Anti-tumor effects of progesterone in human glioblastoma multiforme: Role of PI3K/Akt/mTOR signaling

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A R T I C L E   I N F O

Article history:
Received 2 March 2014
Received in revised form 16 April 2014
Accepted 20 April 2014
Available online 28 April 2014

Keywords:
Progesterone
Glioblastoma
Tumor
Treatment

A B S T R A C T

Glioblastoma multiforme (GBM) is an aggressive primary brain tumor with a mean patient survival of 13–15 months despite surgical resection, radiation therapy and standard-of-care chemotherapy. We investigated the chemotherapeutic effects of the hormone progesterone (P4) on the growth of human GBM in four genetically different cell lines (U87MG, U87dEGFR, U118MG, LN-229) in vitro and in a U87MG subcutaneous xenograft mouse model. At high concentrations (20, 40, and 80 μM), P4 significantly (P < 0.05) decreased tumor cell viability in all cell lines except LN-229. This effect was not blocked by the P4 receptor antagonist RU468. Conversely, at low physiological concentrations (0.1, 1, and 5 μM) P4 showed a proliferative effect in all cell lines which was blocked by RU486. In nude mice, P4 (100 and 200 mg/kg) inhibited tumor growth significantly (P < 0.05) over 5 weeks of treatment and extended survival time of tumor-bearing mice by 60% without signs of systemic toxicity. P4 suppressed tumor vascularization as indicated by the expression of CD31, vascular endothelial growth factor and matrix metalloproteinase-9. Apoptosis in tumor tissue was detected by the expression of cleaved caspase-3, BCl-2, BAD and p53 proteins and confirmed by TUNEL assay. P4 treatment also suppressed PI3K/Akt/mTOR signaling, which regulates tumor growth, as demonstrated by the suppression of proliferating cell nuclear antigen. Our data can be interpreted to suggest that P4 suppresses the growth of human GBM cells both in vitro and in vivo and enhances survival time in mice without any demonstrable side effects.

This article is part of a Special Issue entitled ‘Sex steroids and brain disorders’.

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1. Introduction

Glioblastoma multiforme (GBM) is the most common and one of the deadliest malignant brain tumors in adults. Median survival is only about 13–15 months. In the United States, 12,000 new patients are diagnosed with GBM each year (http://www.cancer.gov/ncicancerbulletin/090412/page5). Interestingly, there is a sex difference in the onset of the disease: primary GBM develops more frequently in males than in females (3:1 ratio), while secondary GBM is commoner in females [1]. The Central Brain Tumor Registry of the United States estimated that 13,630 males and 10,990 females would be diagnosed with primary malignant brain and CNS tumors in 2013 in the United States (www.cbtrus.org).

No safe and effective treatment for GBM exists. The standard therapy is surgical resection followed by radiotherapy with concomitant chemotherapy with temozolomide (TMZ) and subsequent adjuvant chemotherapy with TMZ. GBM tumors recur virtually 100% of the time, and these recurrent tumors are much more difficult to treat, especially if the patient has already undergone the combination treatment with surgery, chemotherapy and radiation. Only two agents are currently used standard-of-care pharmacotherapies for GBM: Gliadel and TMZ, each of which separately increases survival by about 2 months [1–3], but both cause severe side effects. A recent randomized, double blind, placebo-controlled trial of bevacizumab for newly diagnosed GBM suggests that first-line use of bevacizumab does not improve overall survival of patients who had centrally confirmed GBM along with radiotherapy and daily TMZ treatment [4]. Moreover, the current standard treatment has several limitations: (1) GBM infiltrates adjacent brain parenchyma, so complete surgical removal of this tumor is practically impossible; (2) residual tumors can behave in a very aggressive and malignant fashion, producing substantial peri-tumoral brain edema, itself a major negative factor in GBM patients that contributes to their high morbidity and mortality [5]; (3) the maximum radiation dose that can be administered to the brain is rarely sufficient to completely eradicate the tumor [6];
(4) surgically acquired motor, language and cognitive deficits can clearly have a negative impact on the GBM patient's survival and quality of life [7,8]; (5) the brain itself presents a multitude of barriers to treatment, such as tumor location, accessibility for surgery, and the blood brain barrier's (BBB) natural protection against therapeutic agents. Thus, an optimal pharmacotherapy for GBM would: easily cross the BBB, reduce peri-tumoral edema, target multiple mechanisms of tumor growth and metastasis, selectively kill tumor cells only, and have no side effects on healthy tissue.

One promising therapeutic agent may be the neurosteroid progesterone (P4). P4 crosses the BBB rapidly and one of its key effects is to reduce the inflammation and cerebral edema that often accompany traumatic brain injuries (TBI) [9]. In over two decades of pre-clinical research, our laboratory has been studying P4 for the treatment of TBI and stroke in laboratory animals. P4 has multiple mechanisms of action and its safety and efficacy have been demonstrated in two independent phase II clinical trials for TBI [10,11]. The outcomes of two national and international Phase III multi-center trials using P4 in the treatment of TBI are being evaluated and the results should be known later this year (http://clinicaltrials.gov/ct2/show/record/NCT0082200 and http://www.synapse-trial.com/). The literature also supports the anti-proliferative and apoptotic effects of P4 on breast, endometrial, ovarian, colon and salivary gland tumors in vitro and in vivo [12–15]. Recently, we reported that high-dose P4 inhibits human neuroblastoma tumor growth in animal and cell culture models [16]. We found that high doses of P4 significantly decreased tumor growth, and did not induce any cell death in healthy primary cortical neurons and human fibroblasts. In nude mice, P4 inhibited neuroblastoma growth by approximately 50% after only 8 days of treatment, inhibited tumor cell proliferation and angiogenesis, induced apoptosis, and showed no drug toxicity.

Loss of phosphatase and tensin homolog (PTEN) and amplification of the epidermal growth factor receptor (EGFR) proto-oncogene are two genetic alterations seen in WHO grade IV GBM [17,18]. The EGFR gene is frequently mutated in GBM, resulting in a truncated ligand-independent EGFRvIII with constitutive activity. These changes/events are believed to enhance the angiogenic phenotype of glioma cells [19,20]. Therefore, in the present study, we evaluated the anti-tumor effects of P4 against the growth of grade IV human U87MG and U118MG (P4 mutant, p53 wild type), U87dEGFR (EGFRvIII mutant) and LN-229 (PTEN wild type, p53 mutant) cell lines in vitro, and in an in vivo subcutaneous xenograft (U87MG) mouse model of GBM. This model has been shown to be very useful and reproducible for chemotherapeutic studies [21–26]. In this report, we address the following questions: (1) Does P4 efficiently reduce GBM growth and enhance survival time in mice? (2) What is the most effective dose of P4? (3) What are the potential side effects of high doses of P4? (4) What are the mechanisms of action of P4's anti-tumor effects? We examined P4's effects on the expression of tumor vascularization markers matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), and CD31; a tumor proliferation marker (proliferating cell nuclear antigen (PCNA)); and apoptosis (cleaved caspase-3, Bcl-2, Bad, p53, TUNEL assay). Finally, we evaluated the effect of P4 on PI3K/Akt/mTOR signaling in tumor tissue.

2. Materials and methods

For the in vivo study, we calculated the starting sample sizes and power needed to reject the null hypothesis (H0) of no effects on tumor growth with a P-value of 0.05 at a power of 0.8, and determined that we needed 7 mice per group (see Section 2.6 for more details). We conducted two independent experiments with n = 8/group/experiment and pooled the data (n = 16) for the final analysis. All tumor volume measurement, drug treatment, and Western blot and immunohistochemical assays were performed independently by a researcher blinded to the experimental conditions (i.e., treatment versus vehicle alone). None of the animals that survived tumor inoculation and drug treatments were excluded from the experiments. The experiments were performed in accordance with the ARRIVE guidelines.

2.1. Cell culture

Human glioblastoma (U87MG, U118MG and LN-229) cell lines were purchased from ATCC (Manasses, VA). The genetically modified human glioblastoma cell line U87dEGFR, which stably expresses the EGFRvIII mutant form of EGFR, was received as a generous gift from Prof. Erwin Van Meir (Department of Neurosurgery, Emory University). Cells were grown in their respective culture media as suggested by the manufacturers at 37 °C in a 5% CO2 environment.

2.2. Experimental design for in vitro cell death studies

Tumor cells were seeded (0.5 × 10^5/well) in a 24-well plate and kept under starvation overnight prior to drug exposure. For all in vitro experiments, P4 (P3972, Sigma Chemicals, St. Louis, MO) stock was prepared in absolute dimethylsulfoxide (DMSO) and further diluted in culture medium. The final concentration of DMSO was kept at <5 μl/ml. Cells were exposed to different concentrations of P4 (0.1, 1, 5, 10, 20, 40, 80 μM) as repeated exposure for 3 and 6 days. For repeated exposure, culture medium was replaced daily and the P4 was added to cells every day. Cell viability was measured at days 4 and 7.

For the receptor study, we combined the P4 receptor (PR) inhibitor RU486 with P4 and tested it in U87MG and U87dEGFR cells as described earlier.

2.3. Experimental design for in vitro protein expression (p53, PTEN) studies

Tumor cells (U87MG, U87dEGFR, U118MG, and LN-229) were seeded (0.5 × 10^5) in petri dishes and kept under starvation overnight prior to drug exposure. Cells were exposed to different concentrations of P4 (0, 1, 5, 40 and 80 μM) for 24h. Protein was isolated using the RIPA lysis buffer system (sc-24948A, Santa Cruz Biotechnology) per manufacturer’s instructions. A western blot assay was performed to examine the effect of P4 on the expression of p53 and PTEN in different GBM cell lines.

2.4. MITT reduction assay

GBM cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The reaction is based on the cleavage of the tetrazolium ring of the pale yellow MTT into dark blue formazan crystals by mitochondrial dehydrogenase enzyme in viable cells. Formazan crystals accumulate within the cells due to their impermeability to the cell membrane and are then solubilized by adding DMSO (50 ml). The intensity of blue-colored formazan solution is directly proportional to the number of surviving cells. Concentrations were determined by photometric analysis. Briefly, 20 μl of MTT solution (5 mg/ml phosphate buffered saline (PBS)) was added per well and incubated at 37 °C for 4 h until a purple precipitate was visible. DMSO (500 μl) was added to solubilize the crystals and the absorbance was read at 570 nm.
2.5. Propidium iodide staining

Propidium iodide (PI) staining of GBM cell cultures was performed after repeated P4 exposure for 3 days as a qualitative measure of cell death as described previously [27]. Cells were incubated for 1 min with 0.02 mg/ml PI (stock solution 1 mg/ml, 1:50) in medium with gentle shaking and rinsed once with PBS. Conditioned medium was reapplied and phase contrast and fluorescent pictures were taken immediately with an inverse fluorescence microscope attached to a digital camera.

2.6. Experimental design for in vivo studies

The in vivo chemotherapeutic effect of P4 on U87MG xenografts was studied in adult male athymic nude mice (Hsd: Athymic Nude-Foxn1nu; Harlan, Indianapolis, IN). Protocols (204–2009) were approved by the Institutional Animal Care and Use Committee (IACUC), Emory University. U87MG cells (3 × 10⁶) were mixed 1:1 with Matrigel (BD Biosciences, San Jose, CA) and injected into the right flanks of the mice. The tumors were allowed to grow for 5 days after inoculation and before any treatments were applied. When tumor induction had been confirmed by visual analysis, tumor-bearing mice were randomly separated into three groups (n=16 each): Vehicle; P4 (100 mg/kg, P100); and P4 (200 mg/kg, P200). Two groups (n=6 each) of non-tumor-bearing mice served as drug controls for two different doses of P4 (100 and 200 mg/kg) and to assess drug toxicity based on survival, body weight and activity. We selected these doses of P4 on the basis of our previous work [15] where we used 50 and 100 mg/kg doses of P4 and observed similar effects on tumor growth. Thus, in addition to the 100 mg dose, we selected one higher dose (200 mg/kg) to test for a possible stronger cell death-inducing effect of P4 on GBM growth.

P4 was prepared in 30% 2-hydroxypropyl-beta-cyclodextrin (HBC) solution for in vivo experiments and a single subcutaneous injection of either P4 or vehicle alone was given daily at the same approximate time for 34 days. On day 35, all vehicle-treated mice were euthanized because they showed the maximum permitted tumor burden limit according to Emory IACUC guidelines. Eight mice from each P4-treated group were euthanized along with the vehicle group. The remaining 8 mice/group were kept alive for survival studies. The endpoint criterion for the survival study was the maximum tumor burden allowed by the IACUC, Emory University. These animals continued receiving P4 injections until they reached the maximum tumor burden limit.

2.7. Tumor measurement and tumor counting

Tumors were monitored by caliper measurement daily and tumor volume (V) was calculated using the formula: V = 0.4 × a × b² where 'a' was the largest tumor diameter and 'b' was the smallest tumor diameter [28]. At the endpoint of the study, we counted the number of excised tumors from each mouse in the different groups.

2.8. Activity scoring

The animals' activity was measured using the following scale: 0 = no activity, responsive to reflex; 1 = light movements/lethargic; 2 = moderately alert, moving around, no standing up; 3 = very active, highly alert, standing up [16].

2.9. Immunohistochemistry

Tumors were removed by excision under deep isoflurane anesthesia, weighed and then cut in half. Half of each tumor was then fixed in 10% buffered formalin and the other half was snap-frozen in liquid nitrogen for Western blot analysis. Samples were fixed for 24 h, embedded in paraffin, sectioned at 5 μm and immunostained for CD31 purchased from Dako (Carpinteria, CA). Sections were deparaffinized, rehydrated and treated with 3% hydrogen peroxide in distilled water for 20 min. The sections were incubated with blocking buffer (1% bovine serum albumin (BSA) in PBS) at room temperature (RT) for 30 min and then incubated with monoclonal mouse anti-human CD31, endothelial cell, clone JC70A (Dako) primary antibody for 1 h at RT. The sections were then washed three times in PBS and incubated with goat anti-mouse immunoglobulin G (IgG) labeled with Alexa Fluor 594 (f(ab) fragment (A-11071, Molecular Probes, Carlsbad, CA) for 1 h at RT, rinsed with PBS for 3 × 5 min, and then covered with a mounting medium with diaminodiphenylindol (DAP). The sections were examined with a fluorescence microscope and pictures were taken using Image-Plus software©.

2.10. TUNEL assay

Detection of apoptosis in GBM tumor tissue was done using an in situ cell death detection kit, fluorescein (11684795910; Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions.

2.11. Western blot analysis

Protein was extracted from tumor tissue using T-per extraction buffer (Pierce, Rockford, IL) with protease inhibitors and assayed for protein concentration by BCA (bicinchoninic acid assay) microplate protein assay (Pierce, 23225). Protein samples (50 μg) were separated under reducing and denaturing conditions by 4–20% acrylamide Criterion gel (BioRad, Hercules, CA) at 200 V for 1 h and transferred to a polyvinylidene difluoride (PVDF) membrane at 100 V for 30 min. The non-specific binding sites of the membrane were blocked with 5% non-fat dry milk in PBS-T (PBS containing 0.05% Tween-20). The membranes were probed with the following primary antibodies overnight at 4°C: VEGF (A-20), MMP-9(C-20), PCNA (PC-10), and Bcl-2(C-2) purchased from Santa Cruz Biotechnology; cleaved caspase-3 (Asp175), phosphor-Akt(Ser473), p53, and Bad purchased from Cell Signaling Technology (Danvers, MA); mTOR (Y391) from Abcam Inc. (Cambridge, MA); CD31 from Dako (A/S); and β-Actin (AC74) from Sigma. Membranes were then incubated in their respective horseradish peroxidase (HRP) – conjugated secondary antibodies. Blots were developed using a chemiluminescent substrate (Pierce) for 5 min. Chemiluminescent bands were detected on a Kodak autoradiography film in a darkroom and their densities were measured using NIH ImageJ software. In each figure, representative blot images were selected from the same gel.

2.12. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical significance was set at P<0.05. All in vitro data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett’s test. After a power analysis to determine sample size needed to observe a significant difference in tumor growth, the in vivo tumor data were analyzed using repeated measures ANOVA followed by LSD Post hoc test. A two-tailed unpaired t-test was used to analyze Western blot densitometry, tumor weight, tumor frequency and activity data. Analyses were calculated using SPSS™ 21.0 statistical analysis software (IBM, Armonk, NY).
3. Results

3.1. Effect of different concentrations of P4 on the viability of human GBM cell lines

U87MG, U87dEGFR, U118MG and LN-229 cell viability was evaluated by MTT assay. We observed significant (P < 0.001) cell death in U87MG, U87dEGFR and U118MG cells after 3 days of P4 exposure at high concentrations (10, 20, 40, and 80 µM), whereas lower concentrations of P4 (0.1, 1, and 5 µM) did not induce any cell death (Fig. 1A). After 6 days of exposure, this cell death-inducing effect was more pronounced than after 3 days in the U87MG, U87dEGFR and U118MG cell lines. At lower concentrations (0.1, 1, and 5 µM), we observed a significant (P < 0.05) cell proliferation effect of P4 during 6 days of exposure in U87MG, U87dEGFR and U118MG cell lines. Interestingly, P4 could not induce cell death in LN-229 cells at any concentration after 3 and 6 days of exposure. Also, in the LN-229 line we observed a non-significant cell proliferative response at different concentrations (Fig. 1A).

Fig. 1B shows a qualitative evaluation of cell death by PI-staining in different GBM cell lines following P4 exposures at high concentrations (20, 40, and 80 µM) for 6 days. A marked increase in PI-positive dead/dying U87MG and U87dEGFR cells was observed in P4-treated groups compared to the control group. No change in PI-positive LN-229 cells was observed in any of the P4-treated groups compared to the control group, suggesting that there was no cell death following repeated P4 treatment.

3.2. GBM cell death induced by high P4 concentrations is not PR-dependent

To evaluate whether P4-induced cell death in U87MG, U87dEGFR and U118MG cells is PR-dependent, we examined the effect of the PR-antagonist RU486 in combination with P4 on the viability of those cell lines (Fig. 2). All the cell lines showed a significant (P < 0.05) increase in cell viability/proliferation following repeated exposures to P4 alone at low concentrations (0.1, 1 and 5 µM) for 6 days. This proliferative effect was blocked when P4 was combined with RU486 at low concentrations. Conversely, RU486 could not block the cell death-inducing effects of high concentrations of P4 at 3 and 6 days in any tested cell line. Interestingly, RU486 alone showed a P4 mimetic effect at high concentrations in all cell lines, but when P4 and RU486 were combined, a marked enhancement of the toxic effect was observed.

3.3. Effect of P4 on the expression of p53 and PTEN

Densitometric analysis of Western blot data revealed a significant (P < 0.05) increase in the expression of tumor vascularization markers VEGF and MMP-9 in both P4-treated groups compared to vehicle (Fig. 5A and B). P4 at both doses showed similar inhibitory effects on VEGF and MMP-9 expression and no significant difference was observed. Immunohistochemistry revealed a marked decrease in CD31-positive cells in both P4-treated groups compared to vehicle (Fig. 4D). These data suggest that high-dose P4 treatment has an anti-angiogenic effect on GBM growth in vivo.

3.5. P4 inhibits GBM vascularization by modulating the expression of MMP-9, VEGF and CD31

Densitometric analysis of Western blot data revealed a significant (P < 0.05) decrease in the expression of tumor vascularization markers VEGF and MMP-9 in both P4-treated groups compared to vehicle (Fig. 5A and B). P4 at both doses showed similar inhibitory effects on VEGF and MMP-9 expression and no significant difference was observed. Immunohistochemistry revealed a marked decrease in CD31-positive cells in both P4-treated groups compared to vehicle (Fig. 4D). These data suggest that high-dose P4 treatment has an anti-angiogenic effect on GBM growth in vivo.

3.6. Apoptosis-inducing effects of P4 on GBM tumors in vivo

We evaluated the protein expression of cleaved caspase-3, Bcl-2, Bad and p53 in tumor tissue to determine whether P4 treatment induces apoptosis in GBM tumors. Densitometric data revealed a significant (P < 0.05) increase in cleaved caspase-3, Bcl-2 and p53 expression in both P4-treated groups compared to vehicle (Fig. 6A–C). A significant (P < 0.05) decrease in the expression of the anti-apoptotic protein Bcl-2 was observed in both P4-treated groups compared to vehicle (Fig. 6D). No significant difference in protein expression was observed between the two doses of P4. We also performed a TUNEL assay to confirm the apoptotic effects of P4 treatment in GBM tumors and observed a significant (P < 0.05) increase in TUNEL-positive cells in both P4-treated groups compared to vehicle (Fig. 6E). These findings suggest an apoptosis-inducing effect of high-dose P4 on GBM tumors in vivo.

3.7. P4 inhibits GBM cell proliferation and suppresses PI3K/Akt/mTOR signaling in vivo

Western blot data revealed a significant (P < 0.05) decrease in the expression of PCNA in tumor tissue in both P4-treated groups compared to vehicle (Fig. 7A). PI3K/Akt/mTOR signaling plays a critical role in cell proliferation and in blocking apoptosis in GBM cells [29]. Accordingly, we examined the effects of P4 treatment on phospho-Akt and mTOR expression in tumor tissue. Western blot data revealed that P4 at both 100 and 200 mg/kg doses suppressed PI3K/Akt/mTOR signaling as evidenced by a significant (P < 0.05) decrease in the expression of phospho-Akt and mTOR in both P4-treated groups compared to the vehicle group (Fig. 7B and C).
4. Discussion

4.1. P4 treatment induces cell death in grade IV GBM cell lines in vitro: possible role of p53

In this study, our in vitro data can be taken to demonstrate that P4 effectively inhibits the growth of several human GBM tumor cell lines in vitro. P4 also suppresses GBM tumor growth in a xenograft mouse model and enhances the survival time of tumor-bearing mice without showing any drug toxicity as measured by mortality, activity and body weight loss. We also observed that repeated P4 exposures at high concentrations kill U87MG, U87dEGFR and U118MG cells in a concentration-dependent manner. P4 did not show any cell death-inducing effect in LN-229 cells even at very high concentrations, suggesting that not all forms of GBM may be amenable to the salutary effects of high-dose P4 treatment.

At low concentrations, and as might be expected from a potent developmental hormone, P4 showed a proliferative effect in U87MG, U87dEGFR, U118MG and LN-229 cells. These findings are in agreement with our previous work showing that P4 at high concentrations is toxic to SK-N-AS neuroblastoma cells while it remains non-toxic to healthy primary cortical neurons and primary fibroblasts (HFF-1) at the same high concentrations [16]. In the present study we observed no difference in the normal activity of the tumor-bearing mice treated with high doses of P4, whereas mice from the vehicle-treated group showed a significant decrease...
Fig. 2. P4 induces cell death in GBM cells in a PR-independent manner. Effect of repeated exposures of P4, RU486 and their different combinations on the viability of U87MG, U87dEGFR and U118MG cells. Data are expressed as means ± SEM of three independent experiments. Significant difference: *P < 0.05 compared with control (vehicle) group.

Fig. 3. Effect of P4 on the expression of p53 and PTEN in GBM cell lines. Cells were exposed to different concentrations of P4 (0, 1, 5, 40, and 80 μM) for 24 h. Representative Western blot and densitometric analysis of the expression of (A) p53 and (B) PTEN in different cell lines. Data are expressed as means ± standard error of the mean (SEM) from two independent experiments. Significant difference: *P < 0.05 compared with the control “0 μM” group.
in activity compared to control mice, suggesting that P4 remains non-toxic in high doses. Taken together, our current early-stage findings indicate that P4 differentiates between normal healthy cells and tumor cells and specifically induces cell death in tumor cells only, while remaining nontoxic or protective for normal cells. The exact mechanism for this property needs to be explored further.

In contrast to its effects on U87MG, U87dEGFR and U118MG, P4 was ineffective in inducing cell death in the LN-229 cells even at very high concentrations. This finding provides an important piece of information regarding one of the possible mechanisms of P4-induced cell death in GBM cells. Here we propose that the involvement of the p53 tumor suppressor gene could be an important mechanism in mediating the effects of P4. Genetic comparison of U87MG and LN-229 cell lines suggests that both are deleted for p16 and p14ARF tumor suppressor genes. U87MG and U118MG are the wild type for p53 and mutant for PTEN, whereas LN-229 is mutant for p53 and wild-type for PTEN [30]. Therefore, our finding that P4 treatments induced significant cell death in U87MG but not in LN-229 suggests that the p53 tumor suppressor gene is involved. Also, P4 action may not be mediated through PTEN, since U87MG cells are mutant for PTEN while LN-229 is mutant for p53. In contrast, studies on the expression of PTEN and p53 in different GBM cell lines also support this notion. Here, we observed that P4 at high concentrations increased the expression of p53 in U87MG, U87dEGFR and U118MG cells but not in LN-229. Also, PTEN expression was unaffected in all the cell lines after P4 exposure. It has been proposed by others that P4 strongly down-regulates expression of the anti-apoptotic gene Bcl-2 and up-regulates expression of the apoptotic gene p53 at both the transcriptional and protein levels in breast cancer cell lines [13]. This hypothesis may be correct, but further mechanistic studies with transient transfection or siRNA techniques are needed to define the specific relationship of PTEN and p53 with P4 in inducing cell death in GBM cells.

4.2. Non-genomic action of P4 at high concentrations in GBM cell lines

We also examined the role of the PR in P4-induced cell death in GBM cells. We combined different concentrations of P4 with RU486 and then tested the effects in U87MG and U87dEGFR cells. We noted that the cell-proliferative effect of P4 at low physiological concentrations is PR-dependent, since RU486 blocked this effect in combination with P4. However, P4 at non-physiological, high concentrations induces cell death in GBM cell lines in a PR-independent manner and RU486 could not block this effect when given in combination with P4. We take our data to suggest that P4 acts through genomic effects at low concentrations, and through non-genomic effects at high concentrations. Further, the cell death-inducing effect of P4 at high concentrations is not only PR-independent but also glucocorticoid receptor (GR) -independent, since RU486 also blocks the action of the GR. P4 and its metabolites can act at a number of different receptor sites independently of the intranuclear receptor [31]. We and others have previously reported such concentration-dependent, non-genomic effects of P4 [16,32,33]. Interestingly, at high concentrations RU486 alone showed a P4-mimetic effect in both cell lines, and this toxic effect was more pronounced when P4 and RU486 were combined. Our findings are in agreement with a recent study that demonstrated cell death-inducing effects of RU486 in different GBM cell lines that do not express the PR [34]. We have also shown cell death-inducing effects of RU486 alone and in combination with P4 in a neuroblastoma cell line in vitro [16].

On the basis of these findings, it might be argued: why not use RU486 instead of P4, or use them in combination? We suggest opting for P4 because of safety issues surrounding RU486. P4 selectively induces cell death in tumor cells at high concentrations but remains safe in primary cortical neurons and in human primary fibroblasts at the same high concentrations [16]. RU486 is toxic [35]
Fig. 5. P4 modulates GBM tumor vascularization. Representative Western blot and densitometric analysis of the expression of vascularization markers (A) VEGF and (B) MMP-9 in different groups. (C) Tumor tissue sections were fluorescence-stained with anti-CD31 antibody (endothelial marker) and counter-stained with DAPI. Representative photomicrographs (20×) of different groups are shown. Data are expressed as means ± standard error of the mean (SEM) from two independent experiments (n = 8 mice/gp each). Significant difference: *P < 0.05 compared with the vehicle group.

4.3. P4 suppresses GBM tumor growth and enhances survival time in nude mice

We next tested P4’s anti-tumor effects in a subcutaneous xenograft mouse model using the U87 MG cell line. We tested two high doses of P4 on the basis of our in vitro data and our previous findings on neuroblastoma [16]. Our data demonstrate that P4 exposure significantly reduced U87 MG tumor growth in the mice at both 100 and 200 mg/kg doses. No significant difference in efficacy between the two doses was observed. Instead, we found a significant decrease in tumor weight and frequency in P4-treated groups at both doses compared to vehicle-treated mice. P4 at both doses enhanced the survival time of tumor-bearing mice by 60% compared to vehicle-treated mice and did so without showing any toxic side effects that we could observe. These findings strongly suggest the possibility of using P4 in the treatment of human GBM to inhibit tumor growth and extend survival time. To the best of our knowledge, we are the first to report an anti-tumorigenic effect of high-dose P4 against human GBM xenografts of U87MG origin.

4.4. P4 targets hallmarks of GBM development

Human tumor development is a very complex process. According to Hanahan and Weinberg [36], the hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Therefore we examined the effect of P4 treatment on markers of vascularization/angiogenesis (MMP-9, VEGF and CD31), tumor proliferation (PCNA), and apoptosis (cleaved caspase-3, Bcl-2, Bad, p53, TUNEL assay) in tumor tissue extracted from different groups as possible mechanisms of P4’s action against GBM.

4.4.1. P4 modulates GBM tumor vascularization

GBM is characterized by the high expression of VEGF and it has been suggested that levels of VEGF and its receptor correlate with the histologic grade of GBM [37]. MMPs mediate degradation of the extracellular matrix, thereby allowing GBM migration [38]. Several studies have reported high levels of MMP-9 in human GBM tissue [39,40]. MMP-9 overexpression was reported to be associated with slightly shorter overall survival in 163 glioblastoma patients [41]. In this study we observed high expression of VEGF and MMP-9 in our vehicle-treated group. In our hands, P4 treatment at both doses reduced the expression of VEGF and MMP-9. We speculate that P4 reduces tumor growth by depriving it of vascular support. To confirm this notion we examined the expression of an endothelial marker, CD31, and found an inhibitory effect of P4 on CD31 expression compared to the vehicle group, a result in agreement with our previous findings on the inhibitory effect of P4 on VEGF, MMPs and CD31 in human neuroblastoma tumors [16].

even at very low concentrations (0.1 and 1 μM) and non-selectively kills healthy primary cells [16].
4.4.2. P4 suppresses GBM tumor proliferation and induces apoptosis

P4 treatment significantly decreased cell proliferation and induced apoptosis in GBM tumor tissue. P4 decreased the expression of PCNA and Bcl-2 and enhanced the expression of cleaved caspase-3, Bad and p53, suggesting an induction of apoptosis in GBM tissue which was further confirmed by TUNEL assay. Previous studies show that P4 has anti-proliferative and apoptosis-inducing effects in other types of tumors in addition to GBM in vitro and in vivo [15,16,42].

4.4.3. P4 modulates PI3K/Akt/mTOR signaling

We also examined the role of PI3K/Akt/mTOR signaling in P4’s anti-proliferative and apoptosis-inducing effects in GBM tumor tissue. PI3K/Akt/mTOR signaling is highly active in GBM, plays a central role in cell survival, proliferation, and angiogenesis, and is frequently deregulated in human cancer [43]. The PI3K/Akt/mTOR pathway has recently attracted attention because it can promote cell survival even after ionizing radiation therapy, which works on the principle that damage to the DNA of cells leads to apoptosis and thus to tumor regression. It has been reported that the limited effectiveness of TMZ in GBM cells is associated with its inability to induce substantial levels of apoptosis [29,44,45], which in turn has been attributed to constitutive activation of PI3K/AKT/mTOR signaling [29]. PI3K/Akt/mTOR activation has also been reported to suppress TMZ-induced senescence and mitotic catastrophe in cells that avoid G2 arrest, leading to TMZ resistance [46]. In this study we observed high levels of phospho-Akt and mTOR expression in our vehicle group, suggesting a high proliferation rate in tumors which is supported by the high level of PCNA expression and low levels of apoptotic protein expression in this group. Conversely, with P4 treatment, the levels of phospho-Akt and mTOR expression were significantly decreased at both doses. This confirms our observation of decreased levels of PCNA and increased expression of apoptotic proteins in P4-treated groups. Overall, our findings demonstrate a possible role of PI3K/Akt/mTOR signaling in P4’s antitumor effects in GBM of U87MG origin. We have previously reported that Akt signaling may have a role in...
P4's antitumor effects in human neuroblastoma [16]. For future studies, it would be interesting to use specific inhibitors of Akt and mTOR to determine whether P4 exerts its anti-tumor effects through PI3K/Akt/mTOR signaling in a mouse model of GBM.

4.4.4. Are membrane P4 receptors involved in PI3K/Akt/mTOR signaling modulation by P4?

Our in vitro data show that the cell death-inducing effect of P4 at high concentrations is not PR-dependent, whereas the prolife- rative effect of P4 at low concentrations is PR-dependent. P4 also suppresses PI3K/Akt/mTOR signaling in tumor tissue in vivo. Now the question arises: How does P4 affect PI3K/Akt/mTOR signaling through non-genomic action? The classical genomic mechanism of P4 proposes that P4 exerts its effects through the classical nuclear PR which binds to specific P4 response elements within the pro- moter region of target genes to regulate gene transcription [47]. However, P4 has also been shown to act via non-genomic mecha- nisms which may be mediated by distinct PRs, such as the recently discovered membrane-bound PRs (mPRs) and the P4 membrane receptor component (PCMRc). Both m-PRs and PCMRc are widely expressed in the brain, but their functions relevant to P4 effects in the CNS require further elucidation. In humans, three mPR gene isoforms – mPRα, mPRβ, and mPRγ – have been identified [48]. After binding to P4, mPRs rapidly activate G-proteins and their downstream signaling pathways including PI3K/Akt signaling. Membrane-bound PRs, in particular mPRα, have been identified in human breast cancer biopsies and epithelial- derived breast cancer cell lines [49], where they are hypothesized to play a role in cancer development, proliferation, and metastasis. In breast cancer cells, mPRα is thought not only to activate the PI3K/Akt pathway, but also to transactivate EGFR [50]. Membrane-bound PRs are studied primarily in breast carcinoma, and to the best of our knowledge, their expression profile and their interaction with P4 in GBM cells is not yet known. We think that it will be impor- tant to examine the expression and function of mPRs in different GBM cell lines. Considering that mPRs are widely expressed in the human nervous system [46,31], it is possible that P4 sup- presses PI3K/Akt signaling in U87MG tumor cells via mPRs. It is also possible that P4 might have a hormetic effect on mPRs where low and high doses may change the effects of mPR activation. P4 also modulates GABAA receptor-mediated signaling through its metabolite allopregnanolone (ALLO) and, interestingly, ALLO has been reported to interact with mPRα [51]. We have previ- ously reported that P4 induces cell death in human neuroblastoma cells and this effect was not blocked or modulated by the 5α- reductase inhibitor finasteride, which blocks the conversion of P4 into ALLO [16], thus ruling out the involvement of ALLO in P4's effect in neuroblastoma. However, similar studies are needed in GBM cells to rule out ALLO/GABAα signaling from P4's anti-tumor effects.

4.5. Conclusions

Our data show that P4 at high doses inhibits the growth of human GBM and enhances survival time without any side effects in mice. In our mouse xenograft model, P4 works mainly by suppressing tumor cell proliferation and tumor vasculariza- tion/angiogenesis and inducing apoptosis (Fig. 8). Our findings may be clinically relevant. We believe that our data can be taken to suggest that P4 could be used either alone or in conjunction with reduced levels of radiation, surgery and chemotherapy drugs such as TMZ for the treatment of GBM and that this combinatorial ther- apy could improve patient survival, functional outcome and quality of life.

4.6. Limitations and future directions

We recognize that our findings represent but a modest first step in evaluating P4 as an adjunct therapy for GBM. More basic research is needed to clarify the anti-tumor effects of P4 in an orthotopic GBM model using different cell lines and/or primary neurospheres in mice before the hormone can be considered for clinical testing. If P4 proves to be safe and effective as an anticancer therapeutic, it could constitute a far less invasive, easier-to-administer, and less expensive treatment than currently available interventions.

Disclosures

(1) A US patent (# US 8,435,972 B2) was issued to FA and DGS on May 7, 2013 for the use of P4 and compositions related thereto for the treatment of neurogenic tumors, specially neuroblastoma and glioblastoma.

(2) DG Stein receives royalties from products of BHR Pharma- ceuticals Ltd related to the use of progesterone in the treatment of TBI and stroke, and may also receive research funding from BHR Pharmaceuticals, which is developing products related to this research. Some of these royalties are placed into a laboratory account used to support ongoing research. This amounts to 1.5% of royalties. 7.5% went to Dr. Stein for discretionary use. Over 60% of royalties goes to the University senior administration and vari- ous Deans, and another small share to the Chair of the Department of Emergency Medicine. In addition, Stein serves as an occasional consultant to BHR Pharmaceuticals and receives compensation for these services. The terms of this arrangement have been reviewed.
and approved by Emory University, which will continue to receive the largest share of fees and royalties in accordance with its conflict of interest policies.

Acknowledgements

The authors would like to thank Leslie McCann for invaluable editorial assistance. We thank Dr. Gustavo Pradilla in the Department of Neurological Surgery of Emory University for careful review of this manuscript and thoughtful suggestions for improvement. This research was supported in part by gifts from Allen and Company, The Marcus Foundation, The Laney Graduate School of Emory University and the Stein Family Research Fund.

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