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Studies of factors involved in the survival of phorbol ester TPA-treated leukemic HL60 cells

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The aim of this study was to determine the factors responsible for the survival of 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-treated differentiating HL60 cells. After TPA treatment, HL60 cells in nonadherent cell fraction undergo apoptosis, whereas they survive and differentiate when attached to substrate. We have found that the amount of viable TPA-treated cells grown in a shaker decreased greatly in comparison to control cells grown under standard conditions, suggesting the importance of cell adhesion for their survival. Different approaches were used to evaluate the role of the transcription factor NF-źB in the TPA-induced process of cell survival/differentiation. We have found that NF-kB-DNA binding activity was much stronger in TPA-treated differentiating HL60 cells than in apoptotic ones, as was shown by an electrophoretic mobility shift assay (EMSA). The treatments directed to inactivate NF-kB by using the inhibitors of NF-kB signalling pathway, pyrrolidine dithiocarbamate (PDTC) and (E)-3-[(4-methylphenylsulfonyl]-2- propenenitrile (BAY 11-7082), effectively promoted TPA-induced cell death. The possible activator of NF-kB, TNFα, was positively involved both in TPA-induced differentiation and apoptosis of HL60 cells, – monoclonal antibodies against TNFα attenuated TPA-induced HL60 cell differentiation (adhesion to substrate) and the death process in suspension cell fraction. It can be concluded that cell adhesion and NF-kB activity are essential for the survival of TPA-treated HL60 cells.

Key words: TPA, adhesion, survival, NF-kB

INTRODUCTION

Induction of apoptosis and/or differentiation of cancer cells are two desirable goals of anticancer therapy. The increasing knowledge of signalling molecules and delineation of sequences of signalling events leading cell to death or differentiation offer new approaches in cancer treatment. Phorbol ester TPA induces human leukemic HL60 cells to adhere to substrate and to differentiate towards the monocyte / macrophage lineage [1]. In parallel, it induces another process – cells that do not adhere to substrate undergo programmed cell death / apoptosis [2]. There are evidences that TPA has a therapeutic efficacy in controlling myeloid leukemia and other hematological malignancies [3]. In our previous works [4–7], we analysed signalling events involved in TPA-induced apoptosis in promyelocytic HL60 cells. The goal of our current investigation was to ascertain the factors responsible for cell survival after TPA treatment in the fraction of differentiating HL60 cells.

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Signal transduction initiated by interactions between cells or between cells and extracellular matrix is an important factor of survival directed against cell apoptosis. Intramembrane proteins, integrins, are the major family of adhesion receptors regulating anchorage to substrate [8]. Certain specialized cell types deprived of the integrin-mediated survival signals undergo a form of apoptosis, termed “anoikis”. There are data on the protective role of integrins in human promyelocytic cells treated with different phorbol esters [9, 10], although some studies propose that the interaction with a substrate is not necessary for cell survival and differentiation [11].

Little is known about the intracellular signals determining the survival of HL60 cells upon cell attachment to substrate after TPA treatment. One of the possible factors determining the survival of TPA-treated differentiating cells is the transcription factor NF-kB [12]. It is known that β1 and β2 integrins activate NF-kB in human leukemic cells [13]. Another possible activator of NF-kB is the multipotential cytokine tumour necrosis factor α (TNFα). TNFα is known as a potent inducer of NF-kB in HL60 cells [14]. The influence of cell adhesion, of TNFα and of transcription factor NF-kB activity in the survival of TPA-treated cell were studied. The results indicate that adhesion-initiated signalling, including

NF-kB activation, is essential for HL60 cell survival after TPA treatment.

MATERIALS AND METHODS

Cell culture. HL60 cell line was cultured in RPMI-1640 medium supplemented with 10% FCS (foetal calf serum), penicillin (100 U/ml) and streptomycin (100 μg/ml) in 5% CO_2 at 37 ˚C. In special experiments, cells were cultivated under agitation. Cells were treated with a final concentration 30 nM of TPA (Sigma), 10 μg/ml of anti-TNFα antibody (BD Pharmingen), 50 nM of PDTC (Sigma) and 400 nM of BAY 11-7082 (Sigma). Reagents were prepared as stock solutions: 1.5 mM of TPA in DMSO (dimethylsulfoxide), 50 mM of PDTC in DMSO and 100 mM of BAY 11-7082 in DMSO.

Viability assessment using trypan blue dye exclusion test. The number of viable cells was determined by the trypan blue dye (0.4% trypan blue in phosphate buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl)) exclusion test. Cell suspension was mixed with one volume of trypan blue dye solution and the viability was evaluated under a light microscope. The method is based on the penetration of dye into unviable cells.

Apoptosis assay. The apoptotic index was determined using two fluorescent dyes: acridine orange (AO) (Sigma) and ethidium bromide (EB) (Sigma). AO was used to characterize chromatin condensation, and EB characterized membrane integrity. Cells were categorized as follows: 1) VNA – viable with non-apoptotic nuclei (bright green chromatin with organized structure); 2) A – viable and nonviable with apoptotic nuclei (bright green or orange chromatin, i. e. highly condensed or fragmented); 3) O ("other") – necrotic (bright orange chromatin with organized structure) and chromatin-free (cells that have totally lost their DNA content and were exhibiting a weak green-orange staining) [15].

EMSA. 1×10^7 cells were lysed in 50 μl of lysis buffer (10 mM Tris pH 7.8, 3 mM $MgCl_2$, 10 mM NaCl, 0.1% NP-40, 1 mM PMSF, 3 mM DTT, cocktail of inhibitors (2 mM AEBSF, 1 mM EDTA, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin)). Lysates were centrifuged at 500 g for 5 min. The nuclei were washed with 100 μl of buffer (10 mM Tris, pH 7.4, 60 mM NaCl, 1 mM EDTA, 1 mM PMSF), centrifuged at 500 *g* for 5 min, lysed in 25 μl of lysis buffer (20 mM Tris, pH 8.0, 200 mM EDTA, 2 mM EGTA, 20% glycerol, 400 mM NaCl, 3 mM DTT, 1 mM PMSF, cocktail of inhibitors) on ice for 1 h and centrifuged at 14000 *g* for 20 min. An electrophoretic mobility shift assay was performed with equal amounts (10–20 μg) of protein from nuclear extracts. One pmol of γ-[^{32}P]ATP labelled NF-kB consensus sequence (5'-AGTTGAGGACTTTCCCAGGC-3') was incubated with nuclear protein extract in EMSA buffer (0.5 mg/ml BSA, 0.05 mg/ml poly(dI-dC), 10 mM HEPES, pH 7.9, 80 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol) at room temperature for 30 min. The protein–DNA complexes were subjected to native electrophoresis on 6% polyacrylamide gel in 0.5 × TBE buffer (5 mM Tris-borate, 1 mM EDTA). Dried gel was subjected to autoradiography (–70 ˚C).

Statistical analysis. The data are expressed as the representative result or as the mean of at least three experiments ± SD. Means were compared using the t-test. Values were considered significantly different at $p < 0.05$. Statistical analysis was performed using Excel 97 and Sigma Plot-2000 software.

RESULTS AND DISCUSSION

In this study, we examined the role of cell adhesion and the activation of the transcription factor NF-kB on the survival of TPA-treated HL60 cells. After TPA treatment, HL60 cells attached to the substrate experience monocytic differentiation and survive for many days in culture, whereas non-attached cells in suspension continuously undergo apoptosis. To evaluate the role of cell adhesion on cell survival, TPA-exposed HL60 cells were incubated in a shaker. The studies revealed that the amount of viable TPA-treated cells in the agitated population decreased (Fig. 1A) in comparison to cell population grown under standard conditions, and the percentage of apoptotic cells in the culture increased to about 70% (Fig. 1B). Under the same conditions, TPA-untreated HL60 cells were not affected (data not presented). The results obtained with the cells cultured under agitation indicate that the population of HL60 cells is not fenotypically divided into different groups with different fates – to

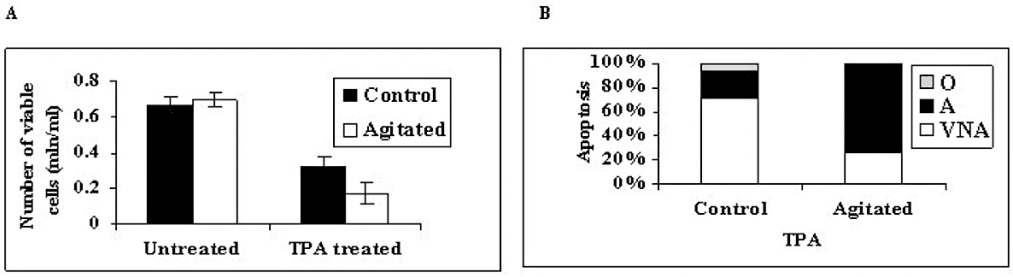

Fig. 1. Effect of adhesion on the survival of TPA-treated HL60 cells. (**A**) Comparison of the decreases in viable cell number in the populations of control and agitated cells. Data are expressed as mean ± SD. The significance of results with TPA-treated cells was $p < 0.05$. (**B**) Distribution in the percentages of cell types in control and agitated cell populations (O – other; A – apoptotic; VNA – viable nonapoptotic) in relation to the total number of TPA (30 nM)-treated cells. Cell number was evaluated at 48 h

survive or to die, determined *a priori*, and that cell adhesion on the substrate is essential in the protection of cells from TPA-induced death.

Little is known about the intracellular signals determining the survival of HL60 cells upon cell attachment to substrate after TPA treatment. It is likely that intracellular molecules induced or activated by cell adhesion may counteract proapoptotic signals induced by TPA. An increase in the expression of antiapoptotic molecules, such as Bcl-2, Bcl-X_L and Mcl-1, was demonstrated in adherent differentiating TPA-treated U937 leukemia cells [16]. The evidence indicates that resistance to apoptosis of bryostatin-1-treated differentiating THP-1 cells might be conferred by enhanced expression of XIAP, and its expression is under the transcriptional regulation of NF-kB [12]. The results obtained by Hida [17] show that TPA-induced differentiation of human leukemia U937 cells correlates with the expression of XIAP, which is under control of NF-kB. Consequently, activation of the transcription factor NF-kB could be a way for the cell to escape apoptosis in a cell fraction adherent to the substrate.

Therefore, next we investigated the role of the NF-kB signalling pathway in TPA-induced apoptosis / survival of HL60 cells. Two approaches were used to evaluate the role of NF-kB in TPA-induced processes. First, we have found that NF-kB–DNA binding activity was higher in TPA-treated differentiating HL60 cells in the adherent cell fraction than in cells undergoing apoptosis, as was determined after 30 h of TPA treatment by electrophoretic mobility shift assay (EMSA) (Fig. 2).Therefore, the effect of NF-kB on HL60 cell survival was evaluated by the use of inhibitors of NF-kB signalling pathway, PDTC and BAY 11-7082. It is known that activation of NF-kB proceeds through the phosphorylation of its inhibi-

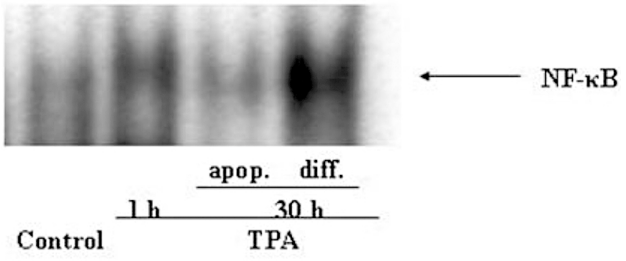

Fig. 2. TPA-induced activation of transcription factor NF-kB. NF-kB activity was examined in apoptotic (suspension) and differentiating (adherent) cells at indicated times after TPA (30 nM) treatment by electromobility shift assay (EMSA) (see Materials and Methods)

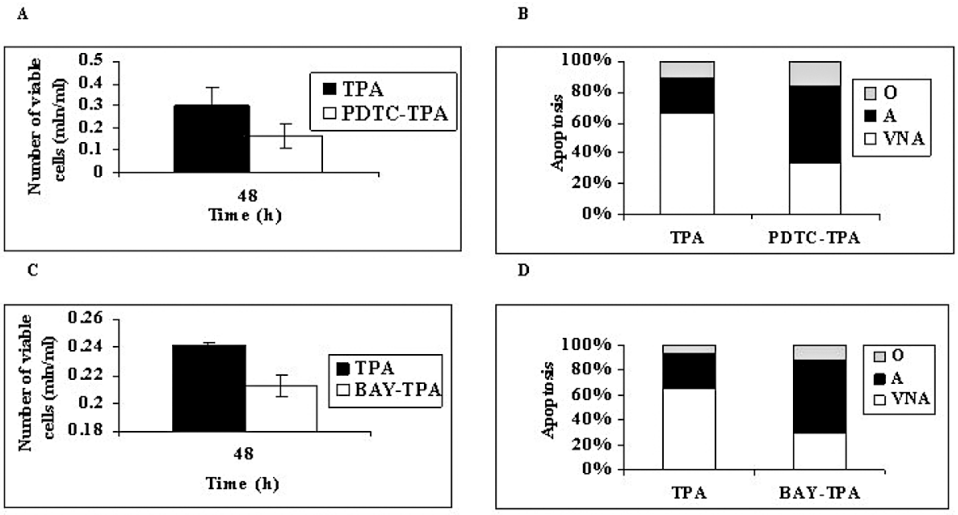

Fig. 3. Effect of NF-kB inhibitors, PDTC and BAY 11-7082, on the survival of TPA-treated HL60 cells. (A), (C) Total number of viable cells (suspension and adherent). Data are expressed as mean ± SD. In both cases $p < 0.05$. (B), (D) Distribution in the percentages of cell types (O – other, A – apoptotic, VNA – viable nonapoptotic) in relation to the total number of suspension cells. Cells were counted at 48 h after TPA treatment. Inhibitors of NF-kB activation, PDTC (50 nM) and BAY 11-7082 (400 nM), were added 1 h before TPA (30 nM) treatment

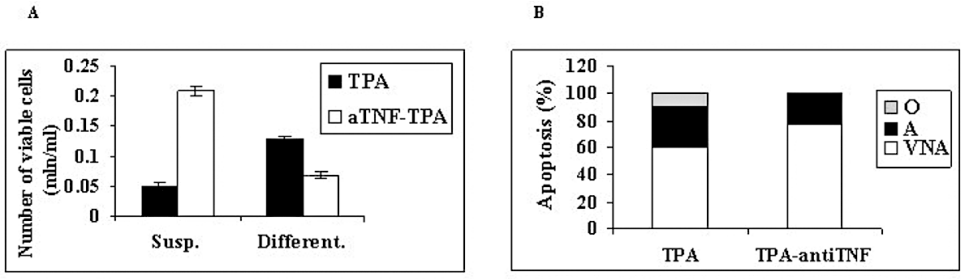

Fig. 4. Role of TNFα in TPA-induced processes in HL60 cells. (A) Suppression of TPA-induced HL60 cell differentiation / adhesion by anti-TNFα monoclonal antibody. Anti-TNFα (10 μg/ml) was applied 1 h before TPA (30 nM) treatment. Cells were counted at 24 h after TPA treatment. (B) Suppression of TPA-induced HL60 cell apoptosis by anti-TNFα monoclonal antibody. After 24 h of TPA treatment, suspension cells were separated and exposed to anti-TNFα (10 μg/ml). Distribution in the percentages of cell types (O – other; A – apoptotic; VNA – viable nonapoptotic) was evaluated at 48 h after the beginning of TPA treatment

tor IB that targets it for polyubiquitination and subsequent degradation by the 26S ubiquitin proteosome. Both inhibitors, PDTC and BAY 11-7082, suppress the phosphorylation of IkB. Since PDTC and BAY 11-7082 demonstrated toxicity towards control HL60 cells, we titrated these drugs to relatively non-toxic doses. At these doses, both inhibitors, in combination with TPA, synergistically enhanced cell death as was judged from the decrease of total cell count as well as from the increase in the percentage of apoptotic cells in overall population (Fig. 3). In parallel, the inhibitors of NF-kB pathway, PDTC and BAY 11-7082, also diminished cell adherence to the plastic after exposure to TPA. Thus, the treatments directed to inactivate NF-kB efficiently promoted TPA-induced cell death. This shows that after TPA treatment NF-kB activation could be an important mediator of cell adhesion / integrin-determined cell survival in HL60 cell fraction adherent to substrate. The involvement of NF-kB in leukemic cell viability after apoptotic treatments was shown by other authors using various experimental approaches [18, 19]. It was shown that in the absence of NF-kB activation, the tumour promoter and survival agent TPA became a strong inductor of apoptosis in Jurkat leukemic T cells. In this instance, TPA-stimulated expression of anti-apoptotic genes was reduced [20].

Besides the integrins, TNFα is another possible activator of NF-kB in a TPA-induced HL60 leukemic cell system. TNFα is an autocrine factor stimulating apoptosis in TPA-treated U937 cells [21] and differentiation in TPA-treated HL60 cells [22]. B. Xie also shows that TNFα is necessary but insufficient for TPA-induced HL60 cell differentiation. TNFα was shown to be a potent inducer of NF-kB in HL60 cells [14]. In this study, we have shown that TNFα is equally involved in both differentiation and apoptosis of TPA-treated HL60 cells, – experiments directed to inhibit TNFα showed its positive role in TPA-induced differentiation/adhesion as well as in TPA-induced apoptosis. As is shown in Fig. 4, monoclonal antibody against TNFα attenuated both TPA-induced HL60 cell adhesion to substrate (Fig. 4A) and the cell death process (apoptosis) in suspension cell fraction (Fig. 4B). The total (adherent and suspension) cell number after treatment with anti-TNFα antibody was substantially greater than that of the TPA alone (data not presented). Therefore, this set of experiments shows that TNFα mediates both TPA-induced processes in HL60 cells and is not the factor responsible for the different fates of TPA-treated cells in the culture. The effects of TNFα could be transmitted through two kinds of receptors – TNFR1 and TNFR2. TNFR1 can signal for apoptosis, whereas TNFR2 is almost exclusively involved in the process of survival / proliferation. There are data on a decrease in the expression of TNFR1 in TPA-induced differentiating HL60 cells [23]. This could be the explanation for the dual effect of TNFa.

In conclusion, the results obtained in this study indicate that cell adhesion to substrate and NF-kB signalling protect HL60 cells from apoptosis induced by TPA.

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FORBOLIO ESTERIO TPA PAVEIKTŲ LEUKEMINIŲ HL60 LĄSTELIŲ IŠGYVENIMĄ LEMIANTYS VEIKSNIAI

S a n t r a u k a

Šio darbo tikslas buvo įvertinti veiksnius, atsakingus už 12-O-tetradekanoilforbolio-13-acetat (TPA) paveiktų besidiferencijuojančių HL60 ląstelių išgyvenimą. Mes nustatėme, kad TPA paveiktų ląstelių auginimas kratyklėje, neleidžiant prilipti prie substrato, mažina jų gyvybingumą. Tai rodo adhezijos svarbą TPA paveiktų HL60 ląstelių išgyvenimui. Tiriant transkripcijos veiksnio NF-kB poveikį ląstelių išgyvenimo/žuvimo procesams, buvo naudoti skirtingi metodai. Elektroforetinio judrumo pokyčio testas rodo, kad TPA paveiktose besidiferencijuojančiose ląstelėse NF-kB ir DNR sąsajos yra didesnės nei apoptozinėse. NF-kB vaidmeniui nustatyti buvo naudoti šio transkripcijos veiksnio signalinio kelio inhibitoriai – PDTC (pirolidinditiokarbamatas) bei BAY11-7082 ((E)-3-[(4-metilsulfonil]-2-propenitrilas). Abu inhibitoriai efektyviai didino po TPA poveikio žūstančių HL60 ląstelių skaičių. Galimas NF-kB aktyvintojas TNFa, kitaip nei NF-kB, dalyvavo ir TPA indukuotame diferenciacijos/išgyvenimo, ir apoptozės procesuose. Antikūnas prieš TNFa slopino ir TPA nulemtą HL60 ląstelių lipimą prie substrato, ir jų žuvimą suspensinių ląstelių frakcijoje.

Gauti rezultatai patvirtina adhezijos bei transkripcijos veiksnio NF-kB svarbą TPA paveiktų HL60 ląstelių išgyvenimui.