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Correlation between chromosome damage and apoptosis induced by fludarabine and idarubicin in normal human lymphocytes

Marcela González-Cid *, Ariela F. Fundia, Maria Teresa Cuello, Irene Larripa

Depto. de Genética, Instituto de Investigaciones Hematológicas 'M. R. Castex', Academia Nacional de Medicina, Bs. As., Pacheco de Melo 3081, Capital Federal 1425, Argentina

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Abstract

Fludarabine (FLU, a fluorinated purine analog) and idarubicin (IDA, a DNA-topoisomerase II poison) are frequently used in cancer chemotherapy. The effects of these drugs on cultured normal human lymphocytes were studied to establish the possible involvement of chromosome damage in the apoptotic program. Chromosome aberrations (CA) were evaluated in first division metaphases and the apoptotic process was measured by morphological and electrophoretical techniques. The percentage of abnormal cells was increased from the doses of FLU 1.0 μg/ml and IDA 0.005 μg/ml ($P < 0.0001$) with an important decrease in the mitotic index (MI) for the highest doses assayed. A significant dose-dependent induction of abnormal cells was observed for both drugs. An increase of apoptotic cells was found at 5.0 and 10.0 μg/ml of FLU ($P < 0.001$) while IDA activated apoptosis at 0.05 μg/ml ($P < 0.01$) and markedly from 0.1 μg/ml ($P < 0.001$). These increments were dose dependent. Apoptotic cell morphology was associated with DNA fragmentation at the highest doses. The increased induction of abnormal cells and the decreased MI were in correlation with the apoptotic index for FLU and IDA, suggesting the role of CA in drug-induced cell death.

Keywords: Fludarabine; Idarubicin; Chromosome aberrations; Apoptosis; Normal human lymphocytes

1. Introduction

Anticancer drugs have the ability to induce many types of DNA lesions. The consequences of this effect could potentially lead to carcinogenesis. The capability of normal cells to recognize and repair different DNA alterations correctly and timely is a key protective mechanism counteracting the generation of oncogenic mutations, from premutational DNA damage (Rajewsky et al., 2000). Some of these lesions could produce chromosome aberrations (CA) that represent a significant damage of the genome, leading to cell death. A particular type of cell death is apoptosis, that is an active process during which the affected cell,

* Corresponding author. Tel.: +54-11-4805-5759x241/291; fax: +54-11-4803-9475.

E-mail address: lacuteci@intramed.net.ar (M. González-Cid).

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either in response to environmental signals or triggered by intrinsic factors, activates and executes a characteristic preprogramed cascade of molecular events which culminates in the cell's physical destruction (Darzynkiewicz and Traganos, 1998).

Two drugs commonly used in cancer chemotherapy are Fludarabine (FLU) and Idarubicin (IDA) (Ferguson and Pearson, 1996). FLU is an adenine nucleoside analog resistant to adenosine deaminase that has been extensively used to treat various hematological malignancies such as B-cell chronic lymphocytic leukemia (B-CLL), lymphomas and acute myeloid leukemia. DNA synthesis inhibition is the most important mechanism of action for FLU, but also inhibits RNA synthesis. Its active metabolite (F-ara-AMP) competes with deoxy-ATP for incorporation into the growing nuclei acid chain causing disruption of nuclei acid synthesis. In the same way, F-ara-AMP interrupts the elongation of the RNA primer strand by DNA primase. (Wright et al., 1994). On the other hand, IDA, a daunorubicin analog, has been used in the treatment of acute myeloid leukemia, lymphomas and breast cancer (Cersosimo, 1992; Hande, 1998). The primary mechanism of tumor cytotoxity appears to be the inhibition of topoisomerase II. IDA stabilizes the enzyme–DNA complexes, preventing the rapid turnover of the protein-cross-linked DNA strand breaks and interferes with processes that require changes in DNA topology, such as DNA replication, repair and transcription. Other mechanisms of IDA include DNA intercalation, nuclear helicases inhibition and free radicals formation (Hande, 1998).

Despite FLU and IDA's increasing use in malignancies, scarce data are available in literature on their potential clastogenicity in normal human cells. Moreover, the relationship of the clastogenic effects of both drugs with the apoptotic program is still not clearly established. Therefore, the aim of this work was to evaluate the in vitro genotoxic and cytotoxic effects induced by FLU and IDA in normal human lymphocytes measuring chromosome damage and apoptotic response for determining the possible correlation between both end-points.

2. Materials and methods

2.1. Chemicals

FLU, 9-β-D-arabinofuranosyl-2-fluoroadenine (CAS no. 75607-67-9) was commercially obtained as Fludara® from Schering, Argentina. IDA, 4-demethoxydaunorubicin, Zavedos® (CAS no. 58957-92-9) was donated by Pharmacia and Upjohn, Argentina.

2.2. Lymphocyte cultures and drug treatments

Blood was obtained from four healthy donors (three women and one man, aged from 32 to 46 years and free of any known exposure to genotoxic agents). Duplicate lymphocyte cultures were set up by adding 0.8 ml of heparinized whole blood in 10 ml F-10 medium containing 15% fetal calf serum, 2% phytohemagglutinin and 10 μg/ml bromodeoxyuridine (BrdU). Peripheral blood lymphocytes (PBL) were treated with 0.1, 0.5, 1.0, 5.0 or 10.0 μg/ml of FLU and 0.005, 0.01, 0.05, 0.1 or 0.5 μg/ml of IDA, 24 h after the start of cultures and left until harvesting. Both chemicals were dissolved in water. The doses assayed were lower than those used clinically. BrdU 50.0 μg/ml and doxorubicin (DOXO) 0.1 μg/ml were included as positive controls. Control and positive cultures were grown under identical conditions. Cells were harvested after 72 h of incubation at 37 °C in complete darkness.

2.3. Chromosome aberrations

Colcemid (0.2 μg/ml) was added for the last 1.5 h of the culture period. Cells were resuspended in a prewarmed hypotonic solution (0.075 M KCl) for 15 min and fixed in methanol/acetic acid (3:1). Differential staining of sister chromatid was carried out by a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). For each dose, at least 50 first division metaphases were examined for the occurrence of different types of structural CA. Gaps were excluded in the calculation of chromosome breakage frequencies. Chromatid exchange configurations and ring chromosomes were scored as two breaks (Auerbach et al., 1981).

Metaphases containing more than ten aberrations were considered as cells with multiple aberrations. Mitotic indices (MI) were evaluated by counting the number of metaphases for 1000 nuclei.

2.4. Morphological characterization of apoptotic cells

Apoptotic cells were determined at the end of each treatment using the acridine orange (AO)/ethidium bromide (EB) assay: 25 μl of the cell suspension was mixed with 1 μl of the staining solution (AO 100 μg/ml + EB 100 μg/ml in PBS), spread on a slide and 200 cells were counted per data point. The percentage of apoptotic cells (apoptotic index, ApI) was calculated as follows: ApI = (number of cells with apoptotic nuclei/number of cells counted) × 100.

2.5. DNA fragmentation assay

Triplicate PBL cultures were treated with FLU (5.0 or 10.0 μg/ml) and IDA (0.05, 0.1 or 0.5 μg/ml) as described above. Untreated PBL were used as controls. Cells were resuspended in lysis buffer (5 mM Tris–HCl, 20 mM EDTA, 0.5% Triton-X 100; pH = 8.0), for 15 min on ice and precipitated with absolute ethanol and 5 M NaCl for 2 h at −70 °C. Pellets were incubated with 5 mg/ml RNase A (37 °C, 30 min) followed by a further incubation with 0.4 mg/ml proteinase K (30 min). After phenol/chloroform/isoamyl alcohol (25:24:1) extraction, supernatants were transferred into new tubes. DNA was reextracted and precipitated overnight at −20 °C. The pellet was washed once with 70% ethanol and resuspended in 10 μl TE buffer. DNA electrophoresis was carried out in a 1.8% agarose gel, stained with EB and visualized under UV light. A 50 bp DNA molecular weight marker (Gibco) was included in each gel.

2.6. Statistical analysis

Differences between the values of abnormal cells and ApI were analyzed by the χ^2 test. MI mean frequencies were evaluated by the Student's t-test. Dose–response relationships were determined by means of the regression coefficients and also linear correlation between CA, MI and ApI was performed (Primer of Biostatistics, version 3.0 by S.A. Glantz, McGraw-Hill, Inc, 1992).

3. Results

Data of structural chromosome abnormalities in PBL treated with FLU and IDA are presented in Table 1. The percentage of abnormal cells was significantly increased from the doses of 1.0 μg/ml of FLU and 0.005 μg/ml of IDA ($P < 0.0001$). A dose-dependent induction of abnormal cells was observed with FLU ($P < 0.0001$) and IDA ($P < 0.01$).

Both drugs mainly induced chromatid and chromosome breaks. Chromatid exchanges were less frequently induced by FLU than IDA-treatment. Pulverizations were produced only by the highest doses of FLU. This drug produced a dose-dependent increase ($P < 0.0001$) of cells with multiple aberrations while no differences were observed with the different concentrations of IDA.

BrdU and DOXO were used to compare the genotoxic effects of chemicals assayed with known clastogenic agents. BrdU did not produce significant increase in CA frequency with respect to control. The percentage of abnormal cells after DOXO treatment was lower than that induced by IDA at the same dose.

The dose of 5.0 μg/ml of FLU produced a decrease in the number of scorable metaphases, this effect was more important at the highest dose. No scorable metaphases were available at 0.5 μg/ml of IDA. MI data showed a significant decrease in the percentage of mitosis in drug-treated cultures compared with controls ($P < 0.005$).

Fluorescent DNA-binding dyes were used to visualize cells with normal or aberrant chromatin organization (Fig. 1). Untreated cells contained nucleus with organized chromatin structure and an intact cytoplasm. In contrast, morphological changes in nucleus and cytoplasm were observed in treated cells. They presented the typical apoptotic features: cell shrinkage, chromatin condensation and margination and nuclear fragmentation.

Table 1
Frequencies of chromosome damage induced by FLU and IDA in human lymphocytes

Chemical (μg/ml)		Cells scored	Abnormal cells (%)	Total structural CA					Total breaks without gaps (%)	Multiple CA > 10 (%)	pvz	Mitotic index (%) (X ± SE)
				Gaps	ctb	csb	cte	ring				
Control		600	8(1.3)	13	7	1	–	–	8(1.3)	–	–	3.3 ± 0.5
FLU	0.1	200	2(1.0)	4	2	–	–	–	2(1.0)	–	–	2.5 ± 0.6
	0.5	300	7(2.3)	3	3	3	1	–	8(2.7)	–	–	2.4 ± 0.3
	1.0	310	25(8.1)[a]	6	15	6	1	–	23(7.4)	6(1.9)	–	2.0 ± 0.5
	5.0	106	72(67.9)[a]	11	48	21	2	–	73(68.9)	27(25.5)	15(14.2)	0.6 ± 0.3[b]
	10.0	14	13(92.9)[a]	5	16	5	–	–	21(150.0)	8(57.1)	1(7.1)	0.3 ± 0.1[b]
BrdU	50.0	250	7(2.8)	7	5	2	–	–	7(2.8)	–	–	2.2 ± 0.5
IDA	0.005	200	51(25.5)[a]	23	103	13	8	–	132(66.0)	–	–	2.4 ± 0.3
	0.01	200	46(23.0)[a]	28	65	13	5	–	88(44.0)	–	–	1.8 ± 0.8
	0.05	240	98(40.8)[a]	60	262	69	30	1	393(163.8)	57(23.8)	–	0.7 ± 0.3[b]
	0.1	182	149(81.9)[a]	192	583	241	13	1	852(468.1)	50(27.5)	–	0.5 ± 0.2[b]
DOXO	0.1	200	125(62.5)[a]	46	302	119	31	1	485(242.5)	4(2.0)	–	1.2 ± 0.4[c]

CA, chromosome aberrations; ctb, chromatid breaks; csb, chromosome breaks; cte, chromatid exchanges; pvz, pulverizations.
[a] $P < 0.0001$, χ^2 test vs. control.
[b] $P < 0.005$.
[c] $P < 0.05$, Student's t-test vs. control.

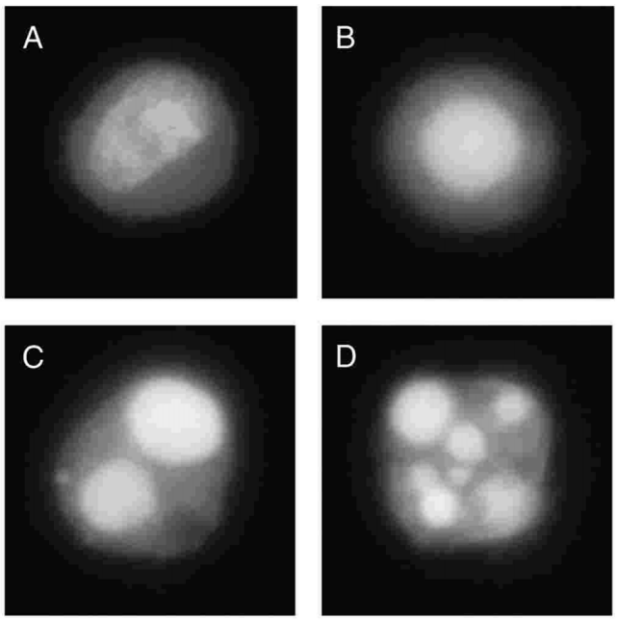

Fig. 1. Morphological detection of apoptotic cells by fluorescence staining. A, control cultures: nucleus with organized chromatin structure; B, C and D, lymphocyte cultures treated with FLU 1.0 μg/ml, B: nucleus, C and D: fragmented nuclei.

At 5.0 and 10.0 μg/ml of FLU and 0.5 μg/ml of IDA, the normally shaped lymphocytes appeared smaller than that of untreated controls.

The percentage of apoptotic cells induced by FLU and IDA is shown in Table 2. A significant increase ($P < 0.001$) of ApI was observed for 5.0 and 10.0 μg/ml of FLU. IDA significantly activated apoptosis at 0.05 μg/ml ($P < 0.01$) and markedly from 0.1 μg/ml ($P < 0.001$). In spite of, similar levels of apoptotic cells were observed in control and lower doses of FLU, a dose-related increase was found for FLU ($P < 0.05$) and IDA ($P < 0.0001$). Untreated controls showed a percentage of necrotic cells of 10.45%, presenting different frequencies in all treated cultures.

Apoptotic cells produced a characteristic fragmentation pattern, the DNA ladder, composed of approximately 180–200 base pairs. Fig. 2 shows this DNA ladder for FLU- (5.0 and 10.0 μg/ml) and IDA-treated cells (0.05, 0.1 and 0.5 μg/ml), while no DNA-fragmentation was detected for untreated PBL cells.

The abnormal cell frequency was compared with the percentage of apoptotic cells induced by both treatments. In each group, the increased induction of abnormal cells was in correlation with ApI for FLU ($r = 0.923$, $P < 0.01$) and for IDA ($r = 0.903$, $P < 0.05$). In addition, an inverse correlation was found between MI and ApI for FLU ($r = -0.890$, $P < 0.02$) and IDA ($r = -0.898$, $P < 0.05$).

Table 2
Percentage of apoptotic cells induced by FLU and IDA in human lymphocytes

Chemical (μg/ml)		Cells scored	Viable cells	Apoptotic cells (ApI)	Necrotic cells
Control		2383	84.10	5.45	10.45
FLU	0.1	1572	79.45	4.58	15.97
	0.5	1326	80.32	5.66	14.02
	1.0	1215	75.31	5.18	19.51
	5.0	779	65.47	16.43**	18.10
	10.0	656	66.77	13.41**	19.82
BrdU	50.0	1430	73.50	3.70	22.80
IDA	0.005	1267	77.43	4.74	17.84
	0.01	722	72.85	6.65	20.50
	0.05	669	77.13	9.87*	13.00
	0.1	488	63.52	12.50**	23.98
	0.5	177	50.28	41.81**	7.91
DOXO	0.1	742	73.32	9.97	16.71

*$P<0.01$; **$P<0.001$, χ^2 test vs. control.

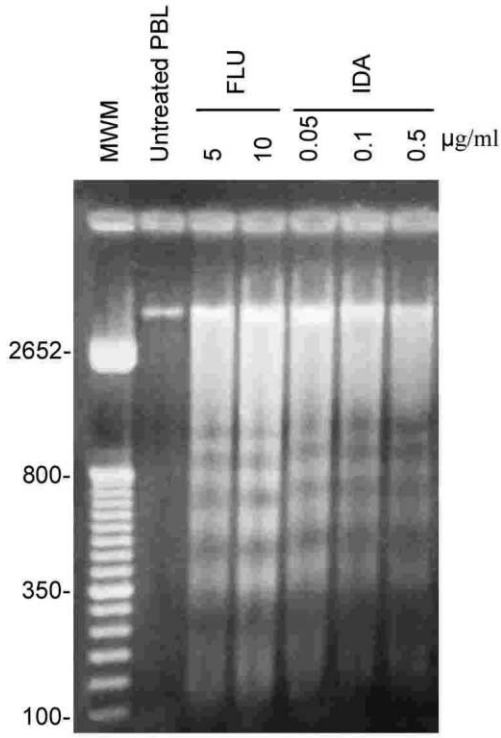

Fig. 2. DNA fragmentation of normal human lymphocytes in agarose gel electrophoresis (1.8%) following treatment with different doses of FLU (5 and 10 μg/ml) and IDA (0.05, 0.1 and 0.5 μg/m). Untreated peripheral blood lymphocytes (PBL) were used as controls. MWM, 50 bp DNA molecular weight marker (Gibco).

4. Discussion

This study addresses the clastogenic effect of FLU and IDA on normal human lymphocytes. CA presented a dose-dependent relationship for both drugs showing an important cytotoxic effect. A higher frequency of chromatid and chromosome breaks in comparison with chromatid exchanges and ring chromosomes was evident in treated cultures presenting major chromosome damage with IDA.

Using the comet assay, Poli et al. (1999) reported that FLU induced, directly or indirectly, strand-breaks on PBL. Treatment of cultured cells with excess DNA nucleoside analogs can induce CA. High levels of adenine produced a clastogenic effect and polyploid metaphases in human cells (Edwards et al., 1995). Other base analogs such as 2′,2′-difluorodeoxycytidine in V79 cells (Auer et al., 1997) or BrdU in CHO cells (San Sebastian et al., 1980), have been found to elevate CA and sister chromatid exchange frequencies. On the other hand, IDA has been reported to increase the frequency of micronuclei in human T-lymphoblastoid CEM cell line (Stopper et al., 1999). Similarly, other topoisomerase II inhibitors such as etoposide, adriamycin, mitoxantrone (Suzuki and Nakane, 1994), DOXO (Tavares et al., 1998) and epirubicin (El-Mahdy Sayed Othman, 2000) increased different types of structural CA in Chinese hamster cell lines.

Morphological features of apoptosis were increased with the drug doses, which correlated well with the presence of DNA fragmentation observed in lymphocytes treated with the highest doses of FLU or IDA. The basal values of apoptosis found (5.45%) were similar to those obtained by Tompa et al. (2000) who reported that the mean spontaneous apoptotic fractions measured by flow cytometry was $6.65 \pm 0.89\%$ in lymphocytes from healthy subjects.

In agreement with our findings, Sandoval et al. (1996) and Consoli et al. (1998) reported that FLU induced apoptosis in normal lymphocytes at similar doses that we used in this work. Apoptosis has also been activated by FLU in B-CLL cells (Zinzani et al., 1994; Bellosillo et al., 1999; Stoetzer et al., 1999) and in different cell lines such as CEM, human monocytic leukemic U937 and myeloid leukemia ML-1 (Huang and Plunkett, 1995; Vrana et al., 1999; Sampath and Plunkett, 2000).

IDA, at similar doses as used in this study, killed normal lymphocytes and blast cells from acute leukemia patients (Belaud-Rotureau et al., 2000), B-cell lymphoma (Smith et al., 1994), leukemic U937 (Vial et al., 1997) and human leukemia HL-60 (Marekova et al., 2000) cell lines. Apoptosis was induced independently of the cell cycle phase after treatment by high doses, whereas after relatively low doses a cell-cycle arrest in G2 phase was observed (Vial et al., 1997; Marekova et al., 2000).

The exact mechanism by which these drugs induced apoptosis is still unknown. FLU incorpo-

rated into DNA inhibits nucleotide excision repair and induces double strand break leading to apoptosis in human lymphocytes (Sandoval et al., 1996). Briefly, it has been suggested that the cytotoxic action of FLU was closely associated with the disruption of DNA replication/repair and inhibition of RNA synthesis (Huang et al., 2000). It is known that the IDA–topoisomerase II-DNA complexes give rise to double strand breaks, that may consequently be expressed as CA (Palitti, 1993), thus triggering cell death process (Binaschi et al., 1997).

In the present study, we have demonstrated that there was a correlation between the CA increase and the MI decrease with the percentage of apoptotic cells, respectively. The use of apoptosis as an indicator of drug action is based on the concept that activation of cellular self-destruction machinery is the main mechanism by which antitumor agents exert their therapeutic effects. FLU and IDA exhibited a direct cytotoxic effect against normal human lymphocytes through the induction of apoptosis in a dose-dependent manner. Probably the increase rate of chromosome damage is incompletely repaired triggering the apoptotic program. In conclusion, the results of the present study suggest that these chemicals especially at high doses could have a significant role in induced cell killing associated to chromosome damage.

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