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The regulation of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ gene expression in response to hydrocortisone, progesterone and ß-estradiol in HL-60 myeloid leukemia cells

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ABSTRACT

Steroid agents have crucial regulatory effects on cell differentiation and proliferation of different cell types. In this study, the influence of progesterone, ß-estradiol and hydrocortisone on the expression of cyclin dependent kinase inhibitor (CDKI) genes, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ was assessed in HL-60 myeloid leukemia cells. Although treatment with these steroid agents did not significantly change the morphology of HL-60 cells, expression levels of p21 and p27 genes were modulated. p57 gene was not expressed in HL-60 cells. The overall data implied that G1/S transition in myeloid leukemia cells may be primarily regulated through $p21^{Waf1/Cip1}$ and with a lesser extent through $p27^{Kip1}$ expression upon incubation with steroids. [Turk J Cancer 2008;38(4):184-189]

KEY WORDS:

HL-60, myeloid leukemia, CDKIs, steroid hormones

INTRODUCTION

HL-60 cell line was derived from a patient diagnosed as acute promyelocytic leukemia (APL) but HL-60 cells does not have t(15;17) chromosomal translocation which is characteristic of APL cells (1,2). As the cells are arrested at the promyelocytic differentiation stage, they correspond to immature myeloid cells (3). Besides, a proportion of HL-60 cells may differentiate spontaneously into more mature cells of the granulocytic and monocytic lineages (2). HL-60 cell line has been a candidate for studying the differentiation of leukemia blasts in the presence of certain agents. However, the resulting mature phenotype may not be identical with normal mature cells (3-5).

Besides their particular morphology, the mature myeloid cells can be distinguished with immunophenotypic changes and with a limited proliferation capacity. The proliferation arrest is mainly observed in cell cycle checkpoints. Several factors known as cyclin dependent kinase inhibitors (CDKIs) function as negative regulators of the cell cycle. There are two families of CDKIs: INK4 inhibitors and CIP/KIP inhibitors. Of the members of

CIP/KIP family $p21^{Waf1/Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ can inhibit cyclin dependent kinases in any phase of the cell cycle, especially during G1/S transition (6,7).

Phorbol esters (e.g. phorbol 12-myristate 13-acetate (PMA), 12-O-tetradecanoyl phorbol-13-acetate (TPA)) are widely used as potent agents for HL-60 differentiation studies. These agents work through protein kinase C (PKC) molecules and activate transcription factors such as AP-1, NF-κB and mitogen-activated protein kinases (MAPKs) (1,8). PMA may induce proliferation in some cell types, but it may lead to growth arrest in others such as HL-60 via blocking the cell cycle progression in G1/S checkpoint utilizing $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ molecules (9).

It is well known that steroid hormones such as progesterone, β-estradiol and hydrocortisone play essential roles in differentiation and proliferation of cancer cells. In breast cancer cell lines, early effect of progestagens is acceleration of G1-S transition; however, following the completion of cell cycle, the cells are arrested at G1 phase via increased expression of $p21^{Waf1/Cip1}$. In addition, prolonged treatment with progesterone can result in overexpression of and leads to differentiation and/or apoptosis in other cell types (10). On the other hand, estradiol regulates cell cycle progression and impairs the $p21^{Waf1/Cip1}$- and $p27^{Kip1}$-associated cell cycle arrest. Therefore, estrogen can reduce the amount of CDKI associated CDKs and promote cell cycle progression (11). Hydrocortisone, acting through glucocorticoid receptors, can interfere with cell proliferation in G1 and S phases by increasing the expression of CDKIs (12).

The aim of the current study was to investigate the effect of the steroid agents, hydrocortisone, progesterone and β-estradiol, on the expression of cell cycle regulatory molecules $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ in acute myeloid leukemia cell line HL-60 in accordance with morphological features.

MATERIALS AND METHODS

Cell culture

The HL-60 cell line is accepted as a convenient model to study human myeloid cell differentiation to granulocyte-like or monocyte-like cells (9). The HL-60 cell line was obtained from the American Type Culture Collection (LGC Promochem, Rockville, MD, USA) and maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37ºC in a humidified 5% CO_2 incubator. The cells were used at seventh passage and were cultured ($2x10^5$) in 24-well tissue culture plates (Nunc, Kamstrupvej, Denmark). For experimental procedures the cells were treated with progesterone, β-estradiol, hydrocortisone and PMA at varying concentrations. Otherwise specified, all reagents were obtained from Sigma (St. Louis, MO, USA).

Assessment of cell morphology and viability

Cell viability was determined according to propidium iodide (PI) exclusion method, and the viable cells were gated for morphological analysis by flow cytometry (EPICS XL-MCL, Beckman Coulter, Fullerton, CA, USA). The size and granularity of the cells were determined with forward scatter (FS) and side scatter (SS) parameters, respectively. The morphology was also confirmed by cytochemical staining using the standard Giemsa procedure.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated with QIAamp® RNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. cDNA was synthesized from 2 µg of RNA, using oligo(dT) primers and RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to manufacturer's instructions. Previously reported primer oligonucleotide sequences were used to amplify $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ genes (13). B-actin gene was amplified as house keeping gene. PCR conditions are shown in Table-1. The cycling conditions for β-actin and $p21^{waf/cip}$ were 30'' at 94°C; 30'' at 58°C; 45'' at 72°C; for $p27^{Kip1}$ were 30'' at 94°C; 30'' at 55°C; 45'' at 72°C, and for $p57^{kip2}$ were 30'' at 94°C; 30'' at 62°C; 45'' at 72°C. RNA samples isolated from peripheral blood mononuclear cells (PBMCs) were used for positive control amplifications.

Preliminary reactions had been set to determine PCR cycle number which meets logarithmic amplification

Table 1
Final concentrations of the reagents used for $p21^{waf/cip}$, $p27^{kip1}$ and $p57^{kip2}$ RT-PCR

Reagent	Final concentration
Taq Buffer	1x
dNTP	0.2 mM
$MgCl_2$	2.5 mM
Primer forward	0.2 µM
Primer reverse	0.2 µM
Taq DNA Polymerase	0.05 U/µL

phase for each primer pair. Three sequential independent PCR amplifications were performed for each gene and reactions were terminated at the predetermined cycle. Products were resolved on 1% agarose gels and visualized with ethidium bromide staining under UV light.

RESULTS

The effect of steroid agents on HL-60 morphology

Differentiation of HL-60 cells can be distinguished with the changes in cell morphology as increase in size and granularity (14). The highest concentrations of the steroid agents not interfering with the viability of the cells were preliminarily determined as 4.0 ug/mL for hydrocortisone, 0.5 ug/mL for progesterone, 50 ng/mL for β-estradiol (data not shown). Following 96 hours of incubation, morphological features of the cells were examined by flow cytometry analysis. There was no significant difference in the cellular morphology between the control group and the steroid-treated groups. However, in comparison with the control and steroid-treated groups; the PMA-treated HL-60 cells were larger in size and their granularity were significantly increased (Figure 1A and B). Therefore, PMA treatment was used as a positive control for morphological differentiation. Moreover, it is found that granularity of the HL-60 cells is more affected than the size by the PMA treatment since there is a higher increase in the SS value than the FS value when compared to control group (Figure 1B).

The expression of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ genes in steroid-treated HL-60 cells

Cellular differentiation is usually accompanied with changes in cell cycle dynamics especially regulated at G1/S transition check point (10). Therefore, following the steroid agent treatments, we analyzed the expression of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ genes (Figure 2).

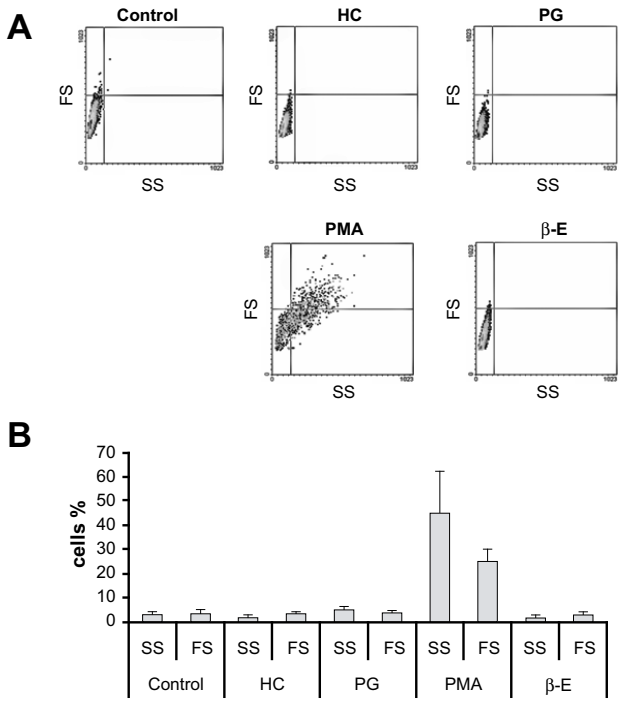

Fig 1(A,B). (A): The morphological features of HL-60 cells treated with hydrocortisone (HC; 4.0 ug/ml), progesterone (PG; 0.5 ug/ml), phorbol 12-myristate 13-acetate (PMA; 10 nM), β-estradiol (β-E; 50 ng/ml) was analysed by flow cytometry. Representative results are shown. (B): Size (FS) and granularity (SS) values of three independent experiments are displayed in percentages

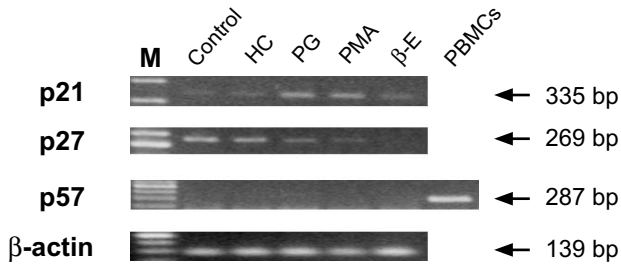

Fig 2. The expression of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ genes in steroid-treated HL-60 cells. cDNA synthesized from PBMCs were used as positive control for $p57^{Kip2}$ RT-PCR
M: 50 bp DNA marker; HC: hydrocortisone; PG: progesterone; PMA: phorbol 12-myristate 13-acetate; β-E: β-estradiol)

In the HL-60 cells which are incubated without steroid agents, $p21^{Waf1/Cip1}$ expression was lower than $p27^{Kip1}$ whereas no $p57^{Kip2}$ expression was observed. Highest expression of $p21^{Waf1/Cip1}$ was detected in progesterone-treated HL-60 cells; on the other hand, $p27^{Kip1}$ was moderately decreased in these cells compared to the control group. All agents caused a decrease in $p27^{Kip1}$ expression with β-estradiol causing the highest decrease. Furthermore, hydrocortisone had the mildest effect on HL-60 cells in terms of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ expression when compared to control cells. As the agent resulting in morphological differentiation, PMA treatment increased the $p21^{Waf1/Cip1}$ expression while decreasing the $p27^{Kip1}$ expression. Collectively, a reciprocal relationship was observed between expression levels of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ as a decline in one molecule's expression was found to be accompanied by an increase in the other, or vice versa. With the exception of the β-estradiol-treated cells, the expression of both genes was relatively decreased. Moreover, no $p57^{Kip2}$ expression was detected in none of the experimental groups whereas it could be amplified in PBMCs.

DISCUSSION

In this study, we assessed the impact of steroid hormones, namely hydrocortisone, progesterone and β-estradiol, on the expression of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ genes in HL-60 myeloid leukemia cells. Possible consequences of steroid incubation on the maturation of these blasts were also monitored in morphological aspects during the experimental process.

Steroids are important regulators in normal development of certain tissues such as mammary, endometrial, alveolar and intestinal epithelia (15). Moreover, progesterone and estrogens are implicated in oncogenesis (16). Glucocorticoids are clinically effective for the treatment of prostate cancer (17). It is well established that the steroids influence the cellular differentiation and proliferation by modulating the activities of cyclins and CDKIs (18).

Cyclins, CDKs and their negative regulators, CDKIs, are responsible for regulating cell-cycle checkpoints (6). The CDKIs analyzed in our study are involved in G1/S checkpoint, the first control step after the cells are induced to proliferate and not to differentiate. HL-60 myeloid leukemia cells have a high proliferation index, however they may be subject to some degree of spontaneous differentiation (19). In our study we used the phorbol ester, PMA, a potent agent differentiating the HL-60 cells through protein kinase C pathway (1,20). The expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ genes are implicated in the PMA-induced cell cycle block (9). Accordingly, in our study, a prominent increase in $p21^{Waf1/Cip1}$ expression was observed in PMA-treated cells, on the other hand, $p27^{Kip1}$ was not augmented at the time of analysis.

Estradiol can modulate the cell cycle indirectly through intracellular signalling pathways and directly as a transcription factor. Estrogen favors cell cycle progression, decreases the formation of $p21^{Waf1/Cip1}$- and $p27^{Kip1}$-cyclinE/Cdk2 complex (11). The reducing effect of β-estradiol on CDKI, especially $p21^{Waf1/Cip1}$, expression in HL-60 myeloid leukemia cells may also indicate an increase in proliferation. Accordingly, the acute myeloid leukemia cases in which estrogen receptors are found to be methylated have a relatively better prognosis (21).

In breast cancer cell lines, progesterone is initially proliferative, however, following the completion of the cell cycle the increased expression of $p21^{Waf1/Cip1}$ leads to an arrest in G1/S transition (10). Besides, prolonged exposure to progesterone cause the overexpression of $p21^{Waf1/Cip1}$ resulting in differentiation and apoptosis of the cells other than breast cancer. For the HL-60 cells treated with progesterone, a prominent increase in $p21^{Waf1/Cip1}$ expression was determined. On the other hand, no obvious change in morphology and viability was noted at 96 hours of incubation.

Glucocorticoid receptors are located intracellularly where they bind hydrocortisone in a cell cycle-dependent way. Glucocorticoids are reported to increase the expression of CDKIs, such as $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, favoring cell cycle arrest in G1 or S phase (12). In response to hydrocortisone, the expression of $p27^{Kip1}$ was higher than $p21^{Waf1/Cip1}$ in HL-60. Nonetheless, although a slight increase was seen in $p21^{Waf1/Cip1}$, the expression pattern was not far from the control cells.

$p57^{Kip2}$ gene is not deleted in HL-60 cell line but it is epigenetically inactivated by DNA methylation (7). The absence of the CDKI $p57^{Kip2}$ may increase the importance of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ in cell cycle regulation. Although deletions have been demonstrated in HL-60 cell line, in genes encoding tumor suppressor protein p53, evidences show that growth factors and differentiation-inducing agents may provide p53-independent induction of CDKIs (e.g. $p21^{Waf1/Cip1}$) as an immediate gene response (1,22).

The overall data implied that G1/S transition in HL-60 myeloid leukemia cells may be primarily regulated through $p21^{Waf1/Cip1}$ and with a lesser extent through $p27^{Kip1}$ expression upon incubation with steroids. In a previous study performed by our group, a similar phenomenon was observed in HL-60 cells grown on fibronectin matrix (19). Although $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ demonstrate redundant effects at the G1/S transition, the reciprocal but alternate change in the levels of expression may indicate their differential regulation upon certain stimuli.

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