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### TUMOR PROMOTER PHORBOL MYRISTATE ACETATE MODULATES KINETICS OF DRUG-INDUCED APOPTOSIS IN HUMAN LEUKEMIA CELLS.

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In recent studies it has been shown that some tumor promoters may suppress apoptosis induced in leukemia cells by tumor necrosis factor or UV irradiation. It follows that environmental tumor promoters may interfere with therapeutic effects of anticancer drugs which exert their activity by inducing apoptosis in tumor cells. In the present study we explored the effects of the tumor promoter phorbol myristate acetate (PMA) on the apoptotic response induced in human leukemia cells by chemotherapeutic agents. To study apoptosis we employed a microculture kinetic (MiCK) assay which allows automated monitoring of apoptosis in suspension cell cultures (Kravtsov and Fabian, *Lab Invest* 74:557, 1996). The MiCK assay detects the size and shape changes of cells undergoing apoptosis and provides a real-time analysis of the apoptotic process rather than using an endpoint analysis as is performed in all other assays of apoptosis. Human promyelocytic (HL60) and lymphoblastic (CEM and CEM/VM1, provided by Dr. W.T. Beck) cells were pretreated with PMA (0.25, 0.5, 1.5 and 10nM) for 2h, distributed in wells of a 96-well microplate and exposed to etoposide (1.5, 10, 25 and 50µM) or doxorubicin (0.1, 1.5, 10, 25µM) continuously for 24h. During that period, apoptosis was monitored using an incubated spectrophotometer. Both HL60 and CEM cells developed an apoptotic response upon exposure to either etoposide or doxorubicin, however the time course and extent of apoptosis varied significantly depending on the cell type and drug concentration. Pretreatment with 0.25, 0.5 or 1nM PMA influenced neither time course nor extent of apoptosis in HL60 cells exposed to any dose of etoposide. Only at 5 and 10nM PMA, doses which induce differentiation in HL60 cells, was a 2 to 3h delay seen in the apoptotic response to 5µM etoposide, but no effect of PMA was seen when apoptosis was induced by 10, 25 or 50µM etoposide. At all doses of doxorubicin in HL60 cells, 1nM PMA caused 2 to 10h delays in apoptosis while 5 and 10nM PMA potentiated the apoptotic response to doxorubicin without changing its kinetics. In CEM cells, none of the tested doses of PMA affected either kinetics or extent of etoposide-induced apoptosis. However, in CEM cells exposed to doxorubicin, PMA significantly delayed and reduced the extent of apoptosis. In CEM/VM-1 cells, which are resistant to topoisomerase II inhibitors, only 25 and 50 µM etoposide and 10 and 25µM doxorubicin induced apoptosis. PMA at 0.5 and 1nM delayed and decreased the extent of apoptotic response to both agents. Our results suggest that the modulating effects of environmental tumor promoters on chemotherapy-induced apoptosis may vary greatly depending upon both the target cells and the chemotherapeutic agents. The continuous readout of the MiCK assay detected modulations in the kinetics of chemotherapy-induced apoptosis that can be easily missed by any of the currently used endpoint assays for apoptosis.

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### ISOTYPE CONVERSION OF CD45RA TO CD45RO AS AN EARLY EVENT IN ARA-C INDUCED APOPTOSIS OF ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) CELLS.

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Leucocyte Common Antigen (LACT/200 or CD45) is a family of cell surface glycoproteins found exclusively on nucleated hematopoietic cells. The restricted pattern of CD45 isoforms on hematopoietic cells has established its role as a marker for leukaemias and lymphomas. The recent discovery that CD45 functions as a transmembrane protein tyrosine phosphatase has attracted attention to the role of this molecule in immunological processes. Merckenschlager and Fisher have suggested that the CD45 isotype conversion from CD45RA to CD45RO is a marker for normal thymocytes destined for apoptosis. Whether chemotherapy induced apoptosis in leukemic cells show the same CD45 isotype conversion is unknown. Four ficolled BM samples of ALL patients with the blasts counts higher than 80% were cultured in serum free medium supplemented with cytokines (IL3 & IL7) without (control) or with ARA-C (10<sup>-5</sup>µM). CD45RA (PE, Becton-Dickinson) and CD45RO (Tri-color, Caltag) were stained at baseline and after 24 hours of culture. Stained cells were analyzed by FACS. At baseline, all four samples expressed CD45RA but not CD45RO. After 24 hours, CD45RO was induced in both control and ARA-C treated cultures. RT-PCR for apoptosis related genes (BCL-2, BCL-XL, BCL-Xs, and BAX) was performed on baseline cells as well as on CD45RA+/CD45RO- and CD45RA-/CD45RO+ cells after FACS sorting. PCR results showed undetectable expression of BCL-Xs and BAX at baseline. At 24 hours, CD45RA-/CD45RO+ cells expressed high level of BCL-Xs and BAX whereas CD45RA+/CD45RO- cells had low expression. BCL-2 and BCL-XL was undetectable at baseline or after 24 hours culture. These preliminary results suggest that there is CD45 isotype switching from CD45RA to CD45RO in cells undergoing ARA-C induced apoptosis. The high expression of apoptosis related genes in the CD45RA-/CD45RO+ cells suggests that perhaps CD45 isotype conversion is an early event of cell death.

### BCL-X<sub>L</sub> IS HETEROGENEOUSLY EXPRESSED BY AML CELLS AND IS ASSOCIATED WITH AUTONOMOUS GROWTH IN VITRO AND WITH P-GLYCOPROTEIN EXPRESSION

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The cells from approximately 70% AML patients exhibit autonomous growth characteristics in vitro, which are associated with a poor response to therapy. We have previously shown that leukemic cells with autonomous growth characteristics express high levels of bcl-2 and are relatively resistant to apoptosis. As bcl-x<sub>L</sub> is a bcl-2 related gene with anti-apoptotic activity which also confers resistance to cytotoxic drugs we have studied its expression in AML in relation to cellular growth characteristics and to the expression of P-glycoprotein. Using the S-18 antibody we have examined bcl-x expression in cells from 16 AML patients by two methods. We used immunoblotting to distinguish bcl-x<sub>L</sub> expression from the smaller, pro-apoptotic form (bcl-x<sub>s</sub>), and a reproducible flow cytometric method, standardised with calibrated microspheres, to quantify bcl-x expression. Autonomous growth of AML blasts was assessed by their ability to form colonies within 5-7 days of culture in a medium without added growth factors, as previously reported. Immunoblotting demonstrated bands at 31kD corresponding to bcl-x<sub>L</sub> from the cells of all 16 patients. Bcl-x<sub>L</sub> was not detected in any sample. Bcl-x<sub>L</sub> expression ranged from 0.25 X 10<sup>5</sup> to 4.24 X 10<sup>5</sup> bound FITC molecules, (median 1.43 X 10<sup>5</sup>). AML blasts with autonomous growth characteristics in vitro expressed more bcl-x<sub>L</sub> (median 1.76 X 10<sup>5</sup>) than those which did not (median 0.86 X 10<sup>5</sup>, p=0.01). Quantitative bcl-x expression strongly correlated with that of P-glycoprotein, also measured by quantitative flow cytometry using the MRK-16 antibody (r=0.95, p<0.001), but not with MRP-1. These results provide a further explanation for the poor prognosis associated with autonomous in vitro growth of AML blasts and illustrate that these cells may co-express different modalities of resistance to cytotoxic drug therapy involving both anti-apoptotic pathways (bcl-x<sub>L</sub>, bcl-2) and classic multidrug resistance (MDR1). The implication of these findings is that the use of agents to reverse MDR1 function in AML may be unsuccessful in the absence of strategies to reduce resistance to apoptosis.

### INHIBITION OF PROTEIN KINASE C (PKC) ARRESTS CELL GROWTH, INDUCES APOPTOSIS, AND ALTERS THE EFFECT OF BASIC FIBROBLAST GROWTH FACTOR (bFGF) ON BCL-2 EXPRESSION IN CHRONIC LYMPHOID MALIGNANCIES. A. König,\* M. Schulz-Jander,\* G.K. Schwartz,\* A. Al-Katib, and J.L. Gabrilove, Memorial Sloan-Kettering Cancer Center, New York, NY, and Wayne State University, Detroit, MI

We have previously shown that 1) basic fibroblast growth factor (bFGF) delays fludarabine-induced apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells, and that 2) it induces an increase in bcl-2 expression in cell lines representative of chronic lymphoid malignancies. To investigate whether protein kinase C (PKC) plays a role in bFGF-mediated upregulation of bcl-2, one EBV-transformed B-prolymphocytic cell line (JVM-2), one EBV-transformed B-CLL cell line (I83CLL), and one non-EBV transformed B-CLL cell line (WSU-CLL) were used as targets. Inhibitors of PKC explored included H-7 (nonspecific), Fluvopiridol and UCN-01 (specific). Treatment of the cells with either H-7 (10<sup>-10</sup> - 100µM), Fluvopiridol (100 nM - 400 nM) or UCN-01 (1µM - 100 µM) led to a marked dose- and time-dependent inhibition of cell growth and survival as determined using trypan blue exclusion. The data for mean viability [%] for two of the cell lines were as follows:

PKC-Inhibitor	molar	I83CLL			JVM-2		
		24h	48h	72h	24h	48h	72h
Fluvopiridol	200nM	63±9	39±1	45±5	92±2	74±2	53±2
	400nM	52±8	10±3	17±3	86±1	41±9	47±9
UCN-01	1µM	85±1	38±9	27±3	65±9	69±3	53±2
	10µM	72±6	31±2	13±5	83±1	58±4	42±2
H-7	10µM	93±1	93±1	92±3	92±1	96±1	93±1
	100µM	68±1	27±1	13±2	67±3	55±3	37±1

Morphological analysis revealed characteristic apoptotic changes such as chromatin condensation and fragmentation, membrane blebbing, and formation of apoptotic bodies. These cellular effects were associated with a significant decrease in bcl-2 expression as observed by Northern and Western blotting. The results showed that all three agents downregulate bcl-2 mRNA within 4-8 hours and bcl-2 protein expression within 8-24 hours. The addition of PKC inhibitors to cells treated with bFGF, resulted in a partial abrogation of bcl-2 upregulation by bFGF, suggesting that PKC is involved in bFGF-mediated enhancement of bcl-2 expression. In addition, these data demonstrate that inhibitors of PKC have significant activity alone in these model systems, suggesting that they might be useful as therapeutics in the treatment of chronic lymphoid malignancies.