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ORIGINAL PAPER

Progesterone effects on cell growth of U373 and D54 human astrocytoma cell lines

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Abstract Astrocytomas are the most frequent primary brain tumors and constitute a leading cause of cancer-related deaths. We studied the effects of progesterone and its antagonist, RU486, on cell growth of two human astrocytoma cell lines with different evolution grade (U373, grade III; and D54, grade IV). Progesterone receptor expression was determined by Western blot. The effects of different doses of progesterone and RU486 on cell number, cell cycle, and apoptosis were analyzed for five consecutive days. Progesterone (10 nM) significantly increased the number of D54 cells from the second day of culture, and the number of U373 cells on days 3–5. RU486 (10 μM) blocked progesterone effects in both astrocytoma cell lines. Interestingly, RU486 administered without progesterone significantly reduced the number of cells from the second day of culture in both cell lines. Progesterone increased S phase of cell cycle in U373 cells (61%, on day 5). RU486 blocked the effects of progesterone on cell cycle but administered alone did not significantly change cell cycle profile. DNA fragmentation (TUNEL) assay showed that the diminution in the number of astrocytoma cells produced by RU486 was not by apoptosis. Progesterone receptor isoforms were detected in both cell lines. Our data suggest that progesterone induces cell growth of human astrocytoma cell lines through the interaction with its nuclear receptor.

Keywords Astrocytomas · Cell growth · D54 cells · Progesterone · Progesterone receptor · RU486 · U373 cells

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Introduction

Astrocytomas comprise the most common primary malignant brain tumors in adults, representing between 30 and 40% of all tumors with fatal outcomes in the majority of patients [1, 2]. Astrocytomas are classified according to their histological characteristics in four groups (I–IV) being the fourth group the more malignant characterized by excessive proliferation, neovascularization, and high invasiveness [3].

The treatment given to patients with astrocytomas depends on many factors, including the tumor size and localization, its growth rate, and the symptoms the patient is experiencing. Various strategies have been used to treat astrocytomas including extensive surgical resection, fractionated and focused radiation, and intracavitary and/or intra-arterial chemotherapy that result in prolonged and non always significant survival for patients but compromise brain function [4]. An alternative for the treatment of astrocytomas is hormonal therapy based on sex steroid hormones such as progesterone (P4), which participates in the regulation of cell proliferation of several tumors [5, 6]. It has been reported that a progestin, medroxyprogesterone, inhibits S-phase of C6 rat glioma cells [7], but the anti-progestin RU486 also inhibits the growth of a human astrocytoma cell line injected in nude mice [8]. However, P4 effects on human astrocytomas growth have not been well characterized.

P4 elicits its effects mostly by interaction with its classical progesterone receptor (PR), which is a ligand-activated transcription factor and it is considered as a predictive marker for disease prognosis and for response to hormonal therapy in breast cancer [9]. It has been found that PR expression directly correlates with histological grades of human astrocytomas, suggesting that PR-positive tumors possess a high proliferative potential [10]. PR exhibits two isoforms (PR-A and PR-B) with different function and regulation [11]. Previous studies in our laboratory have reported the expression pattern of PR isoforms in human astrocytomas [12]. We found that the predominant PR isoform expressed in astrocytomas grades III and IV was PR-B. This differential expression is important because P4 can exert different function in a cell depending on the expression of PR isoforms [12, 13].

In spite of the presence of PR in human astrocytomas, the role of P4 in astrocytomas cell growth and the participation of PR in this process are unknown. In this work, we studied the effects of P4 and its antagonist RU486 on cell growth of human astrocytoma cell lines U373 and D54, corresponding to grades III and IV of tumor evolution, respectively. We also determined the presence of PR isoforms in these cells by Western blot analysis.

Results

Effects of P4 and RU486 on the growth of U373 and D54 human astrocytoma cells lines

A time-course study over a 5-day period with different doses of P4 (1 nM–10 μM) was performed in U373 and D54 human astrocytoma cell lines. Although the majority of P4 doses induce a slight increase in cell growth of both cell lines, only the dose of 10 nM significantly increased the number of cells from the second day of culture in D54 cells and from the third day in the case of U373 cells. In both cell lines P4 (10 nM) effect persisted until day 5 (Fig. 1). The treatment with RU486 (10 μM) without P4 for 5 days significantly decreased the number of U373 and D54 cells as compared with vehicle treatment from the second day of the experiment (Fig. 2). RU486 co-administered with P4 significantly blocked the effects of the latter on days 2 and 4 in D54 and U373 cells, respectively (Fig. 2).

Effects of P4 and RU486 on cell cycle and apoptosis

We evaluated P4 and RU486 effects on cell cycle by flow cytometry. P4 produced a significant increase (61% on day 5) in S phase of the cell cycle in U373 cells as compared with cells treated with vehicle (Fig. 3 and Table 1), whereas RU486 administration reduced the number of cells in S phase (16% on day 5) (Fig. 3 and Table 1).

In the D54 cell line no significant changes were observed in the cell cycle during 5 days of culture (data not shown). In order to assess whether apoptosis had a role in the reduction of the number of cells after RU486 treatment, U373 and D54 cells were examined for apoptotic activity using the TUNEL assay. In situ detection of apoptosis cells revealed an absence of apoptotic cells in both cell lines during treatment with RU486 (data not shown). The percentage of cells in apoptosis was <1% in all cases. No cell death or apparent morphological changes were observed during treatment with RU486.

Determination of PR expression

PR expression was determined in U373 and D54 cells by Western blot. PR-A and PR-B isoforms were detected as bands of 94 and 114 kDa, respectively. PR-B presented a higher content than PR-A in U373 cells, whereas PR-A was the predominant one in D54 cells (Fig. 4).

Discussion

In the present study, we examined the role of P4 and its receptors (PR) in the regulation of cell growth of two human astrocytoma cancer cell lines: U373 and D54. The results show that P4 increases cell growth of human astrocytoma cells, whereas its antagonist RU486 blocks P4 effects, and administered alone inhibits cell growth. In other brain tumors P4 has different effects. In cell cultures of meningiomas (intracranial or intraspinal from the arachnoidal layer of meninges) different doses of P4 (1–100 nM) stimulate cell growth [14, 15], but P4 inhibits cell growth of prolactinomas [16].

P4 can exert its effects through genomic or non-genomic mechanisms. Our results suggest that P4 effects on astrocytomas cell growth occurs via a classical genomic mechanism through an interaction with PR, since RU486 blocked P4 effects on cell growth and cell cycle distribution, and PR isoforms are present in U373 and D54 cells. The participation of other mechanisms involving membrane progesterone receptors cannot be discarded, although these receptors have not been described in astrocytomas. It is important to mention that the concentration of P4 (10 nM) that induces a significant increase in the number of astrocytoma cells is found in the luteal phase of the woman menstrual cycle [17].

PR has been detected in several human brain tumors such as meningiomas, chordomas, craniopharyngiomas,

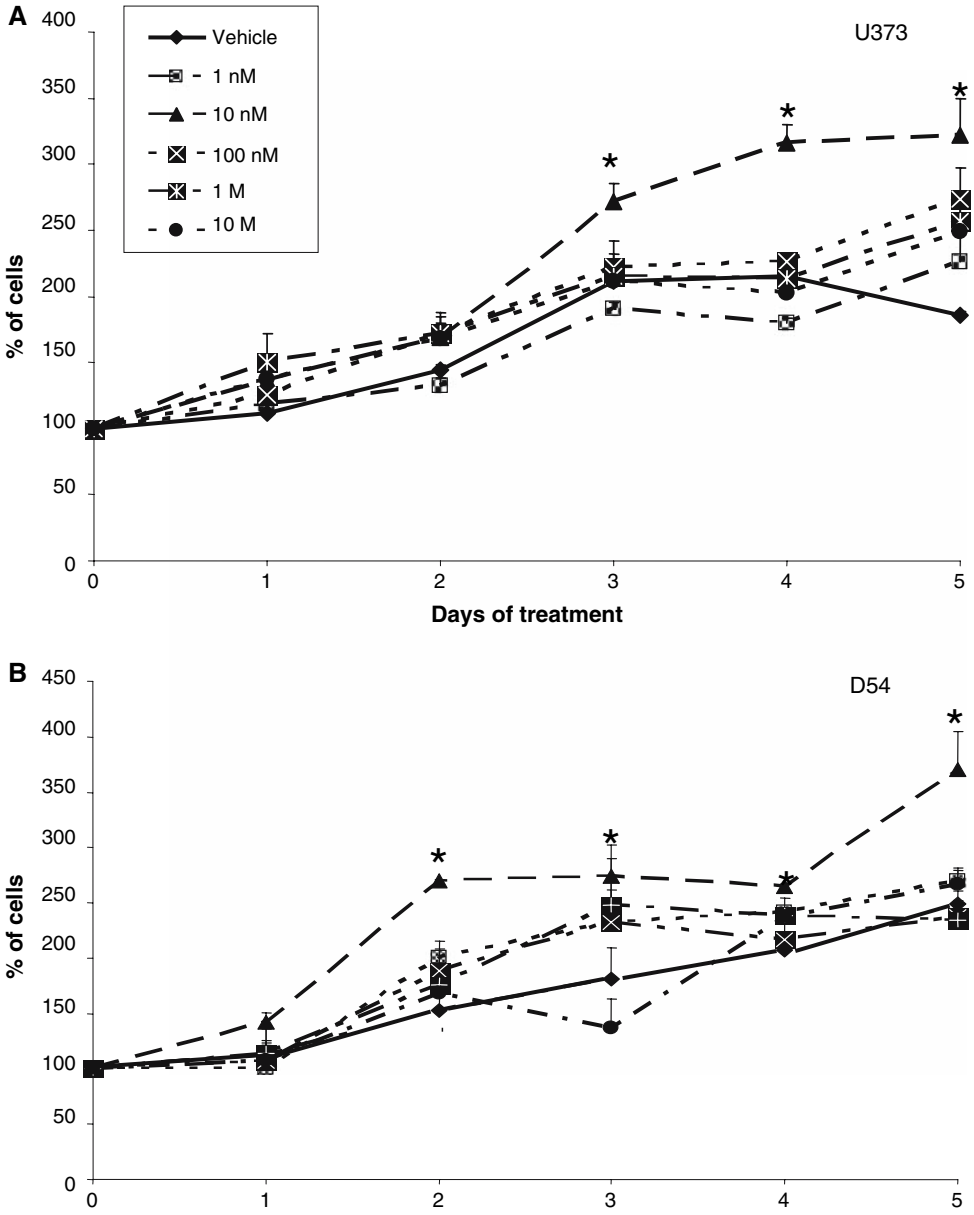

Fig. 1 Effects of P4 on cell growth of U373 and D54 human astrocytoma cell lines. (**a**) U373 and (**b**) D54 human cancer cell lines were treated with different doses of P4 (1, 10, and 100 nM, and 1 and 10 μM) or vehicle (0.02% cyclodextrin in sterile water). Each treatment was performed in six different experiments, each one by triplicate for 5 days. Every day cells were removed from incubation and the number of cells was measured by trypan blue dye exclusion using an inverted microscope. Data are means ± E.S. $*P < 0.01$ vs. vehicle

and astrocytomas [18–20]. It has been found that PR expression directly correlates with histological grades of human astrocytomas, suggesting that PR-positive tumors possess a high proliferative potential [10, 12]. The expression of PR in U373 and D54 cells (Fig. 4) suggests that P4 and RU486 effects are mediated by this receptor. We observed that PR-B was the predominant isoform in U373 cells, whereas PR-A was the predominant one in D54 cells. Thus, this differential PR isoform expression should be involved in the distinct effects of P4 effects in U373 and D54 cells such as those observed in cell cycle.

Interestingly, RU486 alone significantly diminished the number of U373 and D54 astrocytoma cells. This antagonist, at the same concentration used in our work, has been found to exert antiproliferative activities in vitro in neuroblastoma and meningioma cells [21]. Besides, the proliferation of glioma U87MG cell line grafted in nude mice was reduced by RU486 [8]. Although RU486 can interact with glucocorticoid receptor, it has higher affinity for PR, and it is known that induces a conformational change in the ligand binding domain of PR that does not allow the recruitment of coactivators but facilitates receptor interaction with corepressors [22–24]. The molecular mechanisms involved in RU486 effects in U373 and D54 cells are unknown; however, it is possible that RU486 modifies (diminishing or increasing) the expression of several set of genes in these cells as it has been reported, by using microarrays, in female mice (without P4 treatment) [25, 26].

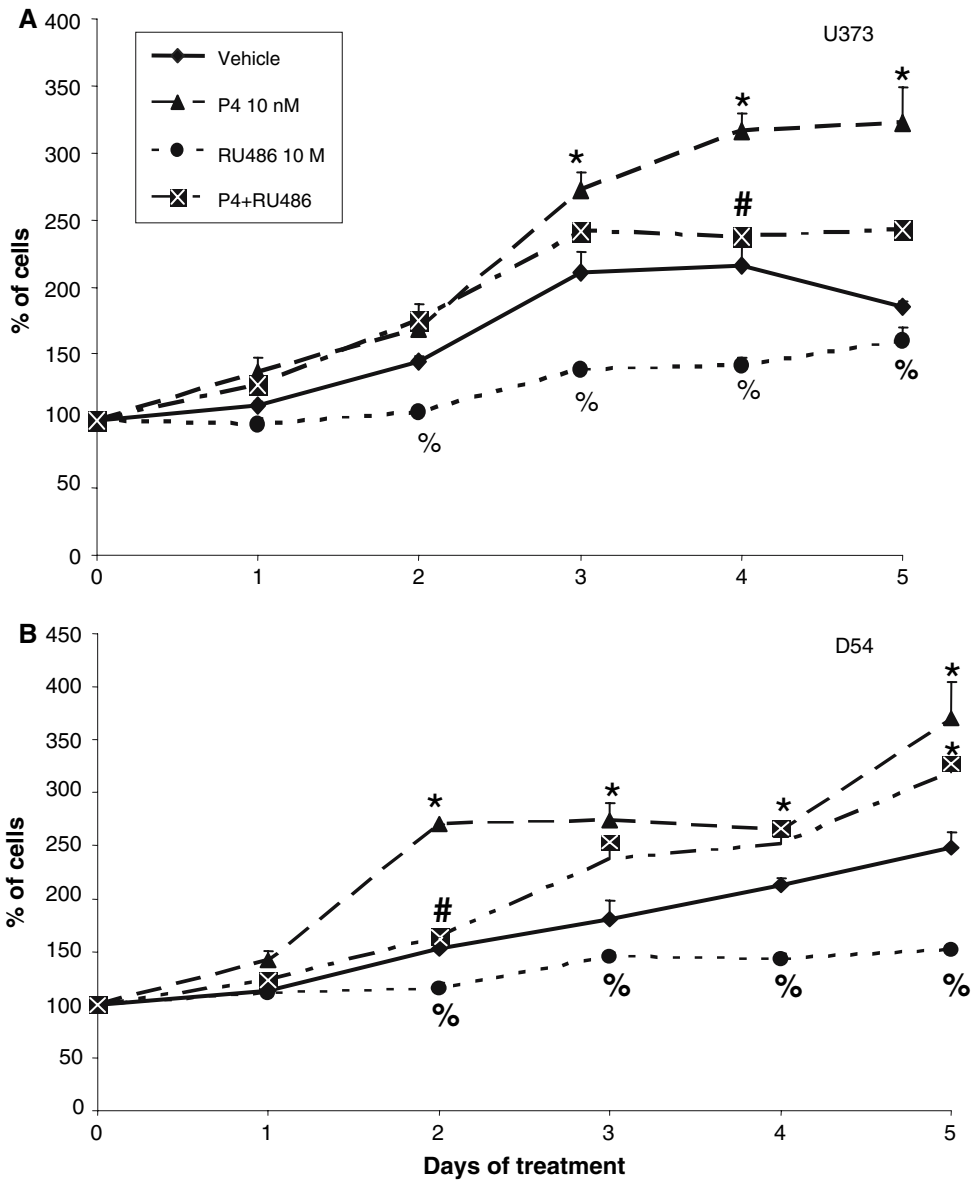

Fig. 2 Effects of P4 and RU486 on cell growth of U373 and D54 cells. (**a**) U373 and (**b**) D54 cells were treated with P4 (10 nM), RU486 (10 μM), P4 + RU486, and vehicle. Cells were analyzed as mentioned in Fig. 1. Data are means ± E.S. $^{*}P < 0.01$ vs. vehicle, $^{\#}P < 0.01$ vs. P4, $^{\%}P < 0.01$ vs. the other groups

Results of DNA fragmentation (TUNEL) assay show that the diminution in the number of U373 and D54 cells observed after RU486 treatment is not due to apoptosis since the number of TUNEL labeled cells did not significantly change during all the experiment (from day 0 to day 5 of culture). Thus, it is possible that RU486 effects should be due to a retardation of DNA replication, thereby inhibiting further progress in the cell cycle. Although P4 and RU486 modified cell cycle profile of U373 cells, this effect was not observed in D54 cells, suggesting that cell cycle regulation by P4 depends on the astrocytomas evolution grade [27].

In conclusion, P4 induces cell proliferation in two human astrocytoma cell lines, U373 and D54 (grades III and IV, respectively), which is blocked by its antagonist RU486, suggesting that P4 effects are mediated by its nuclear PR which is expressed in these cells.

Materials and methods

Cell lines and culture

U373-GB and D54 human astrocytoma cell lines derived from human astrocytomas grades III (ATCC, Manassas, VA) and IV, generously obtained by Dr. Andres Gutiérrez from Dr. Sontheimer (Birmingham, AL) laboratory, and T47D human breast cancer cells (used as positive control of PR expression) were maintained in Dulbecco's modification of Eagle's medium (DMEM) and RPMI medium,

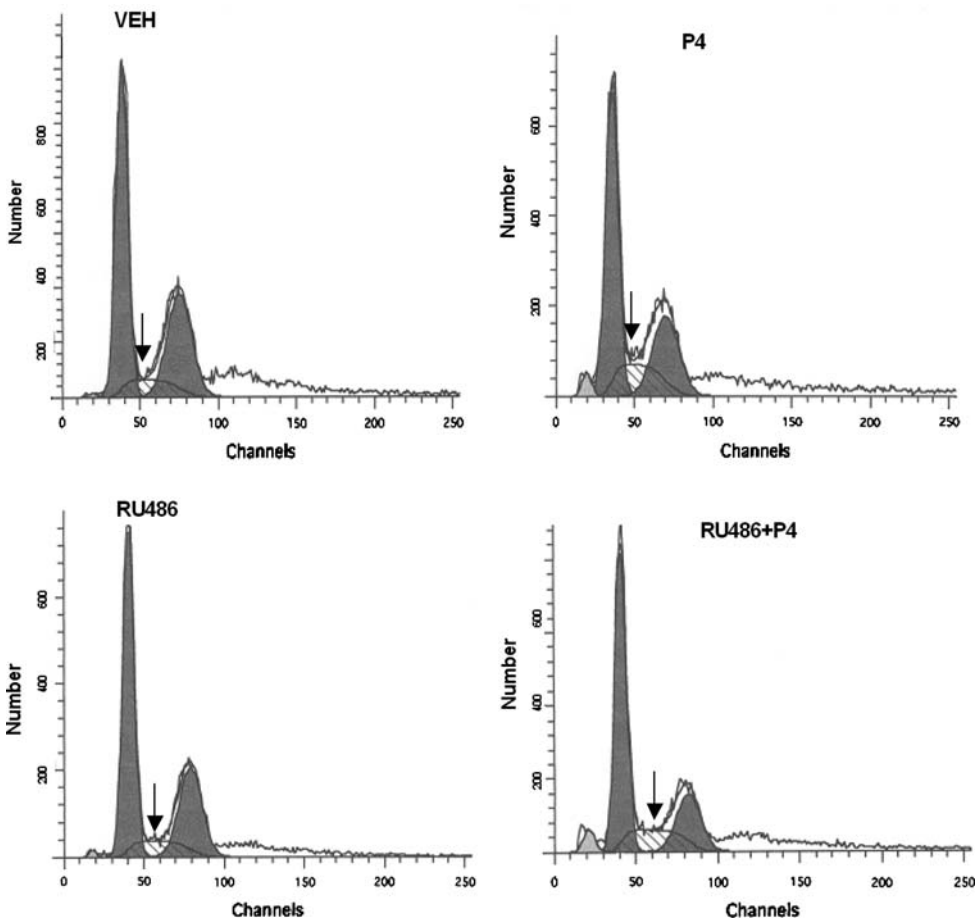

Fig. 3 Cell cycle analysis of U373 cells after P4 and RU486 treatment. Histograms of DNA content show the effects of vehicle (VEH), P4 and its antagonist, RU486, on cell cycle distribution on day 5 (D5). Cells were treated as in Fig. 2. Arrows indicate S phase of the cell cycle. Number in *Y* axis indicates the events (cells) quantified in each phase of the cell cycle

Table 1 Cell cycle analysis of U373 cells exposed to P4 and RU486

Treatment	Days	G0–G1	S	G2/M
Veh	0	42.51 ± 6.0	28.56 ± 6.6	28.92 ± 8.1
	3	56.67 ± 5.5	27.46 ± 7.4	15.86 ± 7.5
	5	58.23 ± 3.7	20.37 ± 4.4	21.39 ± 4.2
P4	0	43.10 ± 5.8	28.63 ± 8.2	28.26 ± 6.9
	3	54.53 ± 1.6	28.58 ± 6.3	16.87 ± 5.7
	5	52.22 ± 8.8	32.89 ± 9.6*	14.90 ± 6.3
RU486	0	42.51 ± 6.0	28.56 ± 6.6	28.92 ± 8.1
	3	59.51 ± 3.4	23.45 ± 3.1	17.03 ± 4.3
	5	58.27 ± 5.4	17.15 ± 5.4	24.58 ± 9.1
P4 + RU486	0	43.10 ± 5.8	28.63 ± 6.9	28.26 ± 8.2
	3	55.72 ± 3.4	26.99 ± 5.8	17.29 ± 5.0
	5	58.95 ± 2.8	18.19 ± 4.0	22.85 ± 4.8

Values are % of cells in each phase of the cell cycle. Data are means ± SE of four experiments per treatment

*$P < 0.05$ as compared with vehicle (Veh) on day 5

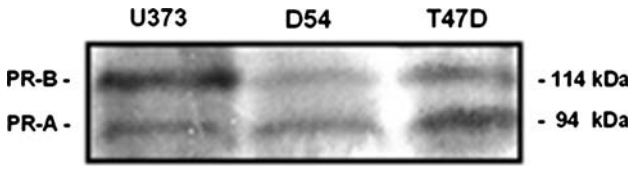

Fig. 4 PR isoforms expression in U373 and D54 human astrocytoma cell lines. D54, U373 and T47D cells were lysed and proteins (70 μg) were separated by electrophoresis on 10% SDS–PAGE. Gels were transferred to nitrocellulose membranes and then incubated with antibodies for PR as described in Materials and methods. The protein-antibody complexes were detected by ECL

respectively, supplemented with 10% fetal bovine serum (FBS) (GIBCO NY), 1 mM pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids (all from GIBCO), at 37°C in a humidified atmosphere with 5% CO_2 were grown as monolayer cultures in 100 cm^2 cell culture dish (Corning, NY).

Hormones

Progesterone-water soluble (cyclodextrin-encapsulated progesterone) and (2-hydroxypropyl)-β-cyclodextrin were dissolved in sterile water and prepared in culture medium DMEM phenol red-free medium. RU486 (Sigma, St Louis) was dissolved in ethanol and prepared in culture medium with a final ethanol concentration of 0.1%.

Treatment

U373 and D54 cell lines were plated on 96-well microtest plates in 250 μl of DMEM with 10% FBS at a cell density of 4×10^3 cells for 24 h. Medium was changed by DMEM or RPMI phenol red-free medium supplemented with 10% FBS without steroid hormones (Hyclone, Utah), 1 mM pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids during 24 h. Then, different doses of P4 (1, 10, and 100 nM, 1 μM, and 10 μM), or hormone vehicle (0.02% w/v cyclodextrin in sterile water) were added to the culture (day 0).

Each dose–response experiment was performed in six independent cultures, each one by triplicate, during 5 days. In other experiments, the effects of P4 (10 nM), RU486 (10 μM), and P4 (10 nM) + RU486 (10 μM) administered at the same time were also evaluated during 5 days.

Cell growth

Cells were harvested from incubation every day during five consecutive days with PBS 1X + EDTA (1 mM). Then, they were centrifuged (1,000 rpm/7 min) and the pellet was resuspended in 10 μl of PBS 1X and 10 μl of Trypan blue. The number of living cells, evaluated by a blind observer, was measured by trypan blue dye exclusion [28] using an inverted microscope (Olympus CKX41, Center Valley, PA).

Cell cycle analysis by flow cytometry

A total of 350,000 U373 or D54 cells were seeded in 100 cm^2 cell culture dishes, cultured, and treated with P4 and RU486 as mentioned above. Cells were collected every day during 5 days after treatments with P4 and RU486. Cells were washed from dishes by incubation in PBS 1X-EDTA for 3 min at 37°C, then they were scraped from dishes, transferred to sterile 15-ml tubes and obtained by centrifugation (1,000 rpm/7 min). Cells were washed twice in PBS 1X, and were fixed with 70% ethanol at 4°C. Next, samples were washed with PBS, centrifuged (1,000 rpm/7 min), and cell pellet was suspended in 1 ml of staining solution (0.02 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1 μg/ml RNase A I free DNAse in PBS). 1×10^6 cells per day and treatment were analyzed at 535 nm on a Becton Dickinson (FACSort) flow cytometer. Data were collected and DNA histograms were analyzed with the program MODFITT (Cell Quest, Ohio).

Detection of apoptosis

Apoptosis was evaluated by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) method [29]. A total of 200,000 U373 or D54 cells were seeded on glass coverslips in 50 cm^2 cell culture dish in DMEM supplemented with 10% FBS for 24 h. Medium was changed by DMEM phenol red-free medium supplemented with 10% FBS without steroid hormones, 1 mM pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids during 24 h. Then, hormonal treatments: vehicle, P4 (10 nM), RU486 (10 μM), and P4 (10 nM) + RU486 (10 μM) were administered on day 0, and apoptosis was evaluated daily for 5 days. Every day medium was retired, cells were washed with cool PBS 1X, and were fixed in 4% paraformaldehyde for 1 h. Cells were washed again with PBS 1X at room temperature and were conserved at 4°C. The In Situ Cell Death detection Kit, fluorescein kit (ROCHE, Basel), was used and the procedure protocol recommended by manufacturer was followed. Briefly, cells were permeabilized with 0.1% Triton X100 in sodium citrate 0.1% solution for 2 min at 4°C. Negative control reaction buffer and positive control reaction treated with Dnase I (Invitrogen, Carlsbad) for 10 min at room temperature were performed. Samples were then subjected to 50 ml of TUNEL reaction mixture in a humidifier chamber at 37°C for 60 min. We detected labeled ends as fluorescent signal (green) under fluorescence microscopy (Nikon eclipse, E600, Road Melville, NY). The apopototic index was estimated by counting the number of stained apoptotic cells using the Metamorph Imaging System (Universal Imaging Corporation, Downingtown, PA) in each culture (three independent cultures in triplicate per experimental condition).

Protein extraction and western blotting

U373, D54 and T47D cells (2×10^6) plated in 100 cm^2 dishes without any treatment were collected and homogenized in TDG lysis buffer with protease inhibitors (10 mM Tris–HCl, 1 mM dithiothreitol, 30% glycerol, 1% Triton X-100, 15 mM sodium azide, 1 mM EDTA, 4 μg/ml leupeptin, 22 μg/ml aprotinin, and 1 mM PMSF). Proteins were obtained by centrifugation at 20,000*g*, at 4°C for 15 min, and quantified by the method of Bradford (Bio-Rad Laboratories, Hercules, CA). Proteins (70 μg) were separated by electrophoresis on 10% SDS–PAGE at 20 mA. Colored and enhanced chemiluminescence markers (Bio Rad, CA and Gibco-BRL, MD) were included for size determination. Gels were transferred 2 h to nitrocellulose membranes (Amersham, NJ) (60 mA, at room temperature in semi dry conditions), which were blocked at room temperature with 5% non-fat dry milk and 0.5% bovine serum albumin for 2 h. Membranes were then incubated with 2 μg/ml of mouse-anti-PR polyclonal

antibody (NeoMarkers RB-1492-P, Fremont, CA), which recognizes both PR isoforms (PR-A and PR-B), at 4°C overnight. Blots were then incubated with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min. Signals were detected by enhanced chemiluminescence (ECL) (Amersham, NJ).

Data analysis

Statistical analysis was performed by SPSS13.0 software for windows (SPSS Inc, Chicago, IL). Data were analyzed by one-way analysis of variance followed by Tukey test for comparison between groups.

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