PATHOLOGY ONCOLOGY RESEARCH
Vol 3, No 2, 1997

ARÁNYI LAJOS
Foundation

ARTICLE

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

Rudolf MIHALIK[1], Ferenc UHER[2], István PETÁK,[1] Anna SEBESTYÉN,[1] László KOPPER[1]

1st Institute of Pathology and Experimental Cancer Research, Semmelweis University Medicine, Budapest,
[2]National Institute of Hematology and Immunology, Budapest; Hungary

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 acic (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 supported by attempts to achive complete remission following administration of all-trans-retionic 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 complexes[9] 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 vulnarable to ETO.[10] ETO can induce cell cyle 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

Received: April 22, 1997, *accepted:* June 12, 1997

Correspondance: prof. László KOPPER, Ist Institute Pathology and Experimental Cancer Research, Semmelweis University of Medicine, Üllői út 26, 1085 Budapest, Hungary; Tel/fax (36)(1)117 0891; e-mail: kopper@korb1.sote.hu

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

 1219-4956/97/020100+06 $ 12.00/0

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 origin[23] was cultured in RPMI-1640 with 10% FCS and gentamycin (0.5 mg/ml). Cultures plated with 2-3x10^5 cells/ml concentration in 24 well plates (Greiner) few hours before treatments. Several times cells were irradiated with ^{60}Co, 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 hidrogenperoxide 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 determinated by fitting to the calibration curve.

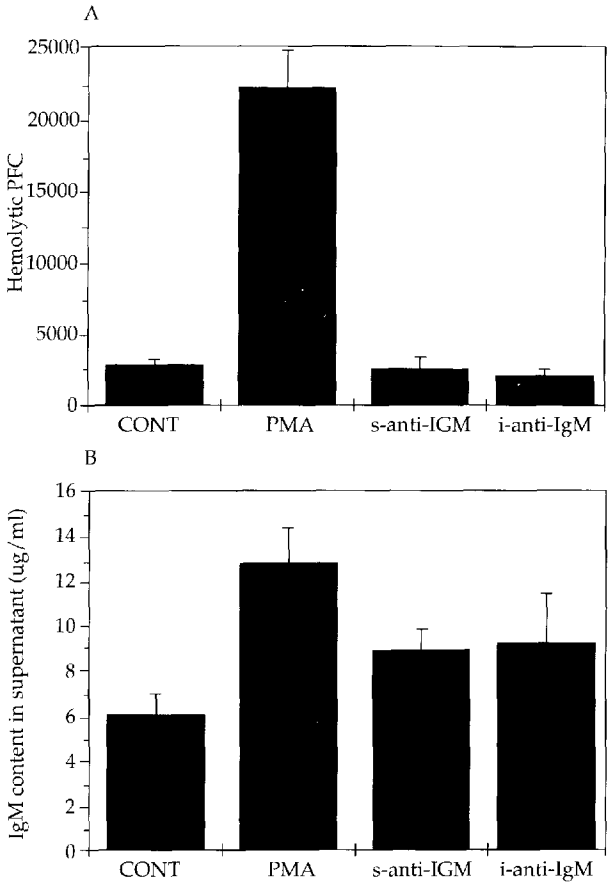

Figure 1. *Differentiation of HT58 lymphoma cells. Cells were exposed to PMA (10 ng/ml) or goat anti-human IgM (5 ug/ml solubilized [s-anti-IgM]) or 20 ug/ml immobilised on plates indirectly [i-anti-IgM]) for 44 hr. Then (A): IgM secreting cells were counted in hemolytic plaque assay and. Mean and SD 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% CO_2 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-

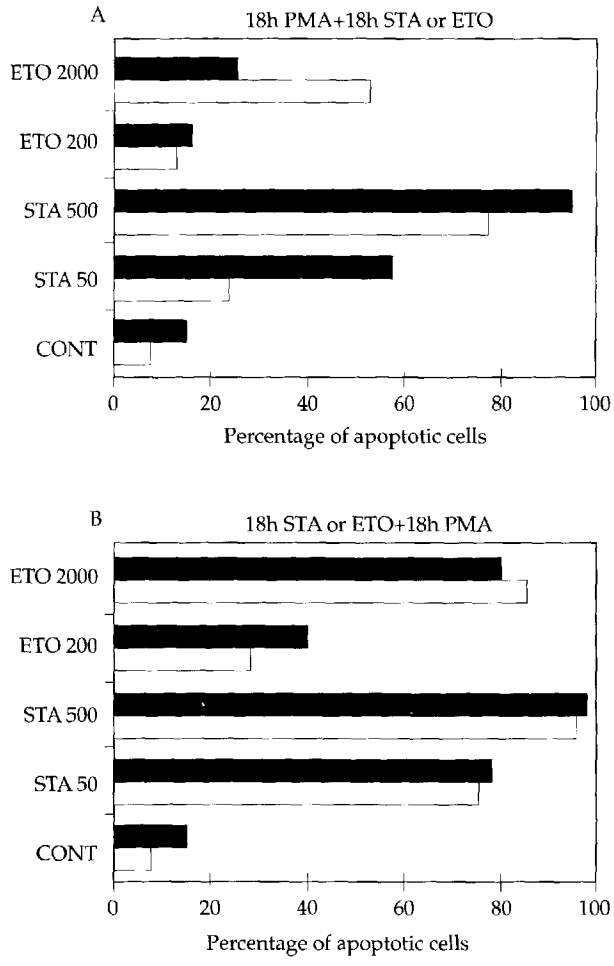

Figure 2. Modulation of drug-induced apoptosis by differentiation with PMA. (A): Cells were treated with PMA (5 ng/ml) for 18 hr followed by addition of STA or ETO (indicated concentrations in nM) for further 18 hr; or (B): cells were pretreated with STA or ETO for 18 hr followed by addition of PMA. 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 ($2x10^5$/ml) were pelleted and resuspended in 900 µl alkaline salt solution (5 mN NaOH in 150 mM NaCl) for 1 min, then neutralized with 50 µl Na_2HPO4 and citric-acid buffer (200 mM, pH 6.5) containing 200 µg/ml PI.

Flow cytometry

DNA content of the cells was determinated 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 µl samples from cultures and diluting by 400 µl ice cold PBS containing 1 µg/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-

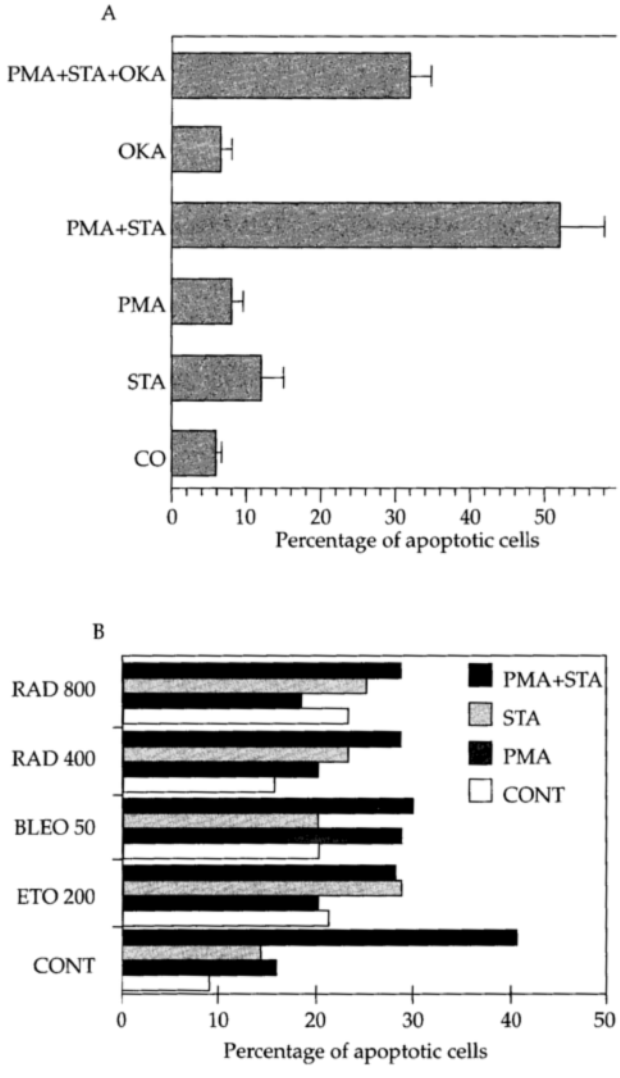

Figure 3. Inhibition of apoptosis induced by STA in differentiation potentiated HT58 cells. Cells were pretreated with PMA (5 ng/ml) for 4 hr then exposed to (A): STA (25 nM) and/or OKA (30 nM) or (B): STA (30 nM) and/or DNA damaging agents (ETO: 200 nM, BLEO: 50 µM, RAD: 400 or 800 cGy) for further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

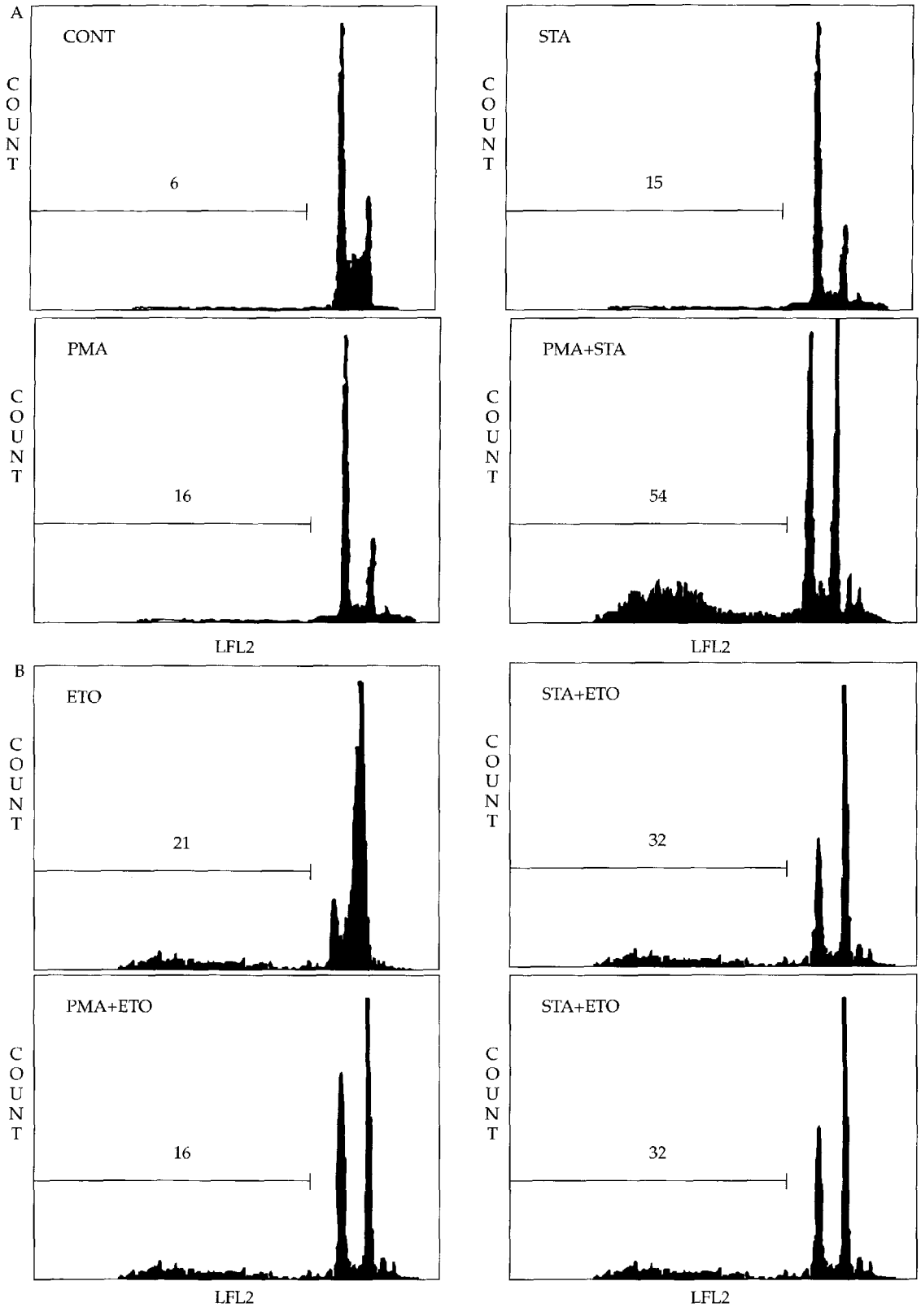

Figure 4. Representative flow cytometric DNA histograms. Cells were treated with (A): PMA and/or STA or (B): PMA and/or STA and/or ETO as described at Fig. 3. Then extracted by alkaline elution and labelled with PI. Percentage of apoptotic cells is shown above the horizontal lines.

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.[27] 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 cystein 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 aquired genetic changes.

Acknowledgement

Parts of this work were presented on the Annual Conference of AACR, 1996, Washington DC, with the support of KV Honn (Detroit, USA) that is highly appreciated by R.M.

References

1. *Kaufmann SH:* Induction of endonucleolitic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res 49:5870-5878, 1989.
2. *Hickman JA:* Apoptosis induced by anticancer drugs. Cancer Metastat Rev 11:121-140, 1992.
3. *Zhivotovsky B, Burgess DH, Vanags DM and Orrenius M:* Involvement of cellular proteolytic machinery in apoptosis. Biochem Biophys Res Comm 230:481-488, 1997.
4. *Darzynkiewicz Z:* Apoptosis in antitumor strategies: modulation of cell cycle or differentiation. J Cell Biochem 58:151-159, 1995.
5. *Warrel RP, Frankel SR, Miller WH et al:* Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retionic acid). New Eng J Med 324:1385-1393, 1991.
6. *Studzinsky GP, Rathod B, Wang QM, et al:* Uncoupling of cell cyle arrest from the expression of monocytic differentiation markers in HL60 cell variants. Exp Cell Res 232: 376-387, 1997.
7. *Bathia U, Traganos F and Darzynkiewicz Z:* Induction of cell differentiation potentiates apoptosis triggered by prior exposure to DNA-damaging drugs. Cell Growth Differ 6:937-944, 1995.
8. *Linette GP and Korsmeyer SJ:* Differentiation and cell death: lesson from the immune system. Curr Opin Cell Biol 6:809-815, 1994.
9. *Liu LF:* DNA topoisomerase poisons as antitumor drugs. Annu Rev Biochem 58:351-375, 1989.
10. *Fry AM, Chresta CM, Davies SM et al:* Relationship between topoisomerase II level and chemosensitivity in human tumor cell lines. Cancer Res 51:6592-6595, 1991.
11. *Del Bino G., Skierski JS and Darzynkiewicz Z:* The concentration-dependent diversity of the effect of DNA topoisomerase I and II on the cell cycle of HL60 cells. Exp Cell Res 195:485-491, 1991.
12. *Enoch T and Norbury C:* Cellular responses to DNA damage: cell-cycle checkpoint, apoptosis and the roles of p53 and ATM. TIBS 20:426-430, 1995.
13. *Kharbanda S, Pandey P, Jin S et al:* Functional interaction between DNA-PK and c-Abl in response to DNA damage. Nature 386:732-735, 1997.
14. *Dubrez L, Savoy I, Hamman A and Solary E:* Pivotal role of a DEVD-sensitive step in etoposide-induced and Fas-mediated apoptotic pathways. EMBO J 15:5504-5512, 1996.
15. *Tamaoki T, Nomoto H, Takahashi I et al:* Staurosporine, potent inhibitor of phospholipide/Ca++ dependent protein kinase. Biochem. Biophys. Res. Comm 135:397-402.
16. *Gadbois DM, Hamaguchi JR, Swank RA and Bradbury EM:* Staurosporine is a potent inhibitor of p34CDC2 and p34CDC2-like kinases. Biochem Biophys Res Comm 184:80-85, 1992.
17. *Yoshida M, Usui T, Tsujimura K et al:* Biochemical difference between staurosporine-induced apoptosis and premature mitosis. Exp Cell Res 232:225-239, 1997.
18. *Mihalik R, Farkas Gy, Kopper L, et al:* Possible involvement of protein kinase C-epsylon in phorbol ester-induced growth inhibition of human lymphoblastic cells. Int J Biochem Cell Biol 28: 925-933, 1996.
19. *Shi L, Nishioka WK, Th'ng J et al:* Premature p34cdc2 activation required for apoptosis. Science 263: 1143-1145, 1994.
20. *Mihalik R, Kopper L and Benczur M:* Modulation of drug-induced apoptosis in a human B-lymphoma cell line (HT58). Immunol Lett 48:17-21, 1995.
21. *Gamard CJ, Blobe GC, Hannun YA and Obeid LM:* Specific role for protein kinase C beta in cell differentiation. Cell Growth Differ 5:405-409, 1994.
22. *Golding B, Pillemer SR, Roussou P et al:* Inverse relationship between proliferation and differentiation in a human TNP-specific B cell line. J Immunol 131:2564-2568, 1988.
23. *Kopper L, Bankfalvi A, Mihalik R. et al:* New in vitro line from a human (B) non-Hodgkin lymphoma. Anticancer Res. 11:1645-1650, 1991.
24. *Kiss K, Uher F and Gergely J:* A natural IgM antiboby does inhibit polyclonal and antigen specific IgM but not IgG B cell responses. Immunol. Lett 39:235-241, 1994.
25. *Gronowicz E, Coutinho A, Melchers F:* A plaque assay for cells secreting Ig of a given type or class. Eur J Immunol 6:588-593, 1976.
26. *Mihalik R, Uher F, Berczi L et al:* Detection of drug-induced apoptosis by flow cytometry after alcaline extraction of ethanol fixed cells. Pathol Oncol Res 2:78-83, 1996.
27. *Coleman TR and Dunphy WG:* cdc2 regulatory factors. Curr Opin Cell Biol 6:877-882, 1994
28. *Wolf CM, Reynolds JE, Morana SJ and Eastman A:* The temporal relationship between protein phosphatase, ICE/CED-3 proteases, intracellular acidification and DNA fragmentation in apoptosis. Exp Cell Res 230:22-27, 1997.
29. *Lock RB:* Inhibition of p34cdc2 kinase activation, p34cdc2 tyrosine dephosphorylation, and mitotic progression in chinese hamster ovary cells exposed to etoposide. Cancer Res. 52:1817-1822, 1992.
30. *Lock RB, Thomson BS, Sullivan DM and Stribinskiene L:* Potentiation of etoposide-induced apoptosis by staurosporine in human tumor cells is associated with events downstream of DNA-protein complex formation. Cancer Chemother Pharmacol 39:399-409, 1997.
31. *Shimizu T, O'Connor PM, Kohn KW and Pommier Y:* Unscheduled activation of cyclin B1/cdc2 kinase in human promyelocytic leukemia cell line HL60 cells undergoing apoptosis induced by DNA damage. Cancer Res 55:228-231, 1995.