The effects of doxorubicin on apoptosis and adhesion molecules of normal peripheral blood leukocytes—an *ex vivo* study

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This *ex vivo* study was designed to evaluate the effect of doxorubicin (Dox) on normal peripheral blood leukocytes (PBL) in terms of apoptosis and membranal expression levels of adhesion molecules. Blood was drawn immediately prior to and after Dox administration from 21 breast cancer patients, and incubated at room temperature for 24 h. Flow cytometry was employed in analysis of apoptosis with Annexin-V and protein membranal expression levels with monoclonal antibodies to CD49d, CD18, CD11a–c and CD63. Dox induced statistically significant apoptosis in all three major PBL subpopulations ($p<0.01$). Between 70 and 90% of samples underwent apoptosis in all PBL subgroups. No significant change was observed in the membranal level of CD63, CD49d and CD11a–c after chemotherapy in any PBL subpopulation. However, a significant reduction in the membranal level of CD18 was demonstrated in polymorphonuclear cells after Dox ($p<0.005$) both in apoptotic and non-apoptotic cells ($p<0.05$), suggesting a direct effect of Dox rather than an apoptosis-associated phenomenon. We observed the expected leukopenia 10 days after Dox administration with no correlation to apoptosis, suggesting that leukopenia by Dox is largely attributed to toxicity of blood progenitors. *Anti-Cancer Drugs* 14:383–389 © 2003 Lippincott Williams & Wilkins.

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Introduction

Accumulating evidence suggests that chemotherapeutic agents exert their cytotoxic effects on tumor cells mainly by inducing apoptosis [1–4]. The lack of cytotoxic selectivity of these drugs results in dose-limiting effects, particularly myelosuppression, manifested by leukopenia and neutropenia associated with life-threatening infections [5]. Doxorubicin (Dox) is known to exert its antineoplastic effect by activating apoptotic pathways involving Fas receptor and caspase-3 modulations [6]. However, only a few reports regarding its effect on normal peripheral blood leukocytes (PBL) have been published [7–9]. Furthermore, the associated leukopenia and neutropenia were attributed mainly to bone marrow stem cell damage, overlooking the possible role of drug-induced apoptosis in PBL [10]. A recent study addressed this aspect studying polymorphonuclear cells (PMN) and established a time-dependent increase in *ex vivo* granulocyte apoptosis, with a maximal effect just preceding the development of neutropenia [11]. However, these reports had major limitations, including the study of combinations of drugs, variance of dosages, heterogeneous patient populations and different methods of apoptosis detection. Studies on the direct effect of a single chemotherapeutic agent in a homogenous patient population are clearly needed.

Adhesion molecules in general, and integrins and tetraspanins specifically, are implicated in signal transduction culminating in apoptosis [12–18]. Moreover, it is becoming more evident that membranal molecules play major roles in sensitivity and resistance to various signals [19,20]. The modulation of adhesion molecule expression in normal PBL subpopulations following chemotherapy was not investigated systematically, and, more specifically, there was no evaluation of their association with drug-induced apoptosis and the expression of adhesion molecules such as CD18 (β subunit of β_2 integrin), CD11a–c (α subunit of β_2 integrin), CD63 (tetraspanin), and VLA-4 ($\alpha_4\beta_1$ integrin).

We designed a single-drug, *ex vivo* study to evaluate the effects of Dox treatment on all three major PBL subpopulations in a homogenous group of breast cancer patients. Our aim was to characterize chemotherapy-induced PBL apoptosis, and to evaluate the participating molecules and pathways involved. Correlations between laboratory findings and clinical data were also assessed.

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Materials and methods

Patient characteristics

The study was comprised of 21 consecutive patients with breast cancer. Institutional and Israeli Health Ministry Helsinki approvals were obtained, and all patients signed an informed consent. Detailed information including demographic data, history and treatment modalities was recorded. Patients were treated with cyclophosphamide 600 mg/m^2, Dox (adriamycin) 60 mg/m^2 ± 5-fluorouracil 600 mg/m^2 (CA/CAF). The drugs were administered in a predefined order so that blood was extracted after Dox alone in the index course, after which the rest of the drugs were infused. No steroids were administered as antiemetics. The patients were assayed after receiving a different number of treatment courses. Due to technical limitations not all samples were included in all aspects of the experiment.

Leukocyte extraction

Blood (5–10 ml) was drawn immediately before and after Dox administration, thereby enabling a minimization of variability by a paired assessment. The sampling after drug infusion was preceded by a saline wash to the i.v. line in order to avoid any sampling bias due to residual drug. Paired samples before and after Dox infusions were both incubated overnight (24 h) 'as are' at 25°C. Leukocyte isolation from each sample pair was achieved by centrifugation for 10 min at 75 *g* followed by hypotonic lysis for 20 s to free the PBL from contaminating red blood cells. NaCl 3.5% stopped the lysis. Supernatant was discarded following another 7-min centrifugation and the cell pellet was resuspended in 1 ml PBS (based on Boyum *et al.* [21]).

Antibodies

Fluorescein isothiocyanate (FITC)–coupled Annexin-V (#1 828 681) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). FITC-coupled monoclonal mouse anti-human IgG1 to CD11a, CD11b, CD11c, CD18, CD49d (α_4 subunit of VLA4) and CD63 were purchased from Caltag (Burlingame, CA).

Flow cytometry

Identification of PBL subgroups

Analysis gates for PMN, monocytes and lymphocytes were established using distinctive forward and side scatter (FSC/SSC) parameters by FACS (EPICS-XL; Beckman Coulter, High Wycombe, UK). Validation of the placing of each major cell group was done with conventional membranal markers associated with each cell population (data not shown).

Analysis of apoptosis

Annexin-V was employed for exposed phosphatidylserine detection according to the manufacturer's instructions. In short, cells were washed in PBS, resuspended (1×10^6/ml), and incubated in a 100 μl solution of HEPES buffer containing 2 μg FITC-labeled Annexin and 0.1 μg propidium iodide (PI) for 15 min at room temperature, further diluted in additional 400 μl PBS and assayed for fluorescence by FACS. Negative control for Annexin was established according to leukocytes of freshly extracted blood and set as background fluorescence. Cells were regarded apoptotic if their assayed fluorescence was higher than the established background. A second approach to verification of apoptosis was analysis of differential cell sizes and refractive/reflective properties established according to FSC/SSC, which allowed discrimination of apoptotic from non-apoptotic cells in each leukocyte subpopulation [22]. Occasional microscopic analysis of cell morphology was used for added verification of the presence or absence of apoptosis.

Determination of surface molecule expression

Surface expression of CD11a–c, CD18, CD49d and CD63 was assessed by direct immunofluorescence using mouse anti-human fluorochrome-coupled monoclonal antibodies. The cells were incubated with saturating amounts of antibodies for 15 min at room temperature in the dark, washed in PBS and diluted in 400 μl PBS. IgG1-matched isotype was used to exclude unspecific binding. Fluorescence was analyzed by FACS. All results were expressed as percent of apoptotic cells relative to the total cell count of the respective sample subgroup. At least 10 000 events were counted in each FACS analysis.

Statistical analysis

Student's paired and unpaired *t*-tests were employed in analysis of differences between cohorts. Correlations were assessed according to Pearson's correlation coefficient. An effect was considered significant when $p \leq 0.05$.

Results

Patients

Twenty-one women participated in the study, four of which were assayed twice in separate courses along the therapy. The mean age was 47.5 years and the mean number of courses of therapy was 3.08. Sixteen of them had surgery (lumpectomy, mastectomy or lymph node dissection), four were treated with Dox as a neoadjuvant and only one patient was previously treated with radiotherapy. Their characteristics are shown in Table 1.

Apoptosis

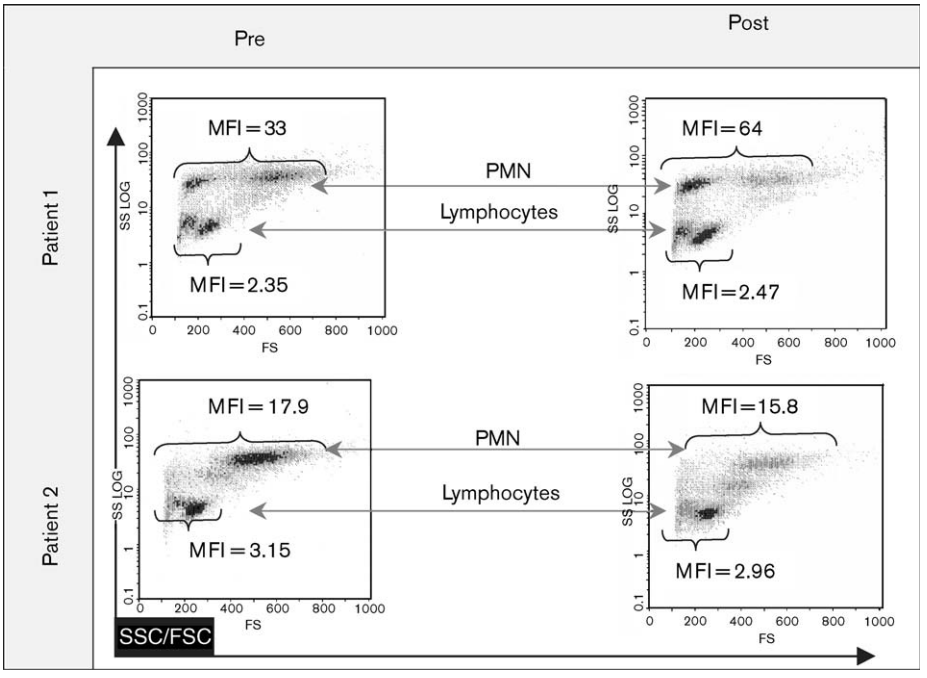
The apoptotic response was measured by FACS. Representative flow scans presentations of the light scatter characteristics and Annexin-V/PI before and after Dox administration are presented (Figs 1 and 2). Dox induced a significant increase in percent of apoptotic cells in all three PBL subpopulations (PMN $p = 0.005$; monocytes

Table 1 Patient characteristics

Patient no.	Age	Protocol	Course no.	Medication	Medical history
1	52	CAF	1		S/P Rt lumpectomy and ALND
2	65	CA	3		
3	76	CA	2		S/P Rt lumpectomy (metastasis)
4	69	CA	1		neoadjuvant
5	54	CA	1		
6	53	CA	4		S/P Lt lumpectomy and ALND
7	69	CA	4		S/P Lt lumpectomy ALND and radiotherapy (metastasis)
8	52	CAF	5		S/P Lt lumpectomy and ALND
9	50	CAF	2		S/P ALND (metastasis)
10	76	CA	6		S/P Lt lumpectomy (metastasis)
11	61	CA	2		(metastasis)
12	47	CAF	1		S/P Rt lumpectomy and ALND
13	44	CA	3		neoadjuvant
14	47	CAF	5		S/P Rt lumpectomy and ALND
15	57	CA	2		S/P Rt mastectomy
16	55	CA	2		S/P Lt lumpectomy and ALND
17	59	AT	1	steriods	neoadjuvant
18	48	CAF	5	G-CSG	S/P Rt lumpectomy and ALND
19	52	CAF	4		S/P Lt lumpectomy and ALND
20	57	CAF	6		S/P Rt lumpectomy and ALND
21	57	AT	1		neoadjuvant
22	47	CAF	2		S/P Rt lumpectomy and ALND
23	43	CAF	5		S/P Lt lumpectomy and ALND
24	51	CAF	5		S/P Lt modified radical mastectomy and ALND
25	50	CAF	4		S/P ALND (metastasis)

ALND, axillary lymph node dissection; Rt, right; Lt, left; G-CSF, granulocyte colony stimulating factor; S/P, state post; AT, adriamycin (Dox), taxol.

Fig. 1



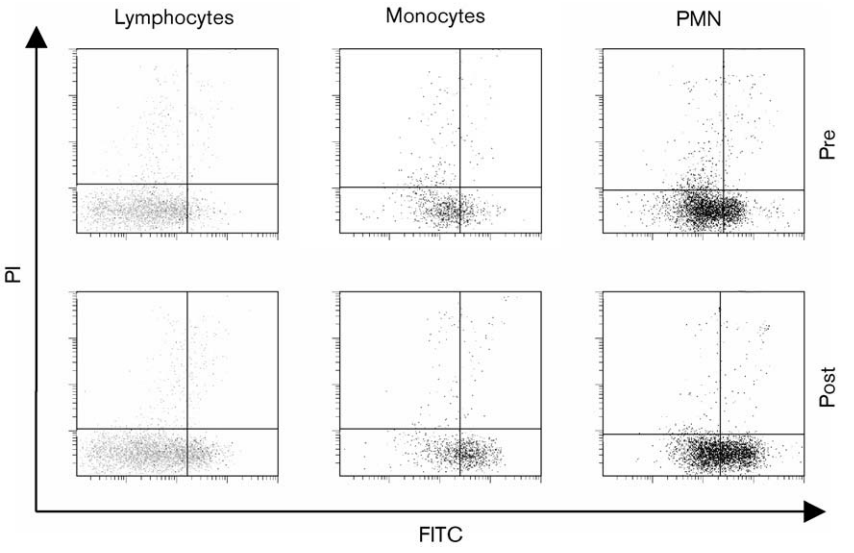
Light scatter characteristics and Annexin-V MFI of PBL before and after Dox administration. The histogram displays the three major PBL subpopulations according to their light scatter characteristics (*x*-axis, FSC; *y*-axis, SSC). Apoptotic cells are smaller and more granular as indicated by decreased FSC and slightly increased SSC. Respective Annexin-V MFI values are indicated. Patient 1 responded to Dox treatment with increased apoptosis demonstrated by both light scatter morphology as well as increased phosphatidylserine exposure, whereas patient 2 did not undergo apoptosis.

$p = 0.001$; lymphocytes $p = 0.01$) (Fig. 3). The apoptotic effect was demonstrated in 70–90% of the patients (Table 2). No correlation was found between the chemotherapy course number and apoptosis.

Adhesion molecules

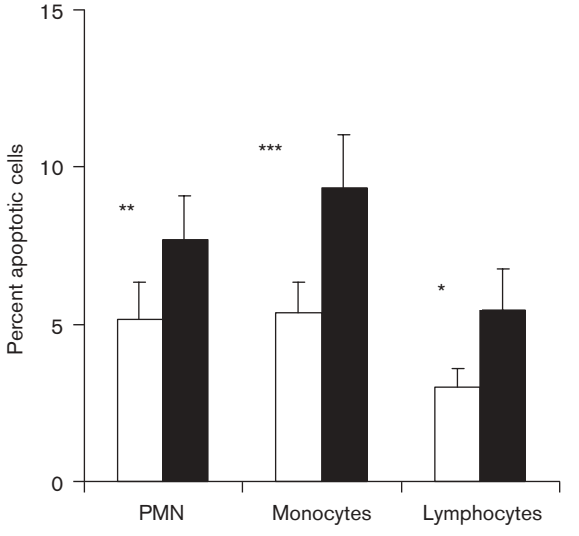
We found a significant reduction in the membranal expression level of CD18 in PMN after Dox treatment ($p < 0.005$), with a mean reduction of 36.64% (24

Fig. 2



Drug-induced apoptosis: representative Annexin-V (FITC) and PI fluorescence of leukocyte subpopulations before and after Dox treatment of a sample with an apoptotic response. All dot-plots display enhanced FITC fluorescence in samples extracted from patients after drug infusion and only a minor increase in PI^+ cells (<5%), supporting an apoptotic rather than a necrotic response.

Fig. 3



Dox-induced apoptosis in PBL subpopulations. Annexin-V indicative of exposed phosphatidylserine was measured by FACS before (white bars) and after (black bars) Dox administration in PMN, monocytes and lymphocytes. Results are expressed as the mean percent of Annexin-V^+ PBL of all evaluated patients ± SE. The increased percent of apoptotic cells was statistically significant in all subgroups ($p<0.05$) (*$p=0.01$; **$p=0.005$; ***$p=0.001$).

patients) (Fig. 4). A significant decrease in CD18 was observed in both apoptotic and non-apoptotic PMN (38.51 and 31.05% decrease, $p < 0.05$). No change in CD18 expression was detected in monocytes and lymphocytes.

There was no significant change in the membranal level of the α chain of the β_2 integrins investigated (CD11a, CD11b and CD11c) on the PBL subpopulations post-chemotherapy (eight patients). No significant change was observed in membranal level of CD63 in any of the three PBL subpopulations after Dox treatment (16 patients) nor was there any variation in the membranal level of CD49d in any of the PBL subpopulations (13 patients) (data not shown).

Clinical outcome

We found a significant drug-induced decrease in counts of leukocytes, monocytes and lymphocytes 10 days after treatment ($p = 0.001$, $p < 0.0001$ and $p = 0.001$, respectively). The decrease in PMN counts did not reach statistical significance ($p = 0.079$). There were no correlations between Dox-induced apoptosis and rate (%) of cell count reduction in any of the subgroups. There were no correlations between age or treatment course and any of the assayed drug response parameters (cell counts, percent apoptosis).

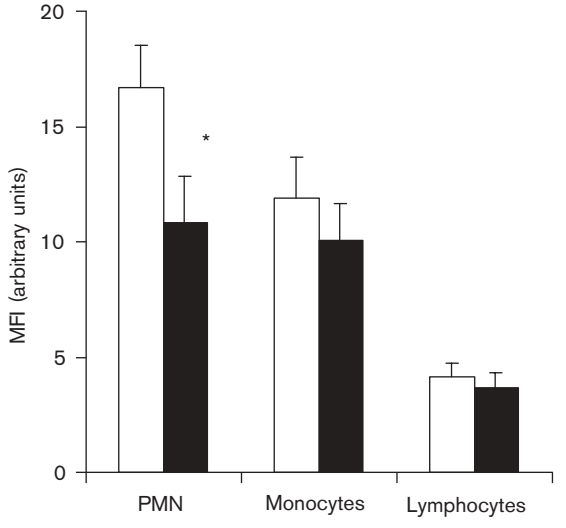
Discussion

In this study we showed that Dox induces a significant *ex vivo* apoptosis of normal peripheral PMN, monocytes and lymphocytes. Approximately 70–90% of samples underwent programmed cell death after Dox treatment (in the respective subgroups). However, the clinical relevance of

Table 2 Apoptosis in PBL subpopulations of Dox-treated breast cancer patients (% Annexin-V$^+$ cells)

Patient no.	PMN		Monocytes		Lymphocytes	
	Pre	Post	Pre	Post	Pre	Post
1	7.6	14	7.7	9.3	4.6	6.4
2	4.75	8.66	8.07	20.3	1.23	6.49
3	1.36	2.19	7.70	4.36	0.38	0.53
4	2.74	0.86	3.28	3.58	0.92	0.32
5	0.91	12.1	1.35	11.9	1.23	19
6	1.73	4.91	1.81	5.46	4.31	6.5
7	2.25	4	2.39	2.67	1.49	4.01
8	1.56	6.44	1.29	8.18	0.94	3.8
9	6.51	9.64	2.11	5.49	2.6	4.39
10	1.48	5.81	15.9	23.6	0.37	3.29
11	22.1	19.6	18.7	21.7	5.1	5.2
12	3.46	2.51	1.79	1.89	2.9	2.89
13	2.69	1.91	2.43	2.58	0.79	0.28
14	1.38	0.21	2.56	2.17	6.28	4.76
15	0.91	1.79	1.28	2.81	0.91	1.44
16	0.56	0.48	0.39	0.67	0.93	0.2
17	6.18	6.71	1.4	2.2	0.42	1.99
18	2.63	4.14	3.23	6.55	2.38	2.54
19	0.56	1.07	3.35	8.45	0.58	0.7
20	3.45	4.3	3.84	3.94	10.3	9.1
21	6.28	15.81	9.48	18.83	3.88	7.53
22	11.0	20.2	7.2	32.9	12.0	30
23	23.05	18.6	15.27	18.47	7.0	8.37
24	6.97	22	5.0	11.0	1.1	5.32
25	0.91	1.89	1.25	2.81	0.91	1.44
Mean	5.17	7.71	5.35	9.34	2.98	5.44
SE	1.18	1.70	0.99	1.70	0.62	1.30

Fig. 4



Dox-induced changes in membranal expression level of CD18. Membranal expression of CD18 was measured by FACS before (white bar) and after (black bar) Dox administration in PMN, monocytes and lymphocytes. Results are expressed as the mean percent change in PBL MFI values of all evaluated patients ± SE. The decrease in membranal expression of CD18 in PMN is statistically significant ($p = 0.002$) (*$p < 0.05$).

these findings is questionable in spite of statistical significance due to the limited extent of induced apoptosis, and lack of correlation between apoptosis and cell counts in the treated patients. Our observations are especially interesting since they are the first to demonstrate the effect of a single cytotoxic drug simultaneously on all the normal major leukocyte subpopulations.

Only few studies investigated the effect of chemotherapy on normal PBL, and the results are limited by profound variability of the design of these studies and heterogeneity of administered drugs and PBL populations. Ferraro *et al.* investigated the effect of anthracyclines *in vitro* on lymphocytes of healthy donors, and showed rapid and massive peripheral T and B cell depletion by apoptosis [8]. Provinciali *et al.* observed a post-treatment apoptotic effect *in vivo* of lymphocytes of women with solid tumors treated with combination chemotherapy [9]. There was an increased apoptosis of PMN cells following chemotherapy in children with acute lymphocytic leukemia [7]. Thus, a readily available source of normal blood cells for the analysis of the effects of chemotherapeutic agents was clearly underutilized.

Membranal embedded molecules have a vital function in signal transduction and apoptosis; in particular, β_2 integrins are of fundamental importance in leukocytes [13,14]. We found that CD18 underwent significant down-regulation in PMN both in apoptotic and non-apoptotic cells, indicating an effect independent of the apoptotic response. These findings correspond with the published results of Wang *et al.* describing a decreased expression of CD11b/CD18 on PMN post-chemotherapy [23]. Recently, Weimann *et al.* reported that

CD18-deficient mice had increased PMN counts in their peripheral blood due to a reduced rate of apoptosis [24]. The CD18-deficient PMN displayed higher levels of anti-apoptotic Bcl-X_L and depleted levels of the pro-apoptotic Bax compared to normal PMN. A different study showed that by blocking PMN CD18 or CD11b, there was an almost complete prevention of immobilized plasma IgA-induced apoptosis [25]. In additional reports it was shown, on the one hand, that Dox induces apoptosis by generation of hydrogen peroxide and activation of the nitric oxide cascade [26], and, on the other hand, that nitric oxide affects the CD18 expression level on PMN [27]. Hence, we could hypothize that reduction of CD18 expression on PMN upon exposure to Dox promotes cellular resistance to apoptosis. However, reports of increased expression of CD11b/CD18 at PMN nadir post-chemotherapy in patients with hematological malignancies suggest a complex role for this molecule [28]. The α chain of the β_2 integrins was studied as well. We found no significant change in the membranal expression of CD11a–c contrary to an expected parallelism with CD18. Several possible explanations exist that should be further explored: the small study group may account for the lack of statistical significance, the α chain involved may be CD11d, which was not analyzed in our study, and a cumulative down-regulation of several or all α chains may render each one separately not significantly associated with the CD18. Both down-regulation and enhanced expression of CD63 in spontaneously apoptotic PMN were reported [17,18]. In our study no significant change was found in the membranal expression level of CD63 after Dox in PBL. Thus, CD63 expression is not modulated by Dox treatment within the time frame of our study.

VLA-4 ($\alpha_4\beta_1$) has been found to exhibit both cell–cell and cell–matrix adhesion [29]. In our study there was no CD49d (α_4) level modulation with exposure to Dox. In contrast, reduced expression of VLA-4 was observed in B cell chronic lymphocytic leukemia [15] and in non-apoptotic Ramos B lymphoblastoid cells [16], and an overexpression of VLA-4 in Dox-resistant multiple myeloma and breast cancer cell lines was reported [19,20]. The complexity of signaling pathways might necessitate a larger sample group to analyze the role of this molecule in the apoptotic pathways.

Clinically, we observed the expected occurrence of leukopenia, 10 days after administration of Dox. This decrease in white blood cells counts is accounted for mainly by lymphopenia and monocytopenia, and a trend towards significant neutropenia. No correlation was found between this reduction and apoptosis of any PBL subpopulation. Chukhlovin recently found that myeloablative therapy caused a time-dependent increase in *ex vivo* granulocyte apoptosis, being maximal at days 7–9, thus preceding the development of neutropenia. This suggested that PBL apoptosis is a major determinant of chemotherapy-induced leukopenia [11]. It should be emphasized that Chukhlovin's research encompassed combined chemotherapy and multiple regimens. Our results confirmed that Dox does induce apoptosis of normal PBL within 24 h of drug infusion, but this early event is not correlated with the leukopenia that occurs clinically 10 days after administration.

We conclude that Dox induces apoptosis in normal PBL within 24 h of administration. This cellular response to drug administration is associated with the modulation of surface expression of CD18, but not CD63, CD49d and CD11a–c. The lack of correlation between cell counts and the rate of apoptosis suggests that leukopenia by Dox is largely attributed to toxicity of blood progenitors. A further study is to be conducted to evaluate the signal transduction pathways and adhesion molecules involved in Dox-induced leukopenia. This may clarify the modalities that assist normal cells to avoid drug-induced cell death.

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References

1 Hannun AY. Apoptosis and the dilemma of cancer chemotherapy. *Blood* 1997; **89**:1845–1853.
2 Kamesaki H. Mechanisms involved in chemotherapy-induced apoptosis and their implications in cancer chemotherapy. *Int J Hematol* 1998; **68**:29–43.
3 Schmitt AC, Lowe WS. Apoptosis and therapy. *J Pathol* 1999; **187**:127–137.
4 Kaufmann HS, Earnshaw CW. Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 2000; **256**:42–49.
5 Hickman JA. Apoptosis induced by anticancer drugs. *Cancer Metastasis Rev* 1992; **11**:121–139.
6 Wu XX, Mizutani Y, Kakehi Y, Yoshida O, Ogawa O. Enhancement of Fas-mediated apoptosis in renal cell carcinoma by adriamycin. *Cancer Res* 2000; **60**:2912–2918.
7 Leujene M, Ferster A, Cantinieaux B, Sariban E. Prolonged but reversible neutrophil dysfunctions differentially sensitive to granulocyte colony-stimulating factor in children with acute lymphoblastic leukemia. *Br J Haematol* 1998; **102**:1284–1291.
8 Ferraro C, Quemeneur L, Prigent AF, Taverne C, Revillard JP, Bonnefoy-Berard N. Anthracyclines trigger apoptosis of both G_0–G_1 and cycling peripheral blood lymphocytes and induce massive deletion of mature T and B cells. *Cancer Res* 2000; **60**:1901–1907.
9 Provinciali M, Ciavattini A, Di Stefano G, Argentani K, Garrazetti GG. *In vivo* amifostine (WR-2721) prevents chemotherapy-induced apoptosis of peripheral blood lymphocytes from cancer patients. *Life Sci* 1999; **64**:1525–1532.
10 Kim KS, Demetri GD. Chemotherapy and neutropenia. *Hematol Oncol Clin N Am* 1996; **10**:377–395.
11 Chucklovin A. Enhanced *ex vivo* apoptosis of peripheral granulocytes is a sufficient factor of neutropenia following myeloablative chemotherapy. *Leuk Res* 2000; **24**:507–509.
12 Dedhar S. Integrin mediated signal transduction in oncogenesis: an overview. *Cancer Metastasis Rev* 1995; **14**:165–172.
13 Walzog B, Jeblonski F, Zarzewicz A, Gaehtens P. β_2 integrins (CD11/CD18) promote apoptosis of human neutrophils. *FASEB J* 1997; **11**:1177–1186.

14 Ropke C, Gladstone P, Nielsen M, Ledbetter JA, Svejgaard A, Odum N. Apoptosis following interleukin-2 withdrawal from T cells: evidence for a regulatory role of CD18 (beta 2-integrin) molecules. *Tissue Antigens* 1996; **48**:127–135.
15 Baldini LG, Cro LM. Structure and function of VLA integrins: differential expression in B-cell leukemia/lymphoma. *Leuk Lymph* 1994; **12**:197–203.
16 Toledano BJ, Bastien Y, Noya F, Mazer B. Characterization of B lymphocytes rescued from apoptosis by platelet-activating factor. *Cell Immunol* 1999; **191**:60–68.
17 Hart SP, Ross JA, Haeslett C, Dransfield I. Molecular characterization of the surface of apoptotic neutrophils: implications for functional downregulation and recognition by phagocytes. *Cell Death Differ* 2000; **7**:493–503.
18 Beinert T, Munzing Silvia, Possinger K, Krombach F. Increased expression of the tetraspanins CD53 and CD63 on apoptotic human neutrophils. *J Leukoc Biol* 2000; **67**:369–373.
19 Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* 1999; **93**:1658–1667.
20 Nista A, Leonetti C, Bernardini G, Mattioni M, Santoni A. Functional role of alpha4beta1 and alpha5beta1 integrin fibronectin receptors expressed on adriamycin-resistant MCF-7 human mammary carcinoma cells. *Int J Cancer* 1997; **72**:133–141.
21 Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scan J Clin Lab Invest* 1963; **21**:77–89.
22 Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, *et al.* Features of apoptotic cells measured by flow cytometry. *Cytometry* 1992; **13**:795–808.
23 Wang X, Clowes C, Duarte R, Pu QQ. Expression of CD11b/CD18 on neutrophils after consolidation chemotherapy for acute myeloid leukemia and after high dose chemotherapy with autologous stem cell transplantation. *Int J Oncol* 2000; **17**:597–602.
24 Weimann P, Scharffetter-kochanek K, Forlow SB, Peters T, Walzog B. A role for apoptosis in the control of neutrophil homeostasis in the circulation: insight from CD18-deficient mice. *Blood* 2003; **101**:739–746.
25 Schettini J, Salamone G, Trevani A, Gamberale R, Vermeulen M, Giordano M, *et al.* Stimulation of neutrophil apoptosis by immobilized IgA. *J Leukoc Biol* 2002; **72**:685–691.
26 Kalyanaraman B, Joseph J, Kalivendi S, Wang S, Konorev E, Kotamraju S. Doxorubicin-induced apoptosis: implications in cardiotoxicity. *Mol Cell Biochem* 2002; **234/235**:119–124.
27 Sato Y, Walley Kr, Klut ME, English D, D'yachkova Y, Hogg JC, *et al.* Nitric oxide reduces the sequestration of polymorphonuclear leukocytes in lung by changing deformability and CD18 expression. *Am J Respir Crit Care Med* 1999; **159**:1469–1476.
28 Engervall P, Laundahl J. Alterations in adhesion molecule (CD11b/CD18 and CD62L) expression on granulocytes after chemotherapy. *Eur J Clin Invest* 1998; **28**:924–929.
29 Ezioni A. Integrins:the glue of life. *Lancet* 1999; **353**:341.