Archives of Medical Research 47 (2016) 419–426

Archives of Medical Research

ORIGINAL ARTICLE

Intracellular Progesterone Receptor Mediates the Increase in Glioblastoma Growth Induced by Progesterone in the Rat Brain

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Received for publication March 1, 2016; accepted October 5, 2016 (ARCMED-D-16-00125).

Background and Aims. Progesterone (P) is a steroid hormone involved in the development of several types of cancer including astrocytomas, the most common and malignant brain tumors. We undertook this study to investigate the effects of P on the growth and infiltration of a tumor caused by the xenotransplant of U87 cells derived from a human astrocytoma grade IV (glioblastoma) in the cerebral cortex of male rats and the participation of intracellular progesterone receptor (PR) on these effects.

Methods. Eight weeks after the implantation of U87 cells in the cerebral cortex, we administered phosphorothioated antisense oligodeoxynucleotides (ODNs) to silence the expression of PR. This treatment lasted 15 days and was administered at the site of glioblastoma cells implantation using Alzet osmotic pumps. Vehicle (propylene glycol) or P_4 (400 μg/100 g) was subcutaneously injected for 14 days starting 1 day after the beginning of ODN administration.

Results. We observed that P significantly increased glioblastoma tumor area and infiltration length as compared with vehicle, whereas PR antisense ODNs blocked these effects.

Conclusion. P, through the interaction with PR, increases the area and infiltration of a brain tumor formed from the xenotransplant of human glioblastoma-derived U87 cells in the cerebral cortex of the rat.

Key Words: Astrocytoma, Glioblastoma, Progesterone, Progesterone receptor, U87 cells.

Introduction

Astrocytomas are the most common tumors of the central nervous system (CNS). They can arise from astrocytes, glial progenitor cells or cancer stem cells (1–5). The World Health Organization has classified them into four grades (I–IV) according to their histological characteristics (6,7). Glioblastomas (grade IV) are the most prevalent and aggressive primary brain neoplasms, accounting for 45% of malignant tumors in the CNS (8) and remaining as an incurable disease with a median overall survival of 12–14 months (9–11). Current medical treatments such as neurosurgery, radiotherapy and chemotherapy achieve only a modest improvement in patient survival (12–14). In adults, the incidence of glioblastoma is 50% higher in males than in females (8,15), suggesting the participation of a hormonal component in its origin and progression. It has been reported that progesterone (P) is involved in the growth and progression of astrocytomas because its administration *in vitro* increases the number of U373 and D54 cell lines (derived from human astrocytomas grades III and IV,

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0188-4409/$ - see front matter.
http://dx.doi.org/10.1016/j.arcmed.2016.10.002

respectively), whereas the antagonist of its intracellular receptor (PR) RU486 blocked P effects (16). Similar results were observed *in vivo* where U373 cells were implanted in the motor cerebral cortex of the rat. P administration significantly increased both the area and the infiltration length of the tumor, effects that were also blocked by RU486 (17). P exerts many of its effects through the interaction with PR, which is a ligand-activated transcription factor that regulates the expression of several genes involved in development, metabolism and reproduction (18–20). Two main PR isoforms have been reported, PR-A and PR-B, each with differential expression patterns, regulation and functions (21–23). PR expression ratio directly correlates with astrocytoma evolution grade, which suggests that PR-positive tumors present a high proliferative potential (24–26). The effects observed both *in vitro* and *in vivo* in astrocytoma cells after P administration suggests that this hormone exerts its effects through PR; however, due to the fact that RU486 is also an antagonist of the glucocorticoid receptor it is necessary to determine whether P effects on astrocytomas growth are due to its interaction with PR by using other strategies. In this work we studied the participation of PR by silencing its expression in P-induced effects on the growth of a tumor caused by the xenotransplant of U87 human glioblastoma cells in the cerebral cortex of male rats.

Materials and Methods

Cell Line and Culture

U87MG cell line (ATCC, Manassas, VA) derived from a human glioblastoma was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mmol pyruvate, 2 mmol glutamine and 0.1 mmol non-essential amino acids (all from Gibco, Grand Island, NY) at 37°C in a humidified atmosphere of 95% air/5% CO_2. DMEM was changed every 48 h until reaching 70–80% of cellular confluence.

In Vitro *Silencing of PR Using Phosphorothioated Antisense Oligonucleotides (ODNs)*

U87 cells cultured in the conditions previously described were treated with 0.5 μg or 1.0 μg of phosphorothioated sense and antisense ODNs (Sigma-Aldrich, St. Louis, MO) vs. total PR (both isoforms) using the X-tremeGENE 9 DNA transfection reagent (Roche, Basel, Switzerland). The reagent was diluted in phenol red-free DMEM without FBS or antibiotics, maintaining a ratio of 3 μL of reagent per 100 μL DMEM. The solution was stirred gently and mixed with the correspondent ODN, maintaining a ratio of 1 μg of ODN per 3 μL of pre-diluted reagent. As a control we used a solution (mock), which was prepared in the same manner except that no ODNs were added. These mixtures were left to settle at 25°C for 30 min and then added to 1,200,000 U87 cells in phenol red DMEM with 10% FBS and without antibiotics. Cells were incubated at 37°C for 72 h and Western blot was performed to assess the silencing of PR. The sequence of the ODNs was as follows: sense: 5′-T*T*A*T*GCCTTACCATGTG*G*C*A-3′; antisense: 5′-T*G*C*C*ACATGGTAAGGCA*T*A*A-3′. The asterisk indicates the site of the phosphorothioated bond.

Protein Extraction and Western Blot

Cells were lysed with RIPA buffer (50 mmol Tris-HCl, 150 mmol NaCl, 0.1% SDS, 1% Triton X-100, 2 μg/mL aprotinin and 1 mmol PMSF) and proteins were obtained by centrifugation at 12,500 rpm at 4°C for 15 min and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). Proteins (70 μg) were separated by electrophoresis on 7.5% SDS-PAGE at 80 V. Colored markers (Bio-Rad, Hercules, CA) were included for molecular size determination. Gels were transferred to nitrocellulose membranes (Millipore, Bedford, MA) for 7 h (35 mA at room temperature in semi-dry conditions). Membranes were blocked with 2% non-fat dry milk and 3% bovine serum albumin at 37°C for 3 h and incubated with a 1:300 dilution of mouse anti PR (AB-52: sc-810, Santa Cruz Biotechonology, Dallas, TX) at 4°C overnight. Blots were then incubated with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (1:5000) (Santa Cruz Biotechnology) for 1 h at room temperature. PR content was normalized to that of mouse anti α-tubulin, which was used as loading control. Blots were stripped with glycine (0.1 M, pH 2.5, 0.5% SDS) at 50°C for 30 min and incubated with a 1:1000 dilution of mouse anti α-tubulin monoclonal antibody (sc-5286, Santa Cruz Biotechnology) at 4°C overnight. Subsequently, blots were incubated with goat anti-mouse antibody coupled to horseradish peroxidase (1:5000) (Santa Cruz Biotechnology) at room temperature for 1 h. Bands were detected using a manual setting by enhanced chemiluminescence (ECL). Signals were detected exposing membranes to Kodak Biomax Light Film (Sigma-Aldrich, St. Louis, MO) using Supersignal West Femto (Thermo Scientific) as peroxidase substrate. The antigen-antibody complex was detected as the area under the peak corresponding to a band density (area is given in inches with a default scale of 72 pixels/inch) using an HP Scanjet G3110 apparatus (Hewlett-Packard, Palo Alto, CA) and the ImageJ 1.455 software (National Institutes of Health, Bethesda, MD). Four independent experiments were carried out.

U87 Cell Microinjection Into the Rat Brain

Adult male Wistar rats (250–300 g) maintained on a 12:12 h light/dark cycle with food and water ad libitum were intraperitoneally anesthetized with ketamine-xylazine (80/10 mg/kg, respectively) and mounted on a