Proliferation and cyclin D3 protein levels are influenced by PKC inhibition with Ro318220 in Jurkat T lymphocytes

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D-type cyclins are important cell cycle regulators. Their function is controlled by intracellular signal transduction cascades, which are activated by extracellular growth factors. To determine the mechanisms by which intracellular signaling pathways influence the progression through cell cycle and cell cycle control proteins, we have analyzed the effect of PKC inhibition on the protein level of cyclin D3 in Jurkat T lymphocytes. Our results show that inhibition of PKC and its signaling pathway by Ro318220 attenuates DNA synthesis of proliferating Jurkat T lymphocytes and downregulates the protein level of cyclin D3. These results suggest a relation between PKC signaling pathway and the cell cycle control proteins in Jurkat T lymphocytes.

Key words: protein kinase C, cyclin D3, cell cycle, Jurkat T lymphocytes

INTRODUCTION

Cell proliferation is regulated by extracellular factors acting primarily during the prereplicative (G1) phase of cell cycle. Growth-stimulatory and inhibitory signals arising from extracellular stimuli via intracellular signaling pathways converge on the cell cycle control proteins that determine whether cells progress through G1 phase [1, 2]. Cyclins constitute the critical components of the core cell cycle machinery in eukaryotic cells. They function to activate and provide substrate specificity to their partners, the cyclin-dependent kinases (cdks) [3]. D-type cyclins (cyclins D1, D2, and D3) share a very similar amino acid sequence (over 70% identity). Cyclins D2 and D3 are predominantly expressed in T cells [4]. The expression of D-type cyclins is controlled largely and perhaps entirely by extracellular signals. Cyclin D1 in particular is rapidly induced following mitogen challenge; its levels rapidly decline when mitogens are withdrawn or when antimitogens are added. For this reason, D-cyclins are regarded as functional links between the extracellular environment and cell cycle machinery [5]. Some of intracellular signaling pathways influence the regulation of D-type cyclins.

One of the intracellular signaling pathways is mediated by the protein kinase C (PKC) family of serine-threonine kinases (conventional, novel, and atypical PKCs). They are involved in the regulation of a wide variety of fundamental cellular processes, including cell growth and cell cycle progression, differentiation, and apoptosis [6–8]. Recently a functional link between PKC and cell cycle control in yeast has been demonstrated [9] and evidence has began to emerge suggesting a role of PKC in cell cycle control [10–12]. However, the effects of PKC activators on the progression of cell cycle appear to be dependent on the cell type. For example, PMA stimulates DNA synthesis in some cells such as Swiss/3T3 cells and lymphocytes, while it causes inhibition of DNA synthesis in rat 3Y1 cells and vascular smooth muscle cells [13, 14]. In several publications, the altered expression of specific cyclins was corelated with PKC activation. The majority of studies point towards cyclin E and A as targets of PKC regulation during G1/S transition in vascular smooth muscle cells and vascular endothelial cells [15]. Less is known about D type cyclins.

As to T cell activation and proliferation, to date it is known that PKCθ could be involved in transcriptional activation of the IL-2 and IL-2Ra genes via modulation of transcription factors AP1 and NF-κB activity [16, 17]. PKCβ is critical to the export of IL-2 molecules from T cells [18]. PKC enzymes (PKCα and PKCε) were suggested as activators of Raf-1, which would lead to the activation of the Raf/MEK/ERK signaling pathway in T cells [19, 20].

It is known that ERK signal is required for progression through G1 phase and involved in the expression of the cell cycle control proteins [21]. These findings suggest that the PKC signaling pathway has a role in mediating signals for activation and proliferation of T lymphocytes. However, very little is known about the molecular mechanisms that link PKC to cell cycle control in T lymphocytes. In the current study, we examine the role of PKC in the regulation of cyclin D3 protein levels and proliferation of Jurkat T lymphocytes.

MATERIALS AND METHODS

Reagents. Ro318220 was from Alexis (Switzerland). RPMI1640 was from Biochrom (Germany). Anti-cyclin D3 was obtained from Transduction Laboratories (Lexington, KY). FBS was from SumBiotech (USA). BCIP/NBT was purchased from Bio-Rad Laboratories (USA). All other materials were purchased from Sigma (USA).

Cell culture. Jurkat leukemic T cells (E6. 1, ATCC) were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 unitsml penicillin, 100 μgml streptomycin and 2 mM glutamine. Cells were grown in 5% CO_2 at 37 °C.

Flow cytometry. Samples were prepared for flow cytometry following the recommendations of Becton Dickinson. Cells were fixed in 70% ethanol and then stored at 4 °C until use. Cells were then washed with PBS, treated with RNase (50 u/ml) and stained for 30 min at room temperature with propidium iodide (50 μg/ml in PBS). Analysis was performed on a Becton Dickinson FACSCalibur (10000 cells collected). The data were analysed using CellQuest program.

Western blot analysis. Jurkat T cells were cultured in RPMI 1640 medium supplemented with 10% FBS in T25 flasks at a cell density of 7.5×10^6 cells per flask. Cells were treated with 1 μM inhibitor Ro318220 for 1 h, 4 h, 8 h, 16 h, 24 h. Following treatment the cells were washed with ice-cold PBS containing 100 μM Na_3VO_4 and then lysed at 4 °C in a lysis buffer containing 1% Nonidet P-40, 50 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1mM phenylmethylsulphonyl fluoride) and phosphatase inhibitors (1 mM Na_3VO_4, 50 mM NaF). After removal of cellular debris by centrifugation at 14000 g for 14 min at 0 °C, the supernatants were collected and protein concentrations were determined using the Bio-Rad protein assay. Then the lysates were boiled for 5 min in a reducing 2 × × SDS sample buffer (62 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 50 mM DTT; 0.1% bromphenol blue). The lysates were subjected to SDS-PAGE (50 μg of protein per lane), transferred to PVDF membrane and analyzed with anti-cyclin D3.

RESULTS AND DISCUSSION

Cell cycle analysis of Jurkat T cells before and after inhibition of PKC with Ro 318220. As previously described, PKC participates in T lymphocyte activation and proliferation as well as in other cell types [18, 19, 22]. To further define the nature of this PKC actions and to explore the effects on the cyclin D3, we studied the effects of the PKC inhibitor Ro318220 on the proliferative activity of continously growing Jurkat T lymphocytes activated by serum mitogens. According to a review of Way et al. [27], Ro318220 inhibits α, β, γ and ε PKC isoforms. The proliferative activity was monitored by a flow cytometric analysis of propidium iodide incorporation. Cell cycle analysis by flow cytometry of Jurkat T cells before and after inhibition by Ro 318220 at various time points showed that during the first 4 h on addition of PKC inhibitor Ro 318220 there was no detectable changes in DNA content of the performed T cells compared to that of the continously growing cells (Fig. 1A). This time of PKC inhibition was insufficient to inhibit the proliferation of Jurkat T lymphocytes. Examination of the Jurkat T lymphocytes 8 h and 24 h after PKC inhibitor treatment showed that the inhibitor treatment for 8 h slowly reduced the proliferation (Fig. 1B) and after 24 h it was arrested (Fig. 1C), and cells accumulated in the G1 phase. However, after a prolonged treatment with PKC inhibitor some portion of cells became apoptotic. As discussed previously about PKC activities in T lymphocytes, our results confirmed the importance of PKC in T cell proliferation.

Downregulation of cyclin D3 protein level is associated with Ro318220-induced inhibition of proliferation of Jurkat T lymphocytes. G1 progression depends on the sustained expression of D-type cyclins, which in turn depends on continuous mitogenic stimulation, suggesting that the D-type cyclins provide a link between mitogenic signaling and the cell cycle machinery [5]. Therefore, we examined the effects of inactivation of PKC by Ro318220 on protein levels of cyclin D3 in Jurkat T lymphocytes. Whole cell lysates were prepared from continously growing Jurkat T lymphocytes activated with serum mitogens as control, from cells treated with 0.001% DMSO as second control, and from cells treated with PKC inhibitor Ro318220 for 1 h, 4 h, 8 h, 16 h and 24 h. As shown in Fig. 2, the protein level of cyclin D3 did not change significantly upon inhibition of the Jurkat T cells with Ro318220 until 8 h

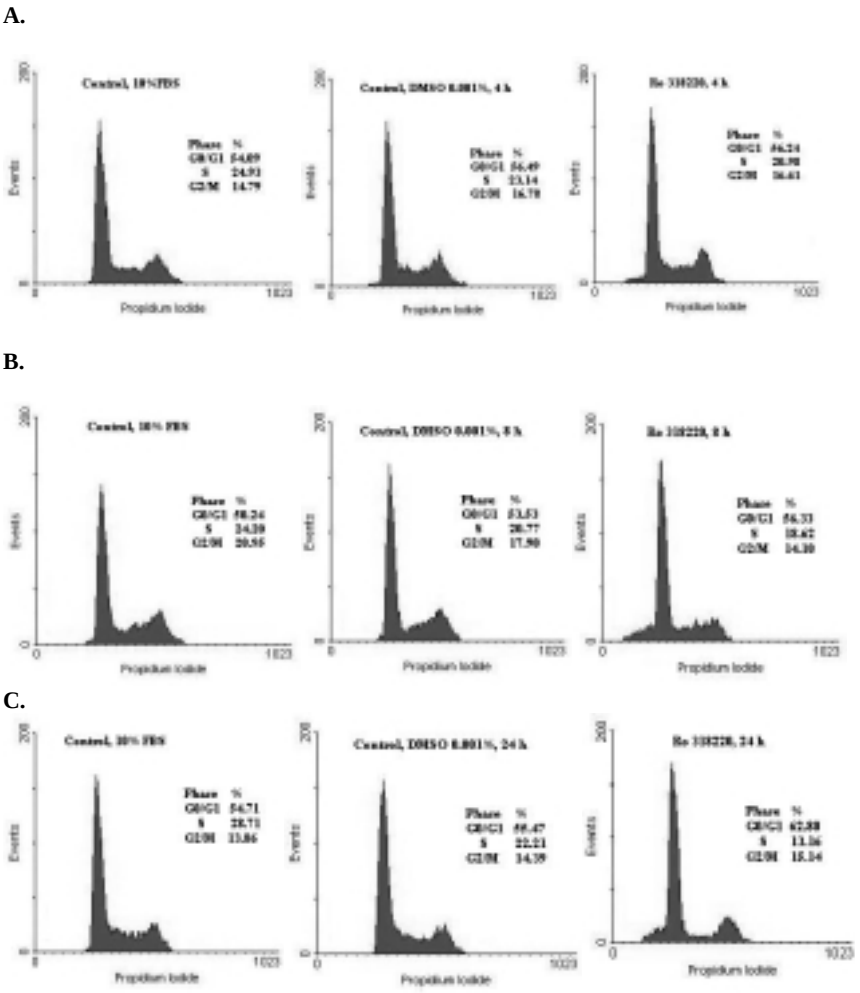

Fig. 1. Cell cycle distribution of Jurkat T cells after treatment with Ro 318220

Jurkat T cells were cultured in tissue culture flasks (1.2×10^6 cells/flask) in the presence of 1 μM Ro 318220 or of its solvent DMSO for the indicated times. Cells were harvested, fixed with ethanol, and stained with propidium iodide. Cell cycle profiles were analyzed by flow cytometry, and the percentages of cells in the G0/G1, S and G2/M phase of the cell cycle are indicated in each histogram

and was downregulated 8 h following treatment with Ro318220. The time of the downregulation of the protein level of cyclin D3 is coincidental with the time of cell cycle arrest by inhibitor Ro318220 (Fig. 1), suggesting that the reduction of protein levels of cyclin D3 could be responsible for the observed G1 arrest. However, we do not know whether the reduction in cyclin D3 protein level occurs through active transcriptional repression or by the post-translational mechanism affecting the protein stability of cyclin D3. On the other hand, it has been demonstrated that PKC modulates the activities of the transcription factors AP-1 and NF-κB, which are required for induction of the transcription of D-type cyclins [23–25] and IL-2, IL-2 receptor alfa genes [26], and our observations on the cell cycle arrest by PKC inhibitor Ro318220 and downregulation of the protein level of cyclin D3 in continously proliferating Jurkat T cells may be related with a reduction of expression of cyclin D3 or IL-2, IL-2 receptor alfa with participating PKC and its signaling pathway. In summary, our data show that the PKC inhibitor Ro318220 attenuates Jurkat T lymphocyte proliferation, and this correlates with the downregulation of protein levels of cyclin D3.

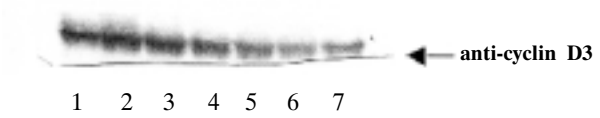

Fig. 2. Effect of PKC inhibitor Ro318220 on the level of cyclin D3 in proliferating T cells

Treatment of cells and preparation of lysates are described in Materials and Methods. Lane 1 – cells growing in RPMI1640 supplemented with 10% FBS; lane 2 – DMSO 0.001%; lane 3 – 1μM Ro318220 1 h; lane 4 – 1μM Ro318220 4 h; lane 5 – 1μM Ro318220 8 h; lane 6 – 1μM Ro318220 16 h; lane 7 – 1μM Ro318220, 24 h.

This is a representative result of experiment performed with three separate cell preparations.

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PKC INHIBAVIMAS RO318220 IR JO ĮTAKA JURKAT T LIMFOCITŲ PROLIFERACIJAI BEI CIKLINO D3 BALTYMO KIEKIUI

S a n t r a u k a

D-tipo ciklinai yra svarbūs ląstelės ciklo reguliatoriai. Jų funkcija yra reguliuojama viduląsteliniais signaliniais keliais, aktyvuojamais išorinių augimo veiksnių. Siekiant išsiaiškinti mechanizmus, kuriais viduląsteliniai signaliniai keliai veikia ląstelės ciklo progresiją ir ciklo kontrolės baltymus, mes tyrėme baltymo kinazės C inhibavimo įtaką Jurkat T limfocitų proliferacijai bei ciklino D3 kiekiui ląstelėse. Nustatėme, kad baltymo kinazės C inhibavimas Ro318220 slopina Jurkat T limfocitų proliferaciją ir sumažina ciklino D3 baltymo kiekį šiose ląstelėse. Gauti duomenys atskleidžia baltymo kinazės C signalinio kelio dalyvavimą Jurkat T limfocitų ląstelės ciklo progresijos ir ciklino D3 baltymo lygio reguliavime.