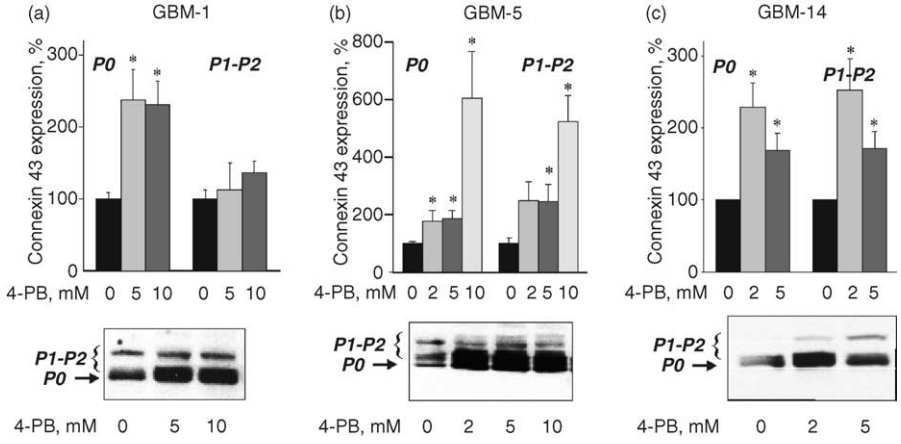

Fig. 6. Western blot of connexin 43 (Cx43) and effects of 4-phenylbutyrate (4-PB). Human glioblastoma primary cell cultures hGBM-1, hGBM-5 and hGBM-14 express phosphorylated isoforms of Cx43. Western blot of Cx43 expression in protein extracts (30 μg/lane) in the presence and absence of 4-PB in hGBM-1 (A) and hGBM-5 (B) and hGBM-14 (C) cells. Anti-Cx43-positive proteins correspond to native unphosphorylated (P0) and phosphorylated (P1-P2) (phosphorylated isoforms grouped together) species of Cx43. Note the marked increase of both Cx43 isoforms in the presence of 4-PB. Statistical significance between means was assessed by Student *t*-test for unpaired values. $*P < 0.05$ relative to control untreated cells ($n = 4$).

gap junctions have to be in the closed state, which makes proliferating cells resistant to growth regulating, mainly inhibitory, effects exerted by neighbouring cells. An upregulation of non-phosphorylated Cx43 might prevent these effects by keeping gap junctions in an open, and thereby functional, state [7]. A deletion mutant of Cx43, lacking the COOH-terminal, has been shown to keep the gap junction open [32], allowing inhibitory compounds from neighbouring cells to attenuate responses to mitogenic stimuli. These observations led us to the conclusion that a functional intercellular conduit could prove to be an effective inhibitor of cell proliferation.

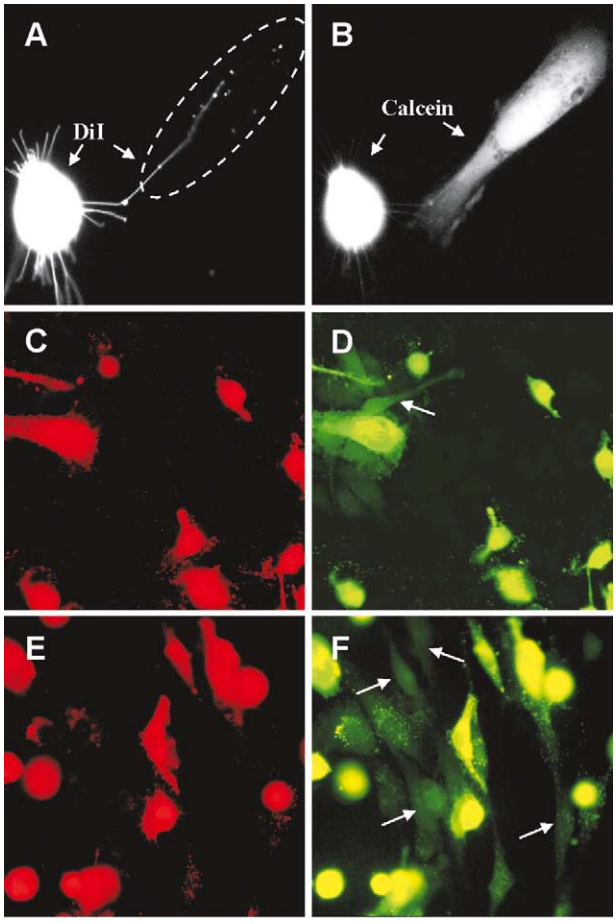

Fig. 7. Gap junction-mediated fluorescent dye transfer in the presence and absence of 4-phenylbutyrate (4-PB) visualised by fluorescence microscopy. Tumour cells (hGBM-1) were preloaded with DiI (red) and calcein (green) fluorescent probes and plated on top of unlabelled cells of the same culture. Labelled cells were allowed to settle. While DiI was retained in the preloaded cells, seen through the red filter (A), green calcein fluorescence, seen through the green filter, had spread to DiI-negative cells, indicating gap junction-mediated transfer (B). In a parallel experiment (C–F), cells preloaded with DiI and calcein were plated on top of unlabelled cells, which had been in contact with 4-PB for 48 h. After 6 h the cells were photographed through the microscope. (C) and (D) show the control experiment without 4-PB, similar to (A) and (B), while (E) and (F) show that a large number of formerly unstained cells had received calcein. By comparing (D) and (F) it is seen that 4-PB had a facilitating effect on gap junction-dependent calcein transfer. White arrows indicate examples of dye transfer.

Elevation of Cx43 protein following 4-PB treatment was demonstrated consistently, in all three GBM cultures studied, by the increased intensity and distribution of epifluorescence, and by a marked increase in concentration as measured by Western blot. It was also evident that Cx43 immunofluorescence in the glioma cells is redistributed, mainly to intercellular contact zones (Fig. 5). As a logical consequence of these observations, we found an increase in intercellular fluorescent dye transfer in 4-PB-treated cells, a sign of enhanced functional GJC.

Although Cx43 is in itself a powerful inhibitor of tumour cell growth and might possibly act as a tumour suppressor [1,33], its induction could be exploited in gene therapies. The main obstacle to efficient gene therapy is the difficulty of reaching a sufficient proportion of cells with the therapeutic gene or gene product. One way to investigate this problem *in vitro* is to enhance the bystander effect by transfection with the *Cx43* gene [34]. The bystander effect is an important mechanism in suicide gene therapy and is dependent on functional GJC [8,35]. HSV*tk*-mediated gene therapy is dependent on the spread of toxic phosphorylated ganciclovir from HSV*tk*-expressing cells to nearby non-expressing cells, causing their death [36]. It is known that recombinant expression of connexin proteins enhances the bystander effect in suicide gene therapy *in vitro* [34] and *in vivo* [35], presumably by upregulation of GJC. In order to relate the bystander effect to the presence of connexins and to cell killing, functional GJC in cultured cells can be assessed by fluorescent dye transfer [37]. Only cell lines sensitive to the bystander effect associated with the HSV*tk*/ganciclovir system show intercellular spread of fluorescent dye [38]. Presumably, 4-PB can mediate enhanced GJC *in vivo* and so might be used in conjunction with suicide gene therapies.

In conclusion, 4-PB is a low molecular-weight, non-toxic agent affecting several important functions of proliferating tumour cells, including inhibition of cell growth, induction of differentiation and apoptosis. Here we demonstrate its ability to increase non-phosphorylated GFAP and to change the subcellular distribution of, and upregulate, the Cx43 protein. Importantly, enhanced functional intercellular communication was also demonstrated. In this context, 4-PB could become a useful and important adjunct to multimodality treatments for malignant glioma.

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