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The role of DNA methylation and histone acetylation in the regulation of progesterone receptor isoforms expression in human astrocytoma cell lines

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ABSTRACT

Many progesterone (P4) effects are mediated by its intracellular receptor (PR), which has two isoforms, PR-A and PR-B, each of them with different function and regulation. Differential PR expression in cancer cells has been associated to a PR isoform-specific promoter methylation. In astrocytomas, the most frequent and aggressive brain tumors, PR isoforms expression is directly correlated to the tumor's evolution grade. However, there is no evidence of the role of epigenetic regulation of PR expression in astrocytomas. We evaluated the effect of the demethylating agent 5-aza-2′-deoxycytidine (5AzadC) and the histone deacetylase inhibitor trichostatin A (TSA) on PR expression in human astrocytoma cell lines U373 (grade III) and D54 (grade IV) by RT-PCR and Western blot. Total PR expression increased with 5 μM 5AzadC treatment, whereas PR-B expression increased with 5 and 10 μM 5AzadC treatment in U373 cells, but not in D54 cells. In U373 cells, PR-A protein content augmented with 10 μM 5AzadC treatment, while PR-B content increased with 5 and 10 μM 5AzadC. PR-B expression was not modified by the TSA concentrations that were used, and the combination with 5AzadC did not change the effects of the latter. The study of 5AzadC effects on the number of astrocytoma cells showed that P4 treatment increased the number of U373 cells, whereas 5AzadC and the combined treatment with P4 reduced it. Our results suggest that PR-B expression is regulated by methylation and not by histone acetylation in U373 cells, and that DNA demethylation reduced the number of U373 cells.

1. Introduction

Astrocytomas are the most common primary brain tumors in humans and are predominantly found in adults between 45 and 79 years old. These tumors are classified according to their histological and molecular characteristics into four grades (I–IV) [1,2]. Grade III and grade IV astrocytomas are defined as high-grade gliomas from which grade IV tumors or glioblastomas are the most frequent and aggressive ones. The survival of patients is inversely related to the tumor's grade. The median survival rate after treatment with surgery or chemo-and radiotherapy could be high after treatment for grade I tumors; about seven years for patients with grade II astrocytomas; three to four years for grade III tumors and nine to eleven months for glioblastomas [3,4].

Progesterone (P4) is involved in the regulation of several physiological and pathological processes, including brain tumor growth [5–8]. This steroid hormone regulates several cell functions through the interaction with its intracellular receptor. Two PR isoforms have been reported: a full-length form PR-B (114 kDa) and an N-terminal truncated one PR-A (94 kDa), whose regulation and function differ from one another. PR isoforms are transcribed from a single gene (11q22-23 in humans) under the control of distinct promoters [9]. Both PR isoforms have been detected in human astrocytomas biopsies and derived cell lines [6,10], and their expression is directly related to the tumors evolution grade [5,11,12]. PR-B is the predominant isoform in grade III and grade IV astrocytomas [6]. *In vitro* studies show that the PR-B/PR-A ratio is 3:1 for a grade III astrocytoma derived cell line (U373) and 0.66:1 for a grade IV derived cell line (D54) [10]. This differential PR isoform expression could be attributable to a PR promoter dependent regulation mechanism [13].

Gene expression can be regulated by epigenetic modifications such as DNA methylation, specifically the addition of a methyl group to the carbon 5 of a cytosine within the dinucleotide CpG. DNA methylation is normally inversely correlated to gene expression and it is strongly associated with CpG islands—DNA regions with a high CpG site frequency located in the 5′ regulatory region

Abbreviations: PR, progesterone receptor; P4, progesterone; 5AzadC, 5-aza-2′-deoxycytidine; TSA, trichostatin A.

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and first exon of genes [14]. Cancer cells show a drastic change in DNA methylation pattern, generally exhibiting global DNA hypomethylation but hypermethylation of certain genes, mainly tumor-suppressor genes [15–17]. In addition to DNA methylation, histone modifications such as acetylation and deacetylation play an important role in gene expression regulation. Histone acetylation favors gene expression by opening chromatin structure [18]. In cancer cells, hypermethylation of tumor-suppressor gene promoters has been associated with the deacetylation of histone H3, which is normally acetylated [19].

Reactivation of epigenetically silenced genes has been possible when using different drugs such as 5-aza-2′-deoxycytidine (5AzadC), which is a well-known demethylating agent that is activated *in vivo* and incorporated into DNA during replication [20]. As a result of 5AzadC treatment, many hypermethylated genes including steroid hormone receptors are re-expressed [21–24]. Furthermore, histone deacetylase (HDAC) inhibitors have become of interest as potential anticancer agents. Trichostatin A (TSA) is a selective inhibitor of class I and II of mammalian HDACs [25] that exerts a potent antitumor activity when treating gliomas, breast, lung, and prostate cancer [26–28].

As other steroid hormone receptors, the PR gene is a DNA methylation target. The PR gene has CpG islands in its first exon and in the promoter region of PR-A, while the PR-B promoter has no CpG islands. In spite of this CpG island distribution, differential PR isoform expression in several cancer cells has been associated to a PR isoform-specific promoter methylation [13,29,30]. In breast cancer cells, the PR-A promoter is hypermethylated, while in prostate cancer cell lines both PR isoforms are silenced by methylation, and the treatment with 5AzadC leads to reactivation of functional PR expression in both types of cancer cells [31–33]. Additionally, both PR gene promoters have been found to be hypermethylated in different leukemia cell lines [34]. Furthermore, in endometrium cancer cells, the treatment with both 5AzadC and TSA had a synergistic effect, restoring and up-regulating PR gene expression [35,36]. However, the regulation of PR isoforms expression by DNA methylation and histone acetylation has not been reported in astrocytomas. The aim of this work was to evaluate the effects of 5AzadC and TSA on PR expression in two human astrocytoma derived cell lines of grade III (U373) and IV (D54).

2. Experimental

2.1. Cell culture and treatments

Human astrocytoma derived cell lines U373-MG (ATCC, Manassas, VA) and D54 (obtained by Dr. Andrés Gutiérrez from Dr. Sontheimer, Birmingham, AL) were used. 5 x 10^5 cells were plated in 10 cm dishes and maintained in DMEM medium (*In Vitro*, S.A., MEX) supplemented with 10% fetal bovine serum, 1 mM pyruvate, 2 mM glutamine, 0.1 mM non essential amino acids (Invitrogen, CA). Cells were maintained at 37 °C under a 95% air and 5% CO_2 atmosphere. Cells were treated with 5 μM or 10 μM 5AzadC (Sigma Aldrich, MO) during 4 days, and the medium was changed with daily supplementation of fresh 5AzadC. HDAC inhibitor TSA was used to treat U373 cells at 250 nM, 500 nM and 1 μM for 24 h. For the combination study, 5AzadC (5 μM) was present in the culture for 96 h, and TSA was added for the last 24 h.

2.2. RNA isolation and RT-PCR

Cells were washed and lysed using a guanidine isothiocyanate solution, and total RNA was isolated as instructed by the RNeasy Mini Kit (Qiagen, CA). 5 μg of total RNA were used for cDNA synthesis, which was carried out using M-MLV reverse transcriptase and oligo dT_{18} primers according to the manufacturer's recommended protocol (Invitrogen, CA). Reverse transcription (RT) was carried out for 60 min at 37 °C and inactivated at 4 °C. After incubation, 2 μl of RT reaction were subjected to PCR in order to amplify a PR fragment common to both isoforms (total PR), a region unique to PR-B, and a region of 18S ribosomal RNA, which was used as an internal control. The sequences of the specific primers for total PR amplification were: 5′-[ACATGGTAGCTGTGGGAAGG]-3′ for the forward (FW) primer and 5′-[GCTAAGCCAGCAAGAAATGG]-3′ for the reverse (RV) primer. The primers used for PR-B amplification were FW-5′-[AGCCGCAGGTCCGTT]-3′ and RV-5′-[CACGTCCGACAGCGACT]-3′; whereas primers for 18S ribosomal RNA amplification were FW-5′-[CGAACGTCTGCCCTATCAAC]-3′ and RV-5′-[TTGGATGTGGTAGCCGTTTC]-3′. The 25 μl PCR reaction was first denatured at 95 °C for 3 min and afterwards 35 cycles were performed. Each cycle was carried out under the following conditions: 95 °C, 0.5 min; 63 °C (total PR) or 56 °C (PR-B), 0.5 min; and 72 °C, 0.5 min. A final extension cycle was performed at 72 °C for 1 min. PCR products were separated on 2% agarose gels and stained with the GelRed Nucleic Acid Gel Stain (Biotium, Inc., CA). The image was captured under a UV transilluminator using a digital Canon camera (PowerShot SD1300IS, USA). The intensity of PR and 18S bands was quantified by densitometry using the ChemiImager 4400 software (ProteinSimple, CA), and total PR and PR-B expression levels were normalized to that of 18S.

2.3. Protein extraction and Western blotting

5×10^5 U373 cells were plated in 10 cm dishes and maintained and treated with 5AzadC as previously described. After 4 days of treatment, cells were homogenized in RIPA lysis buffer with protease inhibitors (1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 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, CA). Proteins (90 μg) were separated by electrophoresis on 8% SDS–PAGE at 20 mA. Coloured markers (Bio Rad, CA) were included for size determination. Gels were transferred to nitrocellulose membranes (Millipore, MA) at room temperature for 7 h at 35 mA in semi dry conditions. Membranes were blocked at 4 °C with a 5% membrane blocking agent (GE Healthcare, UK) for 24 h and then incubated with a mouse monoclonal antibody against both PR isoforms (3 μg/ml, N559 Abcam, MA). Blots were then incubated with a 1:5000 dilution of a secondary antibody (goat anti-mouse IgG, Santa Cruz Biotechnology, Inc., CA) conjugated to horseradish peroxidase for 45 min.

In order to correct the differences in the amount of total protein loaded in each lane, protein content was normalized to that of α-tubulin. Blots were stripped with glycine (0.1 M, pH 2.5, 0.5% SDS) at 4 °C overnight and were reprobed with a 1:5000 dilution of mouse anti-α-tubulin monoclonal antibody (T9026, Sigma Aldrich, MO) at room temperature for 24 h. Blots were incubated with a 1:5000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., CA) at room temperature for 45 min. Signals were detected by enhanced chemiluminescence (ECL) (Thermo Scientific, CA), and films were measured for band density in a semiquantitative way using the HP Scanjet G3110 Photo Scanner (Hewlett–Packard, CA) and ChemiImager 4400 software (ProteinSimple, CA). In order to minimize interassay variations, all Western blots were carried out in parallel.

2.4. Cell number and treatments

1×10^3 U373 cells were grown in 48-well plates and maintained as indicated in the cell culture section. 24 h prior to treatment application, the medium was changed to DMEM phenol red free medium supplemented with 10% fetal bovine serum without