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Progesterone promotes cell migration, invasion and cofilin activation in human astrocytoma cells

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ABSTRACT

Astrocytomas are the most common and aggressive primary brain tumors in humans. Invasiveness of these tumors has been attributed in part to deregulation of cell motility-dependent cytoskeletal dynamics that involves actin-binding proteins such as cofilin. Progesterone (P_4) has been found to induce migration and invasion of cells derived from breast cancer and endothelium. However, the role of P_4 in migration and invasion of astrocytoma cells as well as its effects on astrocytomas cytoskeleton remodeling is not known. In this work we evaluated these aspects in D54 and U251 cells derived from human astrocytomas from the highest degree of malignancy (grade IV, glioblastoma). Our results showed that in scratch-wound assays P_4 increased the number of D54 and U251 cells migrating from 3 to 48 h. Both RU486, a P_4 receptor (PR) antagonist, and an oligonucleotide antisense against PR significantly blocked P_4 effects. Transwell assays showed that P_4 significantly increased the number of invasive cells at 24 h. As in the case of migration, this effect was blocked by RU486. Finally, by Western blotting, an increase in the cofilin/p-cofilin ratio at 15 and 30 min and a decrease at 30 and 60 min in U251 and D54 cells, respectively, was observed after P_4, P_4 + RU486 and RU486 treatments. These data suggest that P_4 increases human astrocytoma cells migration and invasion through its intracellular receptor, and that cofilin activation by P_4 is independent of PR action.

1. Introduction

Gliomas are the most common type of primary malignancy of the Central Nervous System (CNS) that may arise from adult neural stem cells or multipotent neural progenitor cells that persist in proliferative niches in the human CNS [1]. Based on histological characteristics, gliomas can be classified as astrocytomas, oligodendrogliomas or ependymomas, being astrocytomas the most frequent ones [2,3]. The World Health Organization (WHO) classifies astrocytomas on a scale from I to IV according to their increasing degree of malignancy [4,5]. Grade I is known as pilocytic astrocytoma and is a well-circumscribed, non-infiltrative tumor that tends to occur in children and young adults and is often treated with surgical resection. Grade II or diffuse astrocytoma involves a diffuse infiltration of the surrounding tissue by pleomorphic cells with nuclear atypia. Grade III astrocytoma is known as anaplastic astrocytoma, which has a high percentage of cell proliferation and frequent mitotic figures. Grade IV astrocytoma known as glioblastoma multiforme (GBM) is a highly invasive and vascularized tumor, presents hallmarks of vascular endothelial proliferation and wide areas of necrosis [6]. Current medical treatments for astrocytomas are neurosurgery and/or radiotherapy and chemotherapy, however, only a modest increase in the median survival rate of patients has been observed. Despite advances in the diagnosis and treatment of highly malignant astrocytomas, median survival does not exceed 15 months [4]. One of the main reasons for this short survival is related to the fact that at the time of surgery, tumor cells have invaded adjacent healthy brain tissue [7]. It has been observed that more than 50% of the untreated tumors reach the contralateral brain hemisphere [8]. The ability of high-grade astrocytomas to invade healthy brain tissue is one of the most common causes of treatment failure and high mortality.

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Invasion of tumor cells to normal tissue is a multi-factorial process involving the interaction of cells with the extracellular matrix (ECM), contacts between adjacent cells and biochemical processes, which together trigger cell movement. The processes involved in cell motility are controlled in a very rigorous manner; in cancer cells the inhibitory mechanisms controlling cell motility are lost, resulting in a mobile phenotype that facilitates cancer cell migration and invasion [9]. Cell invasion involves penetration through tissue barriers such as the basement membrane and interstitial stroma, and requires adherence, proteolysis of the ECM and migration [10].

In order to migrate, cells need to change their shape and rigidity to interact with surrounding structures of the ECM [11–13]. Migration and cell invasion in physiological and pathological processes such as cancer result from a continuous cycle of five interdependent steps: (1) protrusion of cytoplasm at the leading edge, (2) cell–matrix interaction and formation of focal contacts, (3) recruitment of surface proteases to ECM contacts and focalized proteolysis (4) cell contraction by actomyosin, and (5) detachment of the trailing edge. The first and last steps mainly require the modifications of the actin cytoskeleton which is regulated by actin-binding proteins (ABP) such as cofilin.

Cofilin activity is essential in the process of remodeling actin cytoskeleton during cell motility since it induces the formation of free barbed ends [14,15]. Post-translational modifications such as phosphorylation modulate the capacity of cofilin to interact with actin [16]. Cofilin has been related in tumor invasion and metastasis of various types of tumors [17–19]. In fact, the overexpression of cofilin in U373 cells derived from a human astrocytoma grade III increases cell motility [20]. By using tissue microarrays, it has been found that the expression of cofilin positively correlates with the progression of human astrocytomas [21].

It has been reported that natural sex steroids such as progesterone (P_4) and estradiol (E_2) or synthetic steroids such as medroxyprogesterone acetate, drospirenone or nestorone, are key regulators of morphology and motility of various cell types, including breast cancer cells, endothelial cells and neurons [22–25]. It has been observed that proteins involved in the remodeling of the actin cytoskeleton such as ezrin, moesin or the Arp 2/3 complex are activated by P_4 and E_2 [22,23,25,26]. Besides, P_4 and E_2 have been involved in the formation of focal adhesion (FA) complexes by activation of FA kinase, which are also fundamental in cell motility [24,27]. FA complexes are dynamic protein complexes containing integrins, FA kinase and talin, through which actin cytoskeleton connects the cell with the ECM, [28,29].

Additionally, we have reported that P_4 induces proliferation of U373 and D54 cells derived from astrocytomas grade III and IV respectively, and that the treatment with RU486, antagonist of P_4 receptor (PR), blocks hormone effect, suggesting that PR is involved in this P_4 action [30]. We have also found that in D54 cells, P_4 induces the expression of genes involved in cell cycle regulation, proliferation and angiogenesis such as cyclin D1, EGFR and VEGF [31]. However, the participation of P_4 and PR in migration and invasion of human astrocytomas *in vitro* is unknown. In this work, we studied the effects of P_4 in migration, invasion and activation of cofilin in D54 cells derived from a human astrocytoma grade IV.

2. Experimental

2.1. Cell culture and treatments

D54 cell line generously obtained by Dr. Andrés Gutiérrez from Dr. Sontheimer's (University of Alabama, Birmingham, AL, USA) laboratory and U251 cells obtained from ATCC (USA) both derived from a human astrocytoma grade IV, were used. Cell lines were cultured in 10 cm dishes and maintained in Dulbecco's Modified Eagle medium (DMEM) with high glucose and phenol red, supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 1 mM antibiotics, 0.1 mM non-essential amino acids and 44 mM sodium bicarbonate (Biowest, USA), and grown at 37 °C under a 95% air and 5% CO_2 atmosphere. 24 h before treatments medium was changed by phenol red and hormone-free DMEM medium (In Vitro, S.A., MEX) supplemented with 10% charcoal stripped FBS, 1 mM pyruvate, 1 mM antibiotics and 0.1 mM non-essential amino acids (Biowest, USA). The following treatments were applied for all experiments: vehicle (cyclodextrin 0.02%), P_4 (10 nM), RU486 (10 μM) and the combined treatment P_4 plus RU486 for different periods of time. Cyclodextrin, P_4 and RU486 were purchased from Sigma Aldrich (USA).

2.2. Cell transfection

To verify if the observed effects are conducted through PR, antisense oligonucleotides (ASO) were used to silence it. D54 cells cultured in the conditions described in the previous section, and 24 hours later, cells were incubated with (a) 0.5 μg phosphorothioated sense oligonucleotide (SO) (5′-TGCCACATGGTAAGGCATAA-3′); (b) 0.5 μg phosphorothioated ASO against total PR (5′-TTATGCCTTACCATGTGGCA-3′) (Sigma Aldrich, USA); and (c) the transfection reagent Lipofectamine 2000™ (Invitrogen, USA). Briefly, Lipofectamine 2000 was mixed with phenol red and hormone-free DMEM medium, supplemented with 0.1 mM non-essential amino acids and incubated for 40 min. Meanwhile, SO and ASO were diluted with the medium described above. Then, the diluted SO or ASO and Lipofectamine 2000 were mixed and incubated for 15 min at room temperature. The resultant mixture was added to each well. 72 h after transfection cells were harvested for Western blotting to detect the content of PR or prepared for migration assay.

2.3. Migration assay

To determine the effect of P_4 on cell migration, we used the scratch-wound assay. In 6-well plates 200,000 cells were seeded in complete DMEM medium and grown until reaching 60–70% confluence, then, the medium was changed to hormone-free DMEM as described above. After 24 h (80–90% confluence) a 200 μL pipette tip was used to generate a linear wound. The floating cells were rinsed with PBS, and hormone-free DMEM medium without phenol red and FBS without hormones and with 10 μM cytosine β-D-arabinofuranoside hydrochloride (Ara-C; Sigma Aldrich, USA), a selective inhibitor of DNA synthesis, was added to the plates. One hour later, hormone treatments were added and photographs were taken with an Infinity 1-2C camera (Lumenera, CAN) attached to an inverted microscope (CKX41, Olympus, JPN) at 100× magnification of the scratch area at 0, 3, 6, 12, 24 and 48 h. At 24 h, before taking photographs, fresh medium with Ara-C and steroids were replaced since Ara-C is retained by the cells only for 24 h [32]. The number of migrating cells was counted from 4 randomly selected fields using the public domain program ImageJ1 developed at the National Institutes of Health (NIH, USA).

2.4. Invasion assay

Cells were grown as described in the "Cell culture and treatments" section. Transwell inserts (8.0 μm membrane; Corning, USA) placed in a 6-well plate were covered with 2 mg/mL matrigel (Sigma Aldrich, USA) that was diluted in DMEM red phenol and FBS free. 1 mL of this dilution was placed in the insert and immediately incubated at 37 °C for two hours under a 95% air and 5% CO_2 atmosphere. Then, 300,000 cells suspended in 1.5 mL serum-free