Cell cycle inhibitors in retinoic acid- and etoposide-mediated biological responses

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It was shown that *all-trans* retinoic acid and its isomer 9-cis retinoic acid inhibit the proliferation and induce differentiation of promyelocytic cell line HL-60 and leukemic cells from patients with acute promyelocytic leukemia. Retinoic acid receptors and retinoid X receptors act as ligand-inducible transcription factors – bind to specific DNA sequences that are located in the regulatory regions of gene and in such a way modulate gene activity. Although the effects of retinoic acid on cell differentiation and apoptosis have been largely studied, the mechanism by which retinoic acid induces these effects is not well understood. In the present study we have examined the expression of cell cycle inhibitor proteins ($p16^{INK4a}$, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$) during both retinoic acid-induced differentiation and etoposide-induced apoptosis. We detected some quantitative changes of investigated cell cycle inhibitors in the cytosol and nucleus of HL-60 cells after induction both with retinoic acid and etoposide.

Key words: granulocytic differentiation, apoptosis, p16, p21, p27

INTRODUCTION

All-trans retinoic acid (RA) and its isomer 9-cis RA have been found to mediate the inhibition of proliferation and induction of differentiation of a variety of leukemia cells and other malignant cell types [1–7]. Retinoids act through their binding and activation of specific nuclear receptor family: RA receptors and retinoid X receptors [6–8]. Several authors [9, 10] have confirmed a relationship between differentiation and cell arrest in the G1 phase of the cell cycle.

Commitment to cell division is regulated by the cyclin-dependent protein kinase (CDK) family which is regulated by activating among various proteins and by cyclin-dependent kinase inhibitors (CKI) which in turn could either inhibit or enhance the activity of cyclin–CDK complexes [11]. Two CDK families are known [12]: the first, INK4 family, which consist of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ and the second, Cip/Kip family includes $p21^{Cip}$, $p27^{Kip1}$ and $p57^{Kip2}$. p21 has been found to participate in a number of specific protein–protein interactions and can modulate multiple biological functions [13]. Some of these interactions affect the cell cycle control; others are related to apoptosis. The unique carboxy-terminal domain of $p21^{Cip1/Waf1}$ associates with the proliferating nuclear antigen (PCNA) and can inhibit DNA replication directly [14]. It was also shown that p21 binds to the N-terminus of c-Myc and suppresses c-Myc-dependent transcription [15]. Chang and co-authors [16] have shown that increased p21 expression can induce genes whose products exhibit anti-apoptotic activities. p21 can also play an important regulatory role in differentiation. The biological functions of p27 are not well understood, but they may share some similarity of the p21 functions. The $p16^{INK4a}$ protein was first identified as a tumor suppressor protein and proposed as a negative regulator of cell proliferation. The loss of $p16^{INK4a}$ was shown to facilitate immortalization in many cellular systems [17] and overexpression of $p16^{INK4a}$ in cells with functional Rb results in G1 arrest [18]. However, the mechanisms of p16, p21 and p27 induction during differentiation are largely unknown.

In the present study we examined the expression of p16, p21 and p27 proteins in promyelocytic HL-60 cells induced to granulocytic differentiation by RA and after treatment with etoposide which induces apoptosis.

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MATERIALS AND METHODS

Cell culture. Human promyelocytic leukemia HL-60 cells were cultured in RPMI 1640 medium (Gibco BRL, Life Technologies) supplemented with 10% fetal bovine serum, 100-units/ml penicillin, and 100 μg/ml streptomycin (NordCell, Sweden) in a 5% CO_2-supplemented incubator at 37 °C. Granulocytic differentiation was induced with 700 nM retinoic acid (RA, Sigma). The degree of differentiation was measured by the cellular reduction of nitroblue tetrazolium (NBT) to insoluble blue-black formazan, after stimulation by phorbol myristate acetate (PMA) [4]. Apoptosis was induced with 68 μM etoposide. The proportion of apoptotic cells was quantified using acridine orange and ethidium bromide staining according to Mercille and Massie [19].

Cell cycle analysis. Untreated or drug-treated cells were prepared as described in [20]. Flow cytometric analysis was done by using a FACscan flow cytometer (Becton-Dickinson, USA), LysisII, Ver 1.1 software. The data were registered on a logarithmic scale. Apoptotic cells were detected on PI histogram as a hypodiploid peak. Fixed cells from second aliquot were stained with PI. Samples were analyzed on a flow cytometer.

Isolation of cytosolic and nuclear proteins. Cytosolic and nuclear proteins were isolated as described by Kulyte et al [21].

Gel electrophoresis and immunoblot analysis. Cytosolic and nuclear proteins were resolved by SDS electrophoresis (SDS/PAGE) in a 7–15% polyacrylamide gradient gel in Tris-glycine electrophoresis buffer. After SDS electrophoresis, proteins were transferred to Immobilon™ PVDF transfer membranes (Millipore). Proteins were then blocked and incubated with antibodies against p16, p21, p27 (Santa Cruz, Biotechnology, Inc.) at 1 μg/ml in PBS containing 0.18% Tween-20, 0.35 M NaCl and 1% BSA. The membranes were subsequently washed with PBS-Tween-20 and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (DAKO, A/S, Denmark). Immunoreactive bands were detected by enhanced chemiluminescence (ECL™ Western blotting detection reagents, Amersham, Life Science), according to the instructions of the manufacturer. Reprobing of the membranes was done according to the ECL Western blotting protocols (Amersham, Life Science).

RESULTS AND DISCUSSION

Time-dependent extent of differentiation and apoptosis in RA-induced and etoposide-treated HL-60 cells. HL-60 cells were cultured in the presence of 68 μM etoposide and 700 nM RA and analyzed for differentiation and apoptosis (Fig. 1). Figure 1 represents the time-dependent number of differentiated and apoptotic cells in proliferating, etoposide-treated and RA-induced differentiating cell culture obtained by morphological methods. The data demonstrate that immediately after treatment with etoposide the level of apoptotic cells remains more or less the same as in proliferating culture. Afterwards the percentage of apoptotic cells in the culture increases drastically 35% (2 h) up to 92% (18 h). After induction of HL-60 cells with RA the level of differentiating and apoptotic cells is proportional in the course of time of differentiation. In a differentiating population, half of cells become apoptotic just after 120 h of induction of differentiation. The proportion of apoptotic cells in the differentiating HL-60 cell culture started to increase from the commitment stage (48 h after induction of differentiation) and after 120 h of differentiation it made about 55% of the population. Typically, the number of differentiated cells increased from 6–8% at 24 h to 60–65% at 120 h after induction of differentiation.

A representative example of flow cytometric analysis of control, apoptotic (3 h etoposide) and differentiated for 96 h with 700 nM RA HL-60 cells are shown in Fig. 2. A hypodiploid peak (subG1) represents the amount of apoptotic cells (AP). After

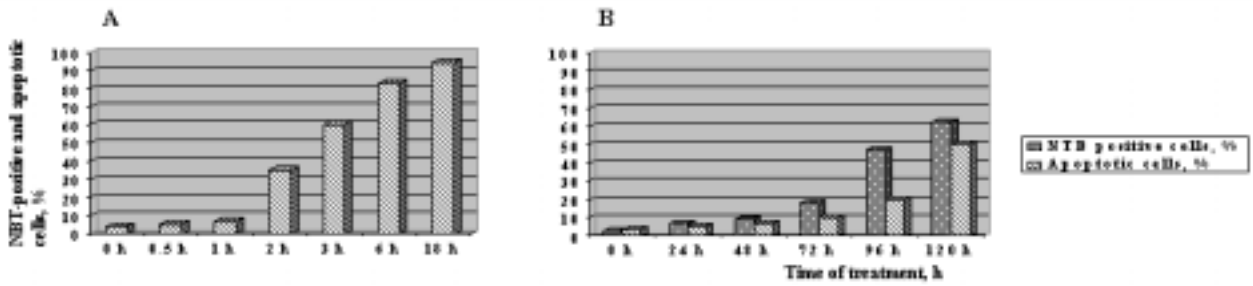

Fig. 1. Kinetics of HL-60 cell differentiation and apoptosis after treatment with etoposide and RA. A – HL-60 cells were treated with 68 μM etoposide for various time (indicated at the upper panel). The apoptotic cells were defined after staining with acridine orange and ethidium bromide. B – HL-60 cells were treated with 700 nM retinoic acid for various time (indicated at the upper panel). The level of differentiated cells was determined by the ability of cells to reduce nitroblue tetrazolium

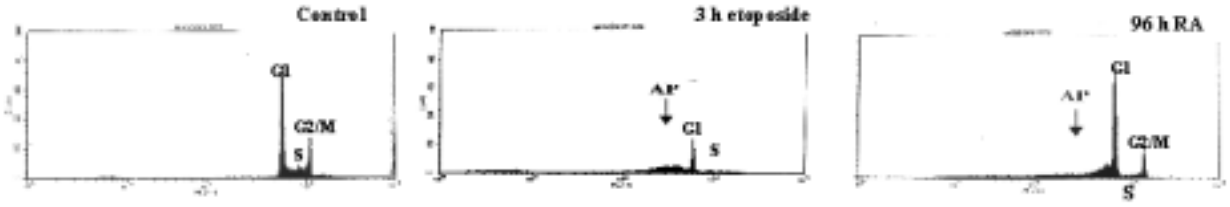

Fig. 2. Flow cytometric analysis of control proliferating, induced to apoptosis and differentiated HL-60 cells. A hypodiploid peak (subG1) represents the amount of apoptotic cells (AP). After 3 h of etoposide treatment the apoptotic cells in the population comprised 60 ± 1.7%. After RA treatment of HL-60 cells for 96 h 62% of differentiated and 50 ± 2.7% of apoptotic cells were detected in the population

exposure of HL-60 cells to etoposide, in a population about 60% of apoptotic cells appear. After cell induction with RA (96 h), the percentage of cells in subG1 phase increased about 10-fold in comparison with control proliferating HL-60 cells. There were 2–3% and 48% of apoptotic and 1–2% and 60–65% of differentiated cells in proliferating and differentiated for 96 h HL-60 cells, respectively. Thus, flow cytometric analysis showed that after induction of differentiation, HL-60 cells started to accumulate in G1 cell cycle phase. It has been shown [22] that the regulation of G1 cell cycle is primarily restricted to the induction of CDKI family members p21, p27, p19 and the reduction of cdk6.

Quantification of cytosolic and nuclear levels of cell cycle control proteins during hematopoietic HL-60 cell apoptosis and differentiation. We investigated changes in the level of cell cycle control proteins during HL-60 cell apoptosis and granulocytic differentiation (Fig. 3). Treatment of cell culture with etoposide causes changes of protein p27 expression both in the cytoplasm and the nucleus – the level of p27 increases immediately during 0.5–2 h of treatment, afterwards it decreases drastically. However, etoposide does not significantly alter the expression of proteins p16 and p21. It slightly increases during the first hours of treatment in the cytosol but not in the nucleus.

A time-course analysis of p16, p21 and p27 shows that RA induces changes in the level of these proteins both in the cytoplasm and in the nucleus. The cytosolic level of p16 slightly increased (36 h) after induction with RA, whereas the nuclear one increased significantly at the same time. It suggests the rapid translocation of this protein into the nucleus. The cytosolic amounts of p21 and p27 proteins were increased (18–36 h) up to six-fold and up to ten-fold, respectively, in comparison with control proliferating cells. The relative amount of p21 and p27 proteins in nuclear extracts increased about 12 h later than that detected in the cytosol of differentiating cells.

In summary, both the cytosolic and the nuclear levels of cell cycle inhibitors p16, p21 and p27 change considerably in differentiating cells cultured with RA but not in apoptotic cells (except p27) treated with etoposide. This suggests that p16, p21 and p27 may have a specific role in differentiation with RA treatment and p27 in apoptotic mechanisms as well.

Fig. 3. Western blot analysis of p16, p21 and p27 proteins in proliferating, differentiated and apoptotic HL-60 cells. Cytosolic (a) and nuclear (b) proteins were isolated from proliferating (C), for various time (0.5, 1, 2, 3, 6, 18 h) treated with 68 μM etoposide and for various time (1, 3, 6, 18, 36 and 96 h) differentiated with 700 nM retinoic acid HL-60 cells and subsequently fractionated by SDS/PAGE as described in Materials and Methods. Immunoblots performed with antibodies against p16, p21 and p27 confirm the expression of these proteins

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LĄSTELĖS CIKLO INHIBITORIŲ ATSAKAS Į RETINOINĘ RŪGŠTĮ IR ETOPOZIDĄ

S a n t r a u k a

Šiame darbe tirtas retinoinės rūgšties ir etopozido poveikis ląstelės ciklo inhibitoriams p16, p21 ir p27 žmogaus promielocitinės leukemijos HL-60 ląstelėse. Srautinės citometrijos duomenimis, diferencijuojančios ir apoptozei indukuotos HL-60 ląstelės kaupiasi G1 ciklo fazėje. Tirtų ląstelės ciklo inhibitorių kiekis citozolyje ir branduolyje kinta priklausomai nuo diferencijuotų ir apoptuotų ląstelių skaičiaus populiacijoje. Manoma, kad p16, p21 ir p27 gali dalyvauti HL-60 ląstelių diferenciacijos, o p27 – ir apoptozės mechanizmuose.