Journal of Steroid Biochemistry and Molecular Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Progesterone arrested cell cycle progression through progesterone receptor isoform A in pancreatic neuroendocrine neoplasm

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ARTICLE INFO

Keywords:
Neuroendocrine neoplasm
Progesterone receptor
Cell cycle
Cyclin
Cyclin dependent kinase
PCR array

ABSTRACT

In pancreatic neuroendocrine neoplasms (Pan-NEN) progesterone signaling has been shown to have both inhibitory and stimulatory effects on cell proliferation. The ability of progesterone to inhibit tumor proliferation is of particular interest and is suggested to be mediated through the less abundantly expressed progesterone receptor (PR) isoform A (PRA). To date the mechanistic processes underlying this inhibition of proliferation remain unclear. To examine the mechanism of PRA actions, the human Pan-NEN cell line QGP-1, that endogenously expresses PR isoform B (PRB) without PRA, was transfected with PRA. PRA transfection suppressed the majority of cell cycle related genes increased by progesterone including cyclin A2 (CCNA2), cyclin B1 (CCNB1), cyclin-dependent kinase 1 (CDK1) and cyclin-dependent kinase 2 (CDK2). Importantly, following progesterone administration cell cycle distribution was shifted to S and G2/M phases in the naïve cell line but in PRA-transfected cells, this effect was suppressed. To see if these mechanistic insights were confirmed in patient samples PRA, PRB, CCNA2, CCNB, CDK1 and CDK2 immunoreactivities were assessed in Pan-NEN cases. Higher levels of cell cycle markers were associated with higher WHO grade tumors and correlations between the markers suggested formation of cyclin/CDK activated complexes in S and G2/M phases. PRA expression was associated with inverse correlation of all cell cycle markers. Collectively, these results indicate that progesterone signals through PRA negatively regulates cell cycle progression through suppressing S and G2/M phases and downregulation of cell cycle phases specific cyclins/CDKs.

1. Introduction

Progesterone Receptor (PR) is a member of steroid hormone family of ligand-activated transcription factors, with known expression in various non-neoplastic and neoplastic tissues [1]. Human PR is present mainly as two isoforms, PRA and PRB, derived from a single gene by alternative transcriptional promoters [2]. These two isoforms play distinctive functional roles including the regulation of different subsets of genes and importantly regulation of each other [3–5]. Currently the prime non-transcriptional function of PRA is thought to be the suppression of the proliferative actions of PRB [4,5]. This suggests a mechanism underlying the observations of overexpression of PRB associating with poor prognosis in endometrial, cervical, and ovarian cancer patients [6,7] and differential physiological responses to progesterone therapy between PRA-negative and positive carcinoma cells [8].

In non-neoplastic pancreatic cells, cell proliferation is negatively regulated by PR [9,10]. A role for PR in the regulation of proliferation in pancreatic cells is also suggested by PR knockout mice where larger mass of pancreatic islets are detected compared to wild-type littermates [11]. PR is expressed in approximately half of pancreatic neuroendocrine neoplasm (Pan-NEN) [9,10,12] and its incidence is significantly higher compared to the same NEN arising in other organs [13]. This suggests the PR expression in NEN to be specific to the tissue of origin, and of interest in developing potential therapeutic interventions. As in the non-neoplastic studies above, in Pan-NEN, PR expression was also inversely correlated with cell proliferation [10] but also associated with disease progression [10] and poor clinical outcome of patients [13]. In

Abbreviations: Pan-NEN, pancreatic neuroendocrine neoplasm; PR, progesterone receptor; PRA, PR isoform A; PRB, PR isoform B; CCNA2, cyclin A2; CCNB1, cyclin B1; CDK1, cyclin-dependent kinase 1; CDK2, cyclin-dependent kinase 2; WHO, World Health Organization

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https://doi.org/10.1016/j.jsbmb.2018.01.003
Received 7 April 2017; Received in revised form 30 November 2017; Accepted 4 January 2018

cell line models of Pan-NEN, marked inhibitory effects of progesterone through PR were also reported [10,13].

One of the traditional models of PR action involves interplay with the estrogen receptor (ER) (e.g. uterus, ovary, breast), however ER expression is extremely low in Pan-NEN [13]. This suggests that any PR effects observed are directly related to PR actions rather than interplay with ER. In actuality, ER-independent inhibitory mechanisms of PR have been identified previously in breast and endometrial cancers and have suggested PR as a therapudic target in these pateints [14–16] this providing precedents and suggested mechanisms for direct targeting and actions of PR in Pan-NENs. These inhibitory actions in breast and endometrial cancers are principally thought to be through the well-documented actions of progesterone on cell cycle genes [15]. In breast carcinoma cell lines expressing PR, cell cycle progression was reported to be accelerated by progesterone administration. However, the increased ratio of cell population in G2/M and S phases was transient and progestin treatment of these cells led to cell arrest in G1 phase and growth inhibition in 22–24 h following the treatment [17]. In addition, progesterone increased the expression of several cell cycle regulators; cyclin B1 (CCNB1), cyclin D1 (CCND1) and cyclin E in endometrial cancer, indicating these cyclins being the key cell cycle regulators implicated in endometrial cell proliferation [18]. Among these cyclins, CCND1 was first reported to be upregulated by progesterone administration in breast cancer [19] and known to regulate cell proliferation during the late G1 phase of cell cycle [20]. Progesterone also drives cell cycle progression by activating other cyclins [19]. We also previously reported that CCND1 abundance was associated with an activation of PRB bound by progesterone in Pan-NEN [10]. However, the association of PR with cell cycle regulation has been virtually unknown in Pan-NEN.

In Pan-NEN, we have recently reported the critical roles of PRA expression in human islets and Pan-NEN cells and the status of PRA was inversely associated with Ki-67 labeling index (LI) and World Health Organization (WHO) grades of individual tumors [10]. In addition, PRA may play a role in inhibition of tumorigenesis in Pan-NEN through the progesterone signaling [10]. However, the molecular mechanisms underlying negative regulation of PRA in the growth of Pan-NEN cells has not been clarified yet. Furthermore, it is still unclear whether the positive regulation of Pan-NEN through progesterone is mediated by cell cycle molecules in a cell cycle phase specific manner. Therefore, in this study, we focused on expression of cell cycle regulator genes, their relevant proteins, proliferative activity of tumor cells and their association with progesterone and PRA status in Pan-NEN. This should provide us with more precise markers for progesterone and PRA actions in Pan-NEN cells and lead to new hormonal therapy in the patients of Pan-NEN.

2. Materials and methods

2.1. Cell line and culture conditions

We employed a human Pan-NEN cell line, QGP-1 (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan). The selection of this particular cell line was based on our previous study; within four different human NEN cell lines (QGP-1; derived from pancreas, COLO 320DM; derived from colon, NCI-H727; derived from lung, KRJ-1; derived from intestine), only QGP-1 demonstrated high expression of PRB and showed a significant difference in the proliferation and CCND1 expression in PRA-transfected cells [10]. While, other human Pan-NEN cell lines are available, it is less certain whether they represent the most common Pan-NENs [21] and thus we did not use them in this study. The neuroendocrine differentiation of QGP-1 cell line has been confirmed previously using immunocytochemistry [10]. The cells were cultured in RPMI 1640 medium (Sigma-Aldrich Co., St. Louis, Mo., USA) containing 10% fetal bovine serum (FBS; Nichirei Co. Ltd., Tokyo, Japan). The cells were maintained at 37 °C, 95% relative humidity and 5% CO2. Forty-eight hours prior to treatment, the medium was replaced. The cells were then incubated with phenol red-free medium containing 10% dextran-coated charcoal-stripped FBS with Progesterone (1 nM, Sigma-Aldrich Co., St. Louis, Mo., USA) or vehicle (0.1% ethanol) for 3, 6, 12, 24 and 48 h [10].

2.2. PRA plasmid construction and transfection

QGP-1 cell line has basal expression of PRB. In order to study the effects of PRA on PRB induced cell proliferation by progesterone, the cells were transfected by exogenous PRA. The main logic for focused on PRA overexpression was based on the fact that in our previous study, we immunolocalized PR isoforms in Pan-NEN cases and the results demonstrated significantly higher expression of PRA in non-neoplastic cases and its expression decreased in NEN with higher grade. Among Pan-NENs, the distribution of PRB expression among different NEN groups did not show any significant differences [10]. From this, we hypothesized that the ratio of PRA/PRB expression could be involved in tumorigenesis of Pan-NEN. However, we did not detect any correlation between PRA/PRB ratio and clinicopathological variables of the cases. We also tentatively classified the cases examined into 4 different groups based on PRA/PRB status as: PRA/PRB both positive, PRA/PRB both negative, PRA positive/PRB negative and PRA negative/PRB positive groups and further evaluated the correlation of these groups with clinicopathological variables. However, we also did not find any correlation in these groups [10]. This made us to specifically focus on the possible role of PRA in tumorigenesis of Pan-NEN patients and we subsequently performed *in vitro* studies using PRA overexpressed QGP-1 cells.

The construction of plasmid encoding human PRA has been described in our previous report [10]. Briefly, a full-length cDNA fragment generated by RT-PCR, was inserted into a pcDNA 3.1 (−) expression vector (Invitrogen, Tokyo, Japan) using the following DNA primers designed for the PRA gene: forward, 5′-AAA GCT AGC GCT CATGAG CCG GTC CGG-3′, and reverse, 5′-AGA AAT CTA GATCAC TTT TTA TGA AAG AGA AGG GG-3′. The construct was transfected into QGP-1 cells using Lipofectamine 2000 reagent (Invitrogen, Tokyo, Japan). pcDNA3.1(–) empty vector was used as a negative control. An immunoblot from nuclear protein was carried out in order to prove and evaluate the transient transfection of PRA.

2.3. Real-time RT-PCR

Total RNA was carefully extracted from QGP-1 cells treated with progesterone for 3, 6, 12, 24 and 48 h using TRIzol method (Invitrogen, Carlsbad, Calif., USA). Reverse transcription kit was used in the synthesis of cDNA (Qiagen, Hilden, Germany) as well as the FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences used in this study are summarized in Table 1. All the samples without PRA transfection, were transfected by pcDNA3.1(–) empty vector. The mRNA levels in each sample were represented as a ratio of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level compared with control levels. T47D breast carcinoma cells were used as positive controls for studying the PR isoforms [10].

2.4. Antibodies

The characteristics of antibodies used for Immunoblotting and immunostaining are summarized in Table 2. Monoclonal antibodies against PRA (hPRa7) and PRB (hPRa2) were purchased from Neo-Markers (Fremont, Calif., USA). The hPRa7 antibody used in this study was reported not to recognize PRB even after antigen retrieval, as demonstrated by the absence of immunoreactivity in the PRB-expressing MDA-MB-231/PRB cell line [22]. This has been proposed to be due to the inaccessibility of the epitope on PRB detected by hPRa7 in 10% formalin-fixed and paraffin-embedded tissue samples [22,23]. PRB