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Allopregnanolone promotes proliferation and differential gene expression in human glioblastoma cells

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ABSTRACT

Allopregnanolone (3α-THP) is one of the main reduced progesterone (P_4) metabolites that is recognized as a neuroprotective and myelinating agent. 3α-THP also induces proliferation of different neural cells. It has been shown that P_4 favors the progression of glioblastomas (GBM), the most common and aggressive primary brain tumors. However, the role of 3α-THP in the growth of GBMs is unknown. Here, we studied the effects of 3α-THP on the number of cells, proliferation and gene expression in U87 cell line derived from a human GBM. 3α-THP (10, 100 nM and 1 μM) increased the number of U87 cells, and at 10 nM exerted a similar increase in both the number of total and proliferative U87 cells as compared with P_4 (10 nM). Interestingly, finasteride (F; 100 nM), an inhibitor of 5α-reductase (5αR), an enzyme necessary to metabolize P_4 and produce 3α-THP, blocked the increase in the number of U87 cells induced by P_4. By using RT-qPCR, we determined that U87 cells express 5α-R isoenzymes 1 and 2 (5αR1 and 5αR2), being 5αR1 the predominant one in these cells. 3α-THP (10 nM) increased the expression of TGFβ1, EGFR, VEGF and cyclin D1 genes. P_4 increased TGFβ1 and EGFR expression, and this effect was blocked by F. These data provide evidence that P_4, through its metabolite 3α-THP, can promote in part cell proliferation of human GBM cells by changing the expression of genes involved in tumor progression.

1. Introduction

Astrocytic tumors (astrocytomas) are the most common malignancies of the Central Nervous System (CNS). According to the World Health Organization (WHO), astrocytomas are classified into four grades (WHO I–IV) of malignancy based on diverse histopathologic and molecular aspects [1]. Astrocytomas grade IV, also known as glioblastomas (GBM), are the most frequent and malignant primary brain tumors. Their malignancy is due to the highly invasive potential, abundant mitotic events, vascularity, and resistance to chemotherapy and radiotherapy. In most cases, it is not possible to surgically remove the tumor [2–5]. In spite of all the recent advances in chemo- and radiotherapy and surgical techniques, the prognosis of patients is still poor, and their survival after diagnosis is about 10–15 months [6,7,3].

There are many factors related to the growth and progression of GBM, such as sex steroid hormones. We and others have reported that progesterone (P_4) promotes cell proliferation, migration, and infiltration of GBM [8–10]. P_4 can be synthesized and metabolized in the CNS, and it is known that their metabolites can prolong, increase or antagonize P_4 effects through different mechanisms of action under physiological or pathological conditions such as cancer [11–13], however, there is no evidence about the effects of P_4 metabolites in GBM growth.

Two primary enzymes are responsible for the metabolism of P_4 and other steroid hormones: 5α-reductase isoforms (5αR1 and 5αR2) reduce P_4 in the position 5 giving rise to 5α-dihidroprogesterone (5α-DHP), then, 3α-hydrosyxteroid dehydrogenases convert 5α-DHP into allopregnanolone (3α-THP). There is evidence that rodent C6 and human 1321N1 astrocytoma cell lines express these enzymes, and use P_4 as a preferential substrate instead of testosterone [14–16].

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3α-THP participates in diverse physiological and pathological processes in the CNS [13,17] through different mechanisms that have not been yet well elucidated. At least three main mechanisms of 3α-THP actions have been identified in the CNS: through the allosteric modulation of $GABA_A$ receptors ($GABA_AR$) in mature neurons and neural progenitor cells; by its binding to the seven transmembrane class of progesterone-binding receptors (mPRs) in glial cells [18–20]; and by its interaction with the pregnane xenobiotic receptor (PXR) expressed in different areas of the CNS [21].

It has been reported that 3α-THP increases the expression of different genes related to regeneration, proliferation, and protection against cell death in diverse CNS cell types such as human and rodent neural progenitor cells or oligodendrocyte precursor cells. These effects occur in a stereospecific way compared with its isomers epiallopregnanolone (5α-pregnan-3β-ol-20-one) and epipregnanolone (5β-pregnan-3β-ol-20-one) [22–25]. Besides, there is a correlation between P_4 α-reduced metabolites and increased malignancy in breast cancer cells [11,26,27]. Despite the protective and proliferative role of 3α-THP in several cell types, there is no information about its relevance in the progression of GBM.

The aim of this study was to know the effects of 3α-THP on cell number, proliferation, and gene expression in U87 human GBM cell line. We also determined if U87 cells expressed 5αR1 and 5αR2, the key enzymes involved in P_4 metabolism and 3α-THP formation.

2. Materials and methods

2.1. Cell culture and treatments

The human glioblastoma cell line U87 (ATCC, VA, USA) was grown in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine, and 0.1 mM non-essential amino acids at 37 °C in a humidified atmosphere with 5% CO_2. For RT-qPCR experiments 3×10^5 cells were plated in 6-well plates, for cell growth experiments 1×10^5 cells were plated in 24-well plates, and for the proliferation assays 5×10^3 cells were plated in 4-well glass slides. The medium was changed by DMEM phenol red free medium supplemented with 10% FBS without steroid hormones under the same conditions as described above during 24 h. For determining 5αR1 and 5αR2 basal expression, no treatments were performed. To evaluate the effects of 3α-THP and P_4 on cell growth, U87 cells were treated with 3α-THP (1, 10, 100 nM and 1 μM), P_4 (10 nM), the 5αR inhibitor finasteride (F, 100 nM), P_4 + F, 3α-THP (10 nM) + F, and vehicle (V; 0.01% DMSO). To determine the effects of P_4 metabolism on cell proliferation and gene expression, cells were treated with: vehicle (V, 0.01% DMSO), 3α-THP (10 nM), P_4, the progesterone receptor antagonist, mifepristone (RU486, 1 μM), P_4 + F, 3α-THP + F, and 3α-THP + RU486. Each experiment was performed in at least three independent cultures. All steroids were purchased from Sigma-Aldrich (MO, USA) and DMSO was purchased from J.T. Baker (PA, USA).

2.2. Cell growth

Treated U87 cells were harvested from incubation every day during five consecutive days with 1 mL PBS 1× + EDTA (1 mM). Then, cells were resuspended in the same solution and added 10 μL of 0.4% trypan blue solution. The number of living cells was determined by the trypan blue dye exclusion assay using a hemocytometer and a microscope (Olympus BX41, PA, USA).

2.3. Proliferation assay

The 5-Bromo-2′-deoxyuridine (BrdU) incorporation kit (11296736001 Roche, IN, USA) was used according to the manufacturer's recommendations to determine U87 cell line proliferation. Cells cultured in the 4-well glass slides were treated for three days and afterward the cell culture medium was replaced by the BrdU labeling medium for 40 min. After cell fixation, BrdU incorporation was detected by immunofluorescence using a monoclonal antibody against it and a fluorochrome-conjugated secondary antibody. Additionally, nuclei were stained with Hoechst 33342 solution (1 μg/mL). Cell nuclei stained with Hoechst and positive BrdU cells were visualized in an Olympus Bx43F microscope (Olympus, PA, USA). Cell counting was done with the ImageJ software 1.45S (National Institutes of Health, USA).

2.4. RNA isolation and RT-qPCR

Total RNA extraction was performed with TRIzol reagent (Invitrogen, CA, USA) according to manufacturer's recommendations. The quantity and purity of RNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA). RNA integrity was determined by electrophoresis of 1 μg of each RNA sample in a 1.5% agarose gel with 0.5× TBE buffer, and only total RNA samples with excellent purity and integrity were used for RT-qPCR. First-strand cDNA was synthesized from 1 μg of total RNA by using the M-MLV Reverse Transcriptase (Invitrogen, CA, USA) and oligo $(dT)_{12-18}$ primers (Sigma-Aldrich, MO, USA) according to its protocol. 2 μL of this reaction were subjected to qPCR in order to amplify 5αR1, 5αR2, TGFβ1, VEGF, EGFR and cyclin D1. Sense and antisense primers of 5αR1 and 5αR2 were purchased from Uniparts (CDMX, MEX) and TGFβ1, VEGF, EGFR, and cyclin D1 primers were purchased from Sigma-Aldrich (MO, USA). Table 1 contains all the information of the primers for qPCR. The results were analyzed by the ΔCt method [28,29], and the 18S ribosomal gene was used as an expression control gene. At least, three independent experiments were performed in duplicate for each sample, and a reaction without RT was used as a negative control for each qPCR.

2.5. Statistical analysis

All data were analyzed and plotted using the GraphPad Prism 5.0 software for Windows XP (GraphPad Software, CA, USA). Statis-

Table 1
Primers used for different gene amplifications.

Gene	Primer sequence	Amplified fragment
5αR1	FW: 5′-CAGTGTATGCTGATGACTGGG-3′	152
5αR1	RV: 5′-GCCTCCCCTTGGTATTTTGT-3′	
5αR2	FW: 5′-CCACAAGGTGGCTTGTTTACG-3′	155
5αR2	RV: 5′-TGGTGGTGAAAAGCTCGCA-3′	
EGFR	FW: 5′-GCCTTGACTGAGGACAGGCAT-3′	152
EGFR	RV: 5′-TGGTAGTGTGGGTCTCTGCT-3′	
Cyclin D1	FW: 5′-TGCATAACCCTGAGCGGTGG-3′	146
Cyclin D1	RV: 5′-TCTCCCGAGACGCCTCCTTT-3′	
VEGF	FW: 5′-CCACACCATCACCATCGACA-3′	153
VEGF	RV: 5′-CCAATTCCAAGAGGGACCGT-3′	
TGFβ1	FW: 5′-CTGGCCCTGTACAACCAGCA-3′	148
TGFβ1	RV: 5′-ACTTGCAGGAGCGCACGA-3′	
18S	FW: 5′-AGTGAAACTGCAATGGCTC-3′	167
18S	RV: 5′-CTGACCGGGTTGGTTTTGAT-3′	

Note: 5αR1 = 5α-Reductase 1; 5αR2 = 5α-Reductase 2; EGFR = Epidermal growth factor receptor; VEGF = Vascular endothelial growth factor; TGFβ1 = Transforming growth factor β1; 18S = 18S ribosomal RNA; FW = forward primer; RV = reverse primer.