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Regulation of Differentiation, Proliferation and Drug-Induced Apoptosis in HT58 Lymphoma Cells

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Recently, it has been suggested, that differentiated cells are more resistant to the apoptotic effect of DNA damaging agents possibly due to the decreased activity of "damage detecting/apoptosis triggering" mechanism. Previously, we have shown, that PMA pretreatment reduced etoposide- (ETO) but enhanced staurosporine- (STA) -induced apoptosis in HT58 cells. Data presented here show that the HT58 human, "mature" B-lymphoma cells

exposed to PMA secrete more IgM into the supernatant indicating commitment of cells to perform differentiated function. The sensitivity of HT58 cells to ETO- or STA-induced apoptosis is influenced diversely with PMA pre- or posttreatment. Interestingly, the DNA damage (gamma radiation, bleomycin, ETO) or okadaic acid (30 nM) reduced the [PMA+STA] - induced apoptosis. (Pathology Oncology Research Vol 3, No 2, 100-105, 1997)

Key words: apoptosis, lymphoma, differentiation, etoposide, staurosporine, PMA

Introduction

There are evidences that chemotherapeutic agents can trigger apoptosis in target cells.^{1,2} Although, there are common biochemical features provoked by the various drugs, several divergent pathways can be also activated.³ Many attempts have been made to potentiate the cytotoxicity of a variety of agents by modulating the sensitivity of the cells towards apoptosis.⁴ Two of these strategies are: *a.* combination of drugs effective at different cell cycle phases and *b.* modulation of sensitivity by differentiation.

The idea that differentiation therapy for cancer can be an alternative to cytotoxic chemotherapy has been sup-

ported by attempts to achieve complete remission following administration of all-trans-retinoic acid.⁵ According to *in vitro* studies myeloid cell lines are useful tools to examine the mechanisms involved in differentiation-potentiated apoptosis.^{6,7} In this respect less is known about lymphoid tumors though differentiation steps are fairly explored in the normal lymphoid cells.⁸

Etoposide (ETO) is characterised as a DNA-damaging agent covalently binding topoisomerase II to DNA in cleavable complexes⁹ and frequently used in antitumor protocols.

It has been shown that cells in S-G2-M cell cycle phases express more topoisomerase II and are usually more vulnerable to ETO.¹⁰ ETO can induce cell cycle perturbation at low (nanomolar) concentrations (G1 and G2 arrest to allow the repair of DNA damage) and apoptosis at higher (micromolar) concentrations.¹¹ Biochemical processes mediating signals from DNA-damage to apoptotic machinery are currently under extensive research.^{12,13} ETO was also applied to explore the components of apoptotic machinery, especially in hemopoietic cell lines.^{3,14}

Staurosporine (STA) is a potent inhibitor of protein kinases with a broad spectrum of activity.^{15,16} Recently, STA has been shown to inhibit cell cycle progression and

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Abbreviations: BLEO: bleomycin; CDK: cyclin dependent kinase; CONT: control; ETO: etoposide; i-anti-IgM: immobilized anti-human IgM antibody; OKA: okadaic acid; PFC: plaque forming cells; PI: propidium iodide; PMA: phorbol 12-myristate 13-acetate; RAD: gamma irradiation; s-anti IgM: solubilised anti-human IgM antibody; STA: staurosporine

to induce apoptosis or premature mitosis in different cell lines.^{17,18} Activation of cyclin dependent kinases (CDK) has been suggested to participate in the STA-evoked cell death effect^{17,19} though CDK1 activity was shown to be blocked at the same concentration of STA.¹⁶

Recently, Darzynkiewicz and his group suggested that DNA-damage induced apoptosis can be potentiated by subsequent but not by prior induction of differentiation.^{4,7} Previously, we have found that STA-evoked apoptosis is enhanced by pretreatment with phorbol ester (PMA) in HT58 lymphoma cells.^{18,20} PMA is a potent and well known inducer of differentiation in various cell types.^{21,22} This paper is concerned with the relationship between the ETO- and/or STA-evoked apoptosis and PMA-induced differentiation in HT58 lymphoma cell line.

Material and Methods

Chemicals

Etoposide, staurosporine, PMA, okadaic acid (dissolved in DMSO), propidium iodide, bleomycin (dissolved in distilled water) were purchased from Sigma.

Cells

HT 58, a human non-Hodgkin lymphoma cell line of B cell origin²³ was cultured in RPMI-1640 with 10% FCS and gentamycin (0.5 mg/ml). Cultures plated with $2-3 \times 10^5$ cells/ml concentration in 24 well plates (Greiner) few hours before treatments. Several times cells were irradiated with ⁶⁰Co, 80 cGy/min, for 5 min.

To induce differentiation, cells were treated with PMA (10 ng/ml), solubilized goat anti-human IgM (5 µg/ml) or plate coated (immobilised) goat anti-human IgM (20 µg/ml) for 44 hr. Supernatant was saved for determination the human IgM content, while cells were used in hemolytic plaque forming assay.

Determination of secreted IgM by ELISA

Sandwich-ELISA test was performed as described elsewhere.²⁴ Briefly: 96-well plates were exposed with a goat anti-human IgM antibody (Sigma) overnight, blocked by 5% dry milk, and dilutions of human IgM antibody (Sigma) and the samples were plated for 1 hr, washed, then biotin conjugated goat anti-human IgM (Sigma), subsequently peroxidase conjugated avidin was added. (Both steps for 1-1 hr.) After washing ortofenilanediamine with hydrogenperoxide was given for a few minutes, the reaction was terminated by 4N sulfuric acid and the extinction was recorded on 492 nm. The quantity of IgM in samples was determined by fitting to the calibration curve.

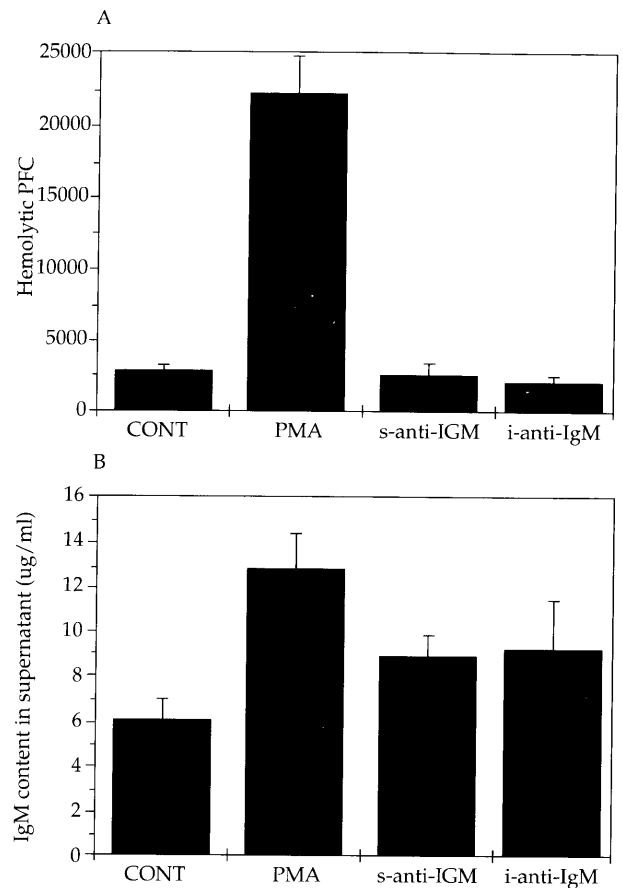


Figure 1. Differentiation of HT58 lymphoma cells. Cells were exposed to PMA (10 ng/ml) or goat anti-human IgM (5 µg/ml solubilized [s-anti-IgM] or 20 µg/ml immobilised on plates indirectly [i-anti-IgM]) for 44 hr. Then (A): IgM secreting cells were counted in hemolytic plaque assay and. Mean and SD of triplicate determination were plotted; (B): human IgM content was determined by ELISA from the supernatant.

Estimation of IgM producing cells (hemolytic PFC assay)

The test was performed according to Gronowicz et al.²⁵ Briefly: Sheep red blood cells were conjugated with protein A and embedded in 0.7% agarose in HBSS at 45°C, mixed with a dilution of anti-human IgM antibody and the treated HT58 cells and spread onto 6-well plates. The plates were incubated for 1 hr in humid, 37°C, 5% CO₂ incubator. Then guinea-pig serum as complement source was added and incubated further for 30-60 min until hemolytic plaques were visible.

Preparation of cells for flow cytometry

Oligonucleosomal DNA was extracted from apoptotic cells as described earlier.²⁴ Briefly: cells were fixed in ethanol (70 % v/v, -20°C), and stored at -20°C until fur-

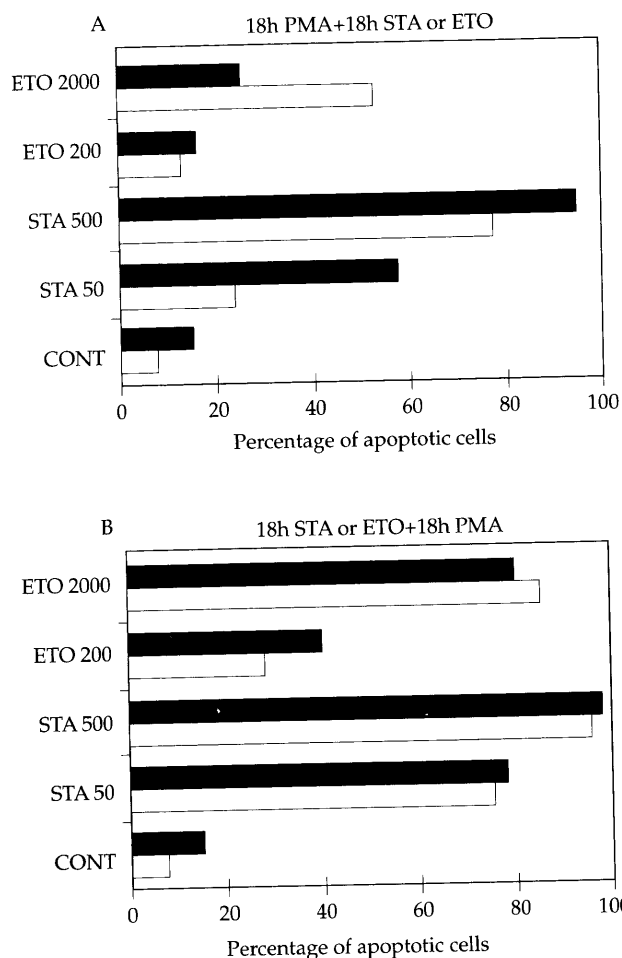


Figure 2. Modulation of drug-induced apoptosis by differentiation with PMA. (A): Cells were treated with PMA (5 ng/ml) for 18 hr followed by addition of STA or ETO (indicated concentrations in nM) for further 18 hr; or (B): cells were pretreated with STA or ETO for 18 hr followed by addition of PMA. Percentage of apoptotic cells were determined by flow cytometry. Mean of two experiment was plotted. Open bars: no PMA; black bars: with PMA.

ther preparation. Fixed cells (2×10^5 /ml) were pelleted and resuspended in 900 μ l alkaline salt solution (5 mM NaOH in 150 mM NaCl) for 1 min, then neutralized with 50 μ l Na_2HPO_4 and citric-acid buffer (200 mM, pH 6.5) containing 200 μ g/ml PI.

Flow cytometry

DNA content of the cells was determined by FACStar Plus (Becton-Dickinson) or Profile II (Coulter) flow cytometers. DNA content was recorded on logarithmic fluorescence scale. Analysis was performed by Winlist software (Verity Software-House). Gating was evaluated as described.²⁴ Viability of cells was determined by taking 200 μ l samples from cultures and diluting by 400 μ l ice

cold PBS containing 1 μ g/ml PI and the proportion of PI negative (as a sign of impermeable plasma membrane) cells were counted.

Results and Discussion

Differentiation of HT58 B lymphoma cells

HT58 is a human lymphoma cell line characterised by mature B cell markers including sIgM and sIgD.²³ First, we studied the potential of PMA and anti-human-IgM to induce differentiation in HT58 cells by estimating the num-

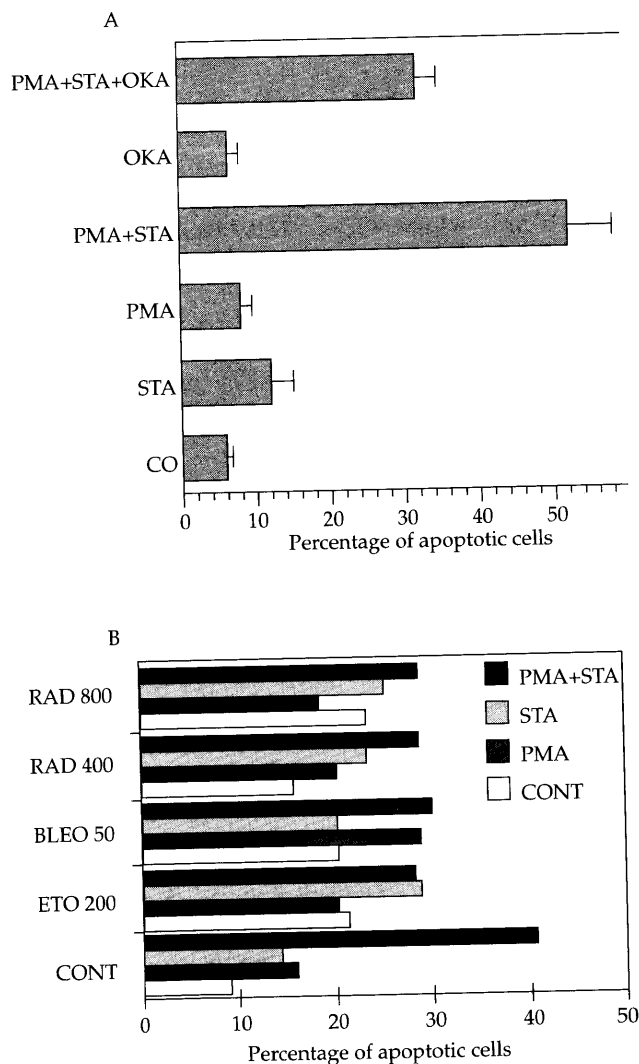


Figure 3. Inhibition of apoptosis induced by STA in differentiation potentiated HT58 cells. Cells were pretreated with PMA (5 ng/ml) for 4 hr then exposed to (A): STA (25 nM) and/or OKA (30 nM) or (B): STA (30 nM) and/or DNA damaging agents (ETO: 200 nM, BLEO: 50 μ M, RAD: 400 or 800 cGy) for further 16 hr. Percentage of apoptotic cells were determined by flow cytometry. Mean of duplicate experiment was plotted.

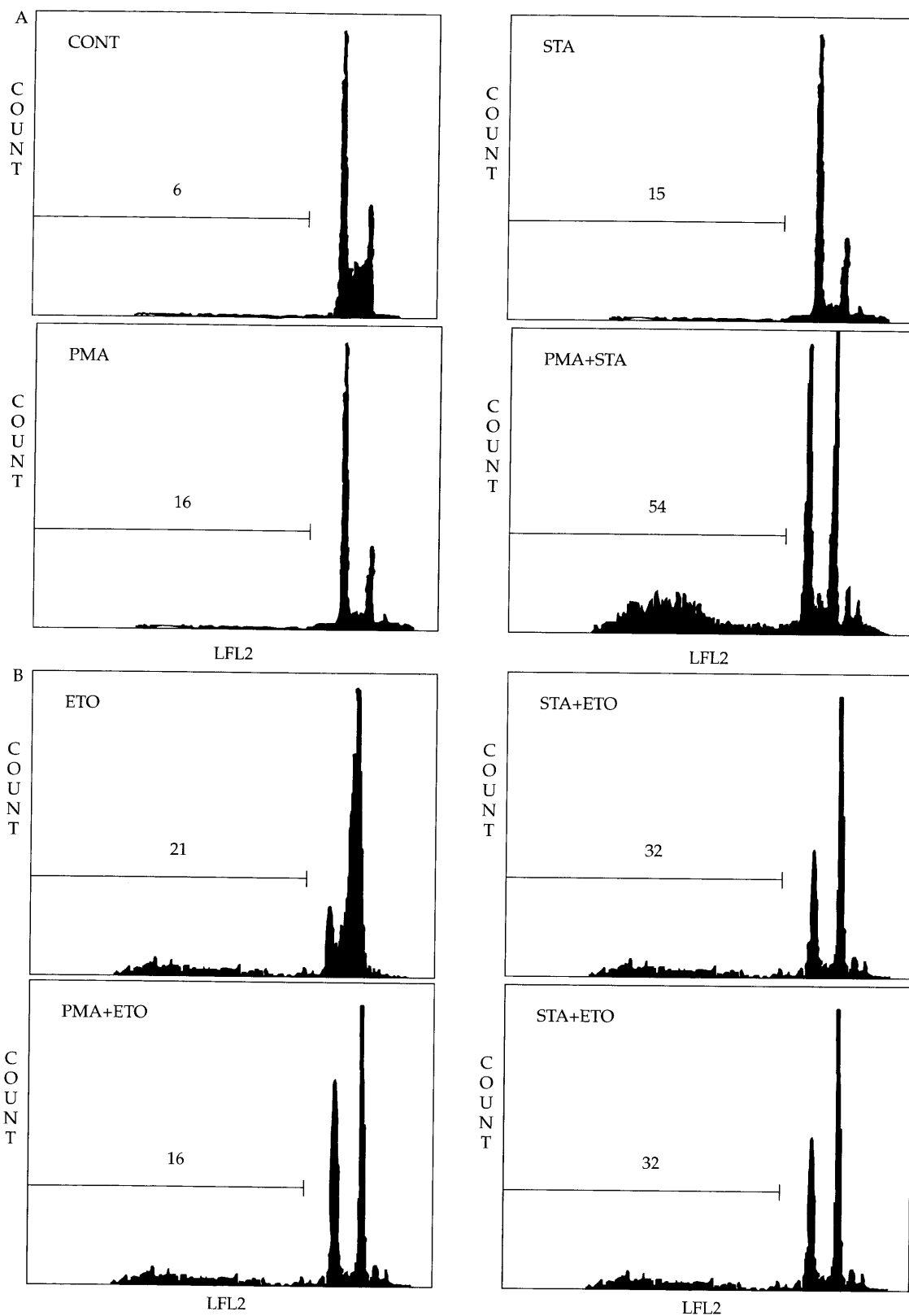


Figure 4. Representative flow cytometric DNA histograms. Cells were treated with (A): PMA and/or STA or (B): PMA and/or STA and/or ETO as described at Fig. 3. Then extracted by alkaline elution and labelled with PI. Percentage of apoptotic cells is shown above the horizontal lines.

ber of IgM secreting cells with hemolytic plaque forming test (*Fig. 1A*) and the IgM content of the culture supernatant with ELISA (*Fig. 1B*). The results indicated that differentiation was induced by PMA but not by anti-IgM stimulus during the 44 hr period in about 10% of HT58 cells.

Differentiation of mature B cell results in loss of transmembrane domain of the antigen receptor (IgM). It is known, that PMA can differentiate immortalised human mature B cells to IgM secreting plasmocytes what correlates with the arrest of cells in G1 phase.²² In HT58 cells PMA also induce the G1 arrest,¹⁸ but only temporarily at least in certain percentage of cells (data not shown). These data suggest that HT58 cells are heterogenous targets to be differentiated successfully by PMA.

Enhanced sensitivity to STA but not to ETO induced by PMA pretreatment

Next, we studied how PMA treatment can modulate the apoptosis inducing capacity of drugs with different target specificity. ETO was used as DNA damaging agent and STA as a protein kinase inhibitor. Results of pretreatment with PMA for 18 hr, followed by various concentrations of ETO or STA for another 18 hr are summarized on *Fig. 2A*. STA-induced apoptosis was enhanced by PMA pretreatment at high (500 nM) and especially at low (50 nM) STA concentrations. In contrast, at low (200 nM) ETO concentration the PMA pretreatment was ineffective while the apoptosis induced by a higher dose (2000 nM) of ETO was decreased by PMA. These data suggest that differentiation caused by PMA pretreatment can oppositely regulate the sensitivity of HT58 cells to perform apoptosis induced by ETO or STA. In parallel experiments the inhibitors were added first for 18 h, followed by PMA to induce differentiation. Results presented on *Fig. 2B* show that PMA posttreatment in contrast to pretreatment had no effect on STA- and high dose ETO-induced apoptosis but enhanced the apoptotic capacity of low dose ETO. This increase in the effectiveness of ETO is in agreement with the results of others.^{4,7} However, the increase of the effectiveness of STA by PMA pretreatment (i.e. differentiation) represents a new observation.

Inhibition of PMA-enhanced sensitivity to STA

In the next experiments we tried to identify some functional and molecular components of PMA enhanced STA-induced cell death. Previously, Greenberg et al showed that the activity of a temperature sensitive mutant of CDK1 is important in the apoptotic signal evoked by STA.¹⁹ Okadaic acid (OKA), a PP1 and PP2A serine phosphatase inhibitor, can promote the activation of CDK1.²⁷ Therefore, we examined the effect of OKA on

the STA-induced apoptosis. OKA induced apoptosis in HT58 cells over 100 nM concentration (not shown) but, contrast to our expectation, inhibited PMA sensitized, STA-induced apoptosis at 30 nM concentration (*Fig. 3A*). This suggests, that low concentration OKA may affect those protein phosphatases which are necessary for apoptosis. Recently, it has been shown that low concentration of OKA inhibits the activation of apoptosis-related cysteine proteases (caspases), but the target phosphatase was not identified.²⁸

ETO and gamma irradiation were shown to diminish CDK1 activation by inhibiting indirectly of its tyrosine dephosphorylation.^{29,30} Therefore, we studied the effect of non-cytotoxic DNA damage on differentiation-sensitized STA-induced apoptosis. Similarly to OKA treatment, in HT58 cells pretreated with PMA (5 µg/ml) for 4 hr, then exposed to STA (30 nM) and DNA damaging agents (ETO (200 nM), BLEO (50 µM), or gamma irradiation (400 or 800 cGy) apoptosis was decreased compared to PMA+STA treatment (*Fig. 3B*).

Representative histograms on *Fig. 4A* show that both PMA (5 ng/ml) and STA (30 nM) treatment result in G1 arrest in the cell cycle. However, after combined treatment with these drugs significantly higher portion of viable cells were in the G2 phase. This may suggest, that the PMA sensitized cells died in G1 or they could not reach G1 again. ETO treatment caused G2 arrest according to the suggested inhibition of CDK1 kinase (*Fig. 4B*). Cells exposed to BLEO and RAD were similarly arrested in G2 (not shown). When cells were treated with PMA+ETO, STA+ETO or PMA+STA+ETO similar G2 block was recorded. This may suggest, that DNA damage can block the cell cycle before cells can reach those phases (G1 or M) where the execution components of STA-induced apoptosis would be fully activated. It is possible, that PMA sensitized STA-induced cell death is not premature mitosis but real apoptosis.

Recently, it was shown that STA induced premature mitosis and apoptosis can be distinguished from each other by several biochemical markers.¹⁷ One of these is the activation of CDK1 in premature mitosis but not in apoptosis. Both phenomena result in cell death but possibly by different mechanism. Recently, HL60 cells were shown to upregulate CDK1 activity after ETO treatment.³¹ All of these indicate that cell cycle and apoptosis have many common regulatory elements and their activity is possibly influenced by the state of differentiation as well as by acquired genetic changes.

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