

A MULTI-BIOMARKER APPROACH TO STUDY THE GENOTOXIC EFFECTS OF IRINOTECAN ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES IN VITRO

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Summary

In the present study a multi-biomarker approach was used to evaluate genotoxic effects of irinotecan administered *in vitro* in its therapeutic dose (350 mg/m²) on non-target cells, peripheral blood lymphocytes. The levels of primary DNA damage in lymphocyte genome and the dynamics of its removal were assessed using the alkaline and neutral comet assay. Lymphocyte viability and the induction of apoptosis following exposure to irinotecan were studied by simultaneous use of a fluorescent assay with ethidium bromide and acridine orange. The levels of residual DNA damage were assessed by SCE assay, while the possible influences of treatment on the progression through the mitotic cycles were studied by analyzing lymphocyte proliferative kinetics. We observed that the percentage of apoptotic cells was higher as compared to necrotic ones in all time-points when irinotecan-treated samples were analyzed. Positive results obtained using both modifications of the comet assay indicate that in lymphocyte DNA following treatment with irinotecan a lot of single and double strand breaks are induced. Dynamics of damage infliction as observed both in alkaline and neutral modification of the comet assay clearly reflects the 'poisoning' of the topoisomerase I, reported as the main mechanism of the irinotecan cytotoxicity. After treatment with irinotecan we observed an almost 7-fold increase of SCE frequency in exposed as compared to untreated lymphocytes that was obviously caused by topoisomerase poisoning in S-phase. Considering the results obtained we can conclude that irinotecan caused a delay of *in vitro* cell proliferation in first mitotic cycle. Despite their limitations, the results of our study indicate that irinotecan in its therapeutic concentrations is able to cause significant amount of primary and residual DNA damage in human peripheral blood lymphocytes. We could assume that the actual levels of DNA damage produced in actively divided cells of patients treated with irinotecan are much higher as compared to those estimated *in vitro*, since DNA damaging potential of irinotecan *in vivo* is up to one thousand times higher due to effectively conversion to its more potent metabolite SN-38. Our results point to the significance of biomarker studies in non-target cells of cancer patients after successful chemotherapy since they could be a good predictive factor to detect sensitive subpopulations of patients with genome instability that have an increased risk for developing of secondary malignancies.

KEY WORDS: *irinotecan, lymphocytes, genotoxicity, DNA damage and repair*

ISTRAŽIVANJE GENOTOKSIČNIH UČINAKA IRINOTEKANA NA LJUDSKIM LIMFOCITIMA PERIFERNE KRVI PRIMJENOM RAZLIČITIH BIOMARKERA U UVJETIMA IN VITRO

Sažetak

Primjenom različitih biomarkera u uvjetima *in vitro* istraženi su genotoksični učinci terapijske koncentracije irinotekana (350 mg/m²) na limfocite periferne krvi. Razine primarnih oštećenja DNA u limfocitnoj DNA i dinamika njihovog

popravka istraženi su primjenom komet testa u alkalnim i neutralnim uvjetima. Preživljenje limfocita i indukcija apoptoze nakon izlaganja stanica irinotekanu istraženi su istodobnom primjenom fluorescencijskog bojenja etidij-bromidom i akridin oranžom. Razine oštećenja DNA procijenjene su i s pomoću testa izmjena sestrinskih kromatida, a mogući učinci tretmana na progresiju mitotičkog ciklusa istraženi su analizom proliferacijske kinetike limfocita. Utvrđeno je da je postotak apoptoza u svim vremenima uzorkovanja i analize bio veći od postotka nekroza. Pozitivni rezultati dobiveni s obje modifikacije komet testa pokazuju da se u limfocitnoj DNA nakon tretmana irinotekomom inducira mnoštvo jednolančanih i dvolančanih lomova. Dinamika nastanka oštećenja uočena primjenom obje modifikacije komet testa, jasno upućuje na disfunkciju enzima topoizomeraze I, koje se navodi kao glavni mehanizam citotoksičnosti irinotekana. Nakon tretmana irinotekomom uočili smo gotovo sedmerostruki porast učestalosti SCE u izloženim limfocitima u odnosu na kontrolu, što upozorava na poremećenu funkciju topoizomeraze u S-fazi. Na osnovi rezultata zaključujemo da irinotekan uzrokuje zastoj proliferacije stanica u prvom mitotskom ciklusu *in vitro*. Dobiveni rezultati pokazuju da terapijske koncentracije irinotekana uzrokuju značajan porast oštećenja DNA i kromosoma u ljudskim limfocitima periferne krvi. Budući da su učinci irinotekana u uvjetima *in vivo* znatno pojačani metaboličkom pretvorbom u aktivni metabolit SN-38, možemo pretpostaviti da su razine oštećenja DNA u aktivno dijelećim stanicama u pacijenata liječenih primjenom irinotekana značajno više nego one utvrđene u uvjetima *in vitro*. Rezultati upućuju i na važnost istraživanja biomarkera u ne-tumorskim stanicama pacijenata koji su liječeni primjenom kemoterapije jer takvi biomarkeri mogu biti dobri pretkazatelji u otkrivanju osjetljivih populacija pacijenata s nestabilnim genomom u kojih je prisutan veći rizik za razvoj sekundarnih karcinoma.

KLJUČNE RIJEČI: *irinotekan, limfociti, genotoksičnost, oštećenje i popravak DNA*

INTRODUCTION

Irinotecan (7-ethyl-10[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin; CPT-11) is clearly one of the most important new anticancer drugs developed in the last few decades. It is a semisynthetic, water-soluble derivative of camptothecin, originally isolated from the Chinese/Tibetan ornamental tree *Camptotheca acuminata* (1, 2). It has undergone extensive clinical investigation worldwide and demonstrated potent activity against many types of human cancer, in particular, gastrointestinal and pulmonary malignancies, but also leukemia and lymphomas, as well as central nervous system malignant gliomas (1-5). Indeed, the addition of irinotecan to first-line therapy with fluorouracil and leucovorin has led to improved survival in patients with advanced colorectal cancer (6).

Irinotecan is a prodrug that is biotransformed by tissue and serum carboxylesterases to an active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin) which has a 100–1,000-fold higher cytotoxic / antitumor activity. Both irinotecan and SN-38 exist in an active lactone form and an inactive hydroxy acid anion form. An acidic pH promotes the formation of the lactone whereas a basic pH favors the hydroxy acid anion form (4, 7, 8).

Generally, camptothecins act by 'poisoning' a nuclear enzyme topoisomerase I. Irinotecan and its active metabolite SN-38 both bind to the

topoisomerase I - DNA complex and prevent religation of single-strand breaks. Topoisomerases are required for the viability of proliferating cells and play essential roles in a number of fundamental nuclear processes. They reduce DNA twisting and supercoiling that occur in selected regions of DNA as a result of essential cellular processes such as transcription, replication and repair recombination. They cleave and reseal the phosphodiester backbone of DNA, and form a covalent enzyme-DNA linkage, which allows the passage of another single- or double-stranded DNA through the nicked DNA. Moreover, topoisomerases are also involved in chromosome organization and segregation (9-11).

Since it interacts with cellular Topo I-DNA complexes, irinotecan has S-phase-specific cytotoxicity (12). However, at higher concentrations, non-S-phase cells can also be killed. The mechanism of non-S-phase cell killing appears to be related to transcriptionally mediated DNA damage, and through the mechanism of apoptosis (13).

The principal dose-limiting toxicity of irinotecan is diarrhea. It can cause either acute diarrhea related to a cholinergic surge from inhibition of acetyl cholinesterase, or a delayed diarrhea syndrome, which is possibly related to the accumulation of the active metabolite of irinotecan in the bowel (14). Other non-hematologic toxicities include nausea, vomiting, anorexia, fatigue, abdominal pain, alopecia, asthenia, and el-

evated alkaline phosphatase and/or hepatic transaminases. The most common hematological toxicity is dose-related neutropenia. Myelosuppression is generally not cumulative, and severe anemia or thrombocytopenia is less common (1, 2, 6, 15).

Genotoxicity of anticancer drugs to normal cells is one of the most serious problems of chemotherapy. The adverse consequences of anti-neoplastic drugs to non-tumor cells and tissues are almost always present and with the number of chemotherapy cycles they are strong determinants of the secondary cancer risk. Whether the treatment with irinotecan is associated with an increased risk of secondary malignancies requires continued study. Considering its mechanism of acting, it is likely that irinotecan is mutagenic and can induce chromosomal aberrations. However, there is a need for the extensive research to clarify and document these effects.

For the assessment of genotoxicity different biomarkers could be employed. The comet assay is a suitable technique to measure various types of DNA damage and their repair. DNA damage is quantified by the proportion of DNA which migrates out of the nuclei toward the anode when individual cells or isolated nuclei, embedded in a thin agarose layer, are subjected to electrophoresis that results in a "comet-like" shape of nuclei (16, 17). The most frequently used alkaline modification of the assay, as developed by Singh et al. (18), enables sensitive detection of single strand breaks and alkali labile sites in DNA. On the other hand, neutral modification allows for the preferential detection of double strand breaks in DNA (19, 20). A variety of possible modifications of the assay also facilitate the detection of incomplete excision repair sites, interstrand cross-links, different DNA repair pathways (base excision and nucleotide excision repair), as well as DNA fragmentation associated with cell death or related to apoptosis (16, 17).

The sister chromatid exchanges (SCE) also afford an excellent parameter for monitoring DNA damage and repair aspects. They have been employed commonly to evaluate cytogenetic responses to chemical exposure, and any discussion of cytogenetics would be incomplete without at least some mention of this endpoint. SCE are the cytological manifestation of a symmetrical exchange of apparently homologous portions

of the chromosomes that involves DNA breakage and reunion mechanisms (21).

In the present study, a multi-biomarker approach was used to evaluate genotoxic effects of irinotecan administered *in vitro* in its therapeutic dose (350 mg/m²) on non-target cells, peripheral blood lymphocytes. Peripheral blood has been an attractive tissue type for biomedical and clinical research, because of its critical role in immune response and metabolism in humans and animal studies, as well as the simplicity and ease of sample collection. All methods used here have been established previously as sensitive biomarkers in many *in vitro* and *in vivo* genotoxicity studies with antineoplastic drugs (22-30). Our goal was to investigate the levels of primary DNA damage in lymphocyte genome and the dynamics of its removal using the alkaline and neutral comet assay. Lymphocyte viability and the induction of apoptosis following exposure to irinotecan were studied by simultaneous use of a fluorescent assay with ethidium bromide and acridine orange. The levels of residual DNA damage were assessed by SCE assay, while the possible influences of treatment on the progression through the mitotic cycles were studied by analyzing lymphocyte proliferative kinetics. The levels of DNA damage recorded in surrogate cells, as peripheral blood lymphocytes, could indicate comparable levels of DNA damage inflicted in other non-target cells, most likely correlated with secondary cancer risk, but also possible levels of desirable DNA damage in target tumor cells, especially those disseminated as micrometastases.

MATERIAL AND METHODS

Blood sampling

Blood sample was obtained from a healthy female donor (age 35 years, non-smoker). The donor had not been exposed to diagnostic or therapeutic irradiations as well as to known genotoxic chemicals for a year before blood sampling. Venous blood (30 ml) was collected in heparinized vacutainer tubes (Becton Dickinson) under sterile conditions.

Lymphocyte isolation

Anticoagulant-treated blood was mixed 1:1 (v/v) with balanced salts solution, layered on the

Ficoll solution (Sigma) and centrifuged at 600 rpm for 40 minutes at room temperature. The layer containing lymphocytes was carefully removed and cells were resuspended in balanced salts solution. They were washed twice by centrifugation at 600 rpm for ten minutes. The final pellet was gently resuspended in culture medium F-10 (Sigma). Viability of cells was checked by supravital staining with 0.1% trypan blue (Sigma).

Antineoplastic drug and treatment *in vitro*

Irinotecan (Figure 1.) was obtained from Aventis Pharma Ltd., as concentrate for solution for infusion (CAMPTO®). The concentrate contains 20 mg/ml irinotecan hydrochloride trihydrate. The dose of antineoplastic drug tested in our *in vitro* experiment was 350 mg/m², recommended as monotherapy in adult patients.

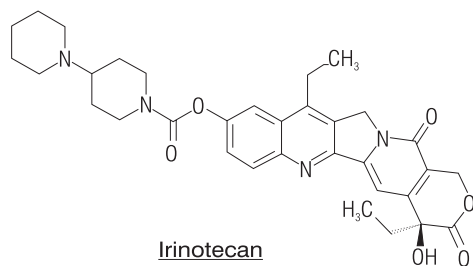


Figure 1. Structure of irinotecan

Four aliquots of isolated lymphocytes were placed in sterile Falcon tubes in final concentration of 2×10^6 cells/ml. Two aliquots were incubated with irinotecan for 2 h at 37° C, while the other two were negative controls. After 2 hours of exposure, culture medium containing irinotecan was carefully removed and the cells were washed twice in F-10 medium. One aliquot of treated lymphocytes and one negative control were incubated *in vitro* at 37° C for 2 hours and used for the alkaline comet assay as well as for the evaluation of the cell viability and apoptosis measurements. Primary DNA damage and cell viability was evaluated immediately after the treatment, as well as 30, 60, 90 and 120 minutes after the treatment. The other aliquot of treated lymphocytes and the other negative control were incubated *in vitro* at 37° C for 72 hours and used for the SCE assay and the cell cycle analysis.

The comet assay

The comet assay was carried out under alkaline conditions, as described by Singh et al. (18) and neutral conditions according to Wojewódzka et al. (20). Two replicate slides per sample per method were prepared. Agarose gels were prepared on fully frosted slides coated with 1 % and 0.6 % normal melting point (NMP) agarose (Sigma). Lymphocyte samples (5 µl) were mixed with 0.5 % low melting point (LMP) agarose (Sigma), placed on the slides and covered with a layer of 0.5 % LMP agarose. The slides were immersed for 1 h in freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1 % Na-sarcosinate (Sigma), pH 10) with 1 % Triton X-100 (Sigma) and 10 % dimethyl sulfoxide (Kemika). Alkaline denaturation and electrophoresis were carried out at 4 °C under dim light in freshly prepared electrophoretic buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13.0). After 20 min of denaturation, the slides were randomly placed side by side in the horizontal gel-electrophoresis tank, facing the anode. Electrophoresis at 25 V (300 mA) lasted another 20 min. Neutral denaturation was carried out in the dark at 8 °C and lasted for 1 h in a buffer containing 300 mM sodium acetate and 100 mM Tris-HCl, pH 8.5. It was followed by the electrophoresis at 14 V and 11-12 mA that also lasted for 1 h. After electrophoresis, the slides were gently washed with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) three times at five-minute intervals. Slides were stained with ethidium bromide (20 µg/ml) and stored at 4 °C in humidified sealed containers until analysis. Each slide was examined using a 250 x magnification fluorescence microscope (Zeiss) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. A total of 100 comets per sample were scored (50 from each of two replicate slides). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells and superimposed comets. Using a black and white camera, the microscope image was transferred to a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd.). To avoid the variability, one well-trained scorer scored all comets. Three main comet parameters were evaluated: tail length

(presented in micrometers), tail intensity (DNA % in comet tail) and tail moment.

Assessment of cell viability, apoptosis and necrosis

For studying cell death and morphological changes in the nuclei, we used dye exclusion method (31) in which viable (intact plasma membrane) and dead (damaged plasma membrane) cells can be visualized by fluorescence microscopy after staining with the fluorescent DNA-binding dyes. Ethidium bromide and acridine orange (1:1) (v/v) were added to the cell suspension in final concentrations of 100 µg/ml and then the cells were incubated at room temperature for 5 minutes. The cellular morphology was evaluated by fluorescent microscopy. Two parallel tests with aliquots of the same sample were performed and a total of 500 cells per sample were counted. Cells were scored as viable, apoptotic or necrotic as judged by nuclear morphology and membrane integrity. Viable normal cells excluded ethidium bromide and their nuclei were bright green with intact structure. Viable apoptotic cells were bright green with highly condensed or fragmented nuclei. Non-viable normal cells had bright orange chromatin with organized structure, while non-viable cells with apoptotic nuclei had highly condensed or fragmented bright orange chromatin. Quantitative assessments were made by determining the percentage of apoptotic and necrotic cells.

SCE assay

Cell cultures were set up by adding 0.5 ml of lymphocyte suspension to F-10 medium (Sigma) supplemented with 20 % fetal calf serum (Sigma), phytohemagglutinin (Murex Biotech Ltd.) and antibiotics penicillin (Crystacillin[®], Pliva) and streptomycin (Streptomycin sulfate, Krka). Bromodeoxyuridine (BrdU; Sigma) was added at a final concentration of 5 µg/ml. For each sample, two replicates were established. The culture vessels were double-wrapped in foil to avoid photolysis and incubated at 37°C *in vitro* for 72 h in complete darkness. Colchicine (Sigma) at a final concentration of 0.4 µg/ml was added 2 hours prior to harvesting. After reincubation, the cells were harvested by centrifugation, resus-

ended in a pre-warmed hypotonic solution KCl (0.075 M) for 20 min and fixed in repeated changes of cold freshly prepared fixative 3:1 methanol and acetic acid, v/v. Preparations were made by pipetting drops of a concentrated lymphocyte suspension onto clean slides, which were allowed to air-dry. To obtain harlequin chromosomes, slides were stained using a modified fluorescence plus Giemsa method (FPG) as described by Perry and Wolff (32). All the slides were coded and analyzed blindly. A total of 100 randomly selected second division metaphases (50 from each of two replicates) per each sample were analyzed. In order to determine the number of SCE per metaphase, we examined only well differentially stained second division metaphases containing 46 clearly visible chromosomes. Every point of exchange was counted as a SCE. Exchanges at the centromere were included only when twisting at this point could be ruled out.

Analysis of the lymphocyte proliferation kinetics

In FPG-stained preparations, cells dividing for the first (M₁), second (M₂) or third time (M₃) in culture containing BrdU were determined by differential staining pattern of sister chromatids. Basically, M₁ cells contain chromosomes with both sister chromatids stained uniformly darkly. M₂ cells contain only harlequin chromosomes, with one chromatid darkly stained and its sister chromatid lightly stained, whereas M₃ cells contain some differentially stained chromosomes and chromosomes with both sister chromatids stained uniformly lightly. Lymphocyte proliferation kinetics was studied on 200 differentially stained metaphases per each blood sample. The proliferation rate index (PRI) was calculated according to the formula: $PRI = (M_1 + 2M_2 + 3M_3) / \text{total number of cells scored}$, as reported by Lamberti et al. (33).

Statistical analysis

Statistical analyses were carried out using Statistica 5.0 for Windows (StatSoft, Tulsa, USA). In order to normalize distribution and to equalize the variances, a logarithmic transformation of data was applied. The level of statistical significance was set at $p < 0.05$. The extent of DNA

damage, as recorded by the alkaline and neutral comet assay, was analyzed considering the mean (\pm standard error of the mean), median and range of the comet parameters measured. Multiple comparisons between groups were done using multifactor ANOVA. Post-hoc analysis of differences was done using the Scheffé test. The comparisons between values obtained for the cell viability, necrosis and apoptosis in irinotecan-treated and control samples were made by χ^2 test. The statistical significance of data obtained by the SCE test was evaluated using Student's t-test. Each sample was characterized for the extent of DNA damage by considering the mean (\pm standard error of the mean), median and range of SCE per cell. Statistical significance of data considering the lymphocyte proliferation kinetics was tested using the χ^2 -test.

RESULTS

Results of the present study are summarized and detailed in Tables 1-4. Appearances of comets in treated and control samples are shown in Figure 2. a-d, while SCEs in second-division lymphocyte metaphase following a 2-h exposure to irinotecan are displayed on Figure 3.

Lymphocyte viability after isolation was 98.8% and their baseline DNA damage as recorded by the alkaline comet assay was as follows: average DNA migration (comet tail length) $13.17 \pm 0.14 \mu\text{m}$ (median $12.82 \mu\text{m}$); mean tail intensity (tail DNA %) 1.04 ± 0.23 (median 0.39), and mean tail moment 0.12 ± 0.02 (median 0.05). The same parameters measured in the neutral comet assay were: mean DNA migration $16.88 \pm 0.22 \mu\text{m}$ (median $16.67 \mu\text{m}$); mean tail intensity 0.85 ± 0.12 (median 0.46), and mean tail moment 0.13 ± 0.02 (median 0.08). The results obtained suggest that procedure of lymphocyte isolation did not induce significant damage or mortality to lymphocytes.

Cell viability, apoptosis and necrosis

The results of cell viability test after incubation of human lymphocytes with irinotecan show that tested compound caused a significant decrease in cell viability as compared to control sample, in each time point following *in vitro*

treatment ($p < 0.05$, χ^2 test; Table 1). According to our observations irinotecan also caused a higher percentage of apoptotic versus necrotic cells in each sample analyzed. However, the differences were not statistically significant when different time-points were compared. Percentages of apoptotic cells with damaged membrane steadily increased in first 60' following treatment.

Viability of lymphocytes in control sample gradually decreased and at time point 120' it was significantly lower as compared to time-points 0' and 30' (Table 1). That was not surprising, considering that lymphocytes were kept in a culture medium without any supplements and growth factors.

Alkaline comet assay

The values obtained for all comet parameters evaluated in irinotecan-treated lymphocytes were significantly increased as compared to control sample, in each time point following *in vitro* treatment ($p < 0.05$, analysis of variance; Table 2). Our results indicate that primary DNA damage has two peaks, first at 60' and second at 120' after treatment with irinotecan. Statistical evaluation confirmed that the mean DNA migration recorded in the sample analyzed at 120' following treatment was significantly increased as compared to samples 0', 30' and 90' (Table 2). Since the comet tail moments are positively correlated with the level of DNA breakage in the cell, it could be concluded that the amount of single strand breaks and alkali labile sites in irinotecan-treated cells steadily increased in first 60' following treatment. There is also a possibility that DNA repair and oxidative processes contributed to the increased level of DNA damage as recorded at time point 120'.

By using the alkaline comet assay we were able to detect even subtle differences in DNA migration in normal untreated lymphocytes. In control sample, the lowest DNA migration was observed in sample 0', indicating that isolated lymphocytes during 2 h of incubation *in vitro* effectively repaired DNA lesions inflicted by isolation procedure. The values of all comet parameters steadily increased in later time-points, but were still significantly lower than in irinotecan treated samples (Table 2).

Table 1.

RESULTS OF THE QUANTITATIVE FLUORESCENT ASSAY FOR THE SIMULTANEOUS IDENTIFICATION OF APOPTOTIC AND NECROTIC CELLS IN ISOLATED PERIPHERAL BLOOD LYMPHOCYTES

Time after treatment	Lymphocytes (%)				
	Viable	Necrotic	Apoptotic		
			Σ	intact membrane	damaged membrane
IRINOTECAN 350 mg/m ² (in vitro treatment for 2 h)					
0'	63.4 ↑	5.4 ↑	31.2 ↑	26.4	4.8
30'	59.6 ↑	8.6 ↑	31.8 ↑	18.2	13.6
60'	63.0 ↑	5.0 ↑	32.0 ↑	16.0	16.0
90'	58.4 ↑	8.0 ↑	33.6 ↑	27.6	6.0
120'	52.2 ↑	12.0 ↑	35.8 ↑	23.4	12.4
CONTROL SAMPLE					
0'	85.8	2.0	12.2	12.2	-
30'	82.4	1.6	16.0	15.2	0.8
60'	77.8	1.2	21.0	17.6	3.4
90'	74.6	2.0	23.4	21.0	2.4
120'	67.2*	3.8*	29.0*	24.2	4.8

Evaluation was made by analyzing 500 cells per sample per each experimental point.

↑ - significantly different as compared to control sample analyzed at the same time;

* - significantly different as compared to samples 0' and 30' ($p < 0.05$, χ^2 test).

Neutral comet assay

Immediately after *in vitro* treatment with irinotecan, the values obtained for all comet parameters were significantly increased as compared to control sample ($p < 0.05$, analysis of variance; Table 3). Differences observed in later time-points are indicated in Table 3. Our results indicate that primary DNA damage in irinotecan-treated lymphocytes, as recorded by neutral comet assay, gradually decreased in first 60' following treatment, but later on increased again and reached its second peak at time-point 120' (Table 3). Statistical evaluation confirmed that the mean DNA migration recorded in the sample analyzed at 30' following treatment was significantly lower as compared to sample 0', while the mean DNA migration recorded in the sample analyzed at 120' following treatment was significantly higher as compared to sample 30' (Table 3).

Since neutral modification of comet assay sensitively detects double strand breaks in DNA, it should be stressed that their amount was the highest immediately after treatment. Because of effective DNA repair, their abundance was lower and therefore a decrease of all comet parameters was observed. However, in later time points due to DNA repair itself and oxidative processes the levels of DNA damage increased again.

Primary DNA damage in control lymphocytes also gradually decreased in first 60', but

later on it increased and reached its peak at time-point 90', when the highest values of all three comet parameters were recorded (Table 3). Observed dynamics of DNA damage in control sample was expected, considering that lymphocytes were kept in a culture medium without any supplements and growth factors.

Appearance of lymphocyte comets as observed in the alkaline and neutral comet assay is

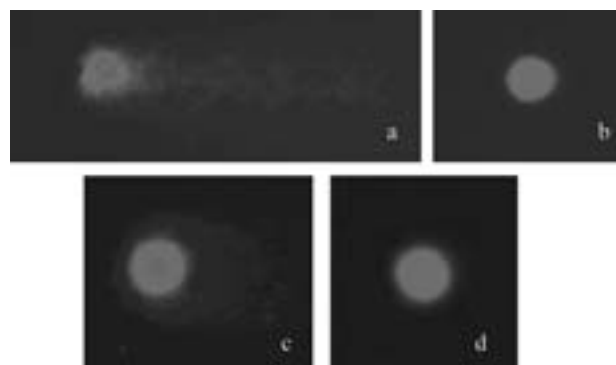


Figure 2. Appearance of lymphocyte comets in the alkaline and neutral comet assay: (a) following *in vitro* treatment with irinotecan (350 mg/m²) many comets detected by alkaline electrophoresis had long tails containing a low fraction of highly fragmented DNA; control lymphocytes (b) had comets without or with very short tails. In the neutral modification of the assay, irinotecan-treated lymphocytes had an appearance of large 'clouds' (c), while control lymphocytes remained almost round shape (d). Preparations were stained with ethidium bromide.

Table 2.

RESULTS OF THE ALKALINE COMET ASSAY ON ISOLATED PERIPHERAL BLOOD LYMPHOCYTES TREATED IN VITRO WITH IRINOTECAN

Time after treatment	ALKALINE COMET ASSAY											
	DNA migration – tail length (µm)				Tail intensity (DNA %)				Tail moment			
	Mean ± S.E.	Med.	Min.	Max.	Mean ± S.E.	Med.	Min.	Max.	Mean ± S.E.	Med.	Min.	Max.
IRINOTECAN 350 mg/m ² (in vitro treatment for 2 h)												
0'	15.88±0.60 ↑	14.10	12.18	61.54	1.28±0.18 ↑	0.51	0.00	9.29	0.17±0.02 ↑	0.07	0.00	1.07
30'	15.57±0.55 ↑	14.74	11.54	57.05	1.13±0.20 ↑	0.51	0.00	11.07	0.15±0.03 ↑	0.07	0.00	1.84
60'	18.47±1.10 ↑	14.74	11.54	74.36	1.98±0.41 ↑	0.38	0.00	25.46	0.36±0.09 ↑	0.06	0.00	6.36
90'	16.87±1.07 ↑	14.74	11.54	85.90	0.98±0.19 ↑	0.24	0.00	9.85	0.16±0.04 ↑	0.03	0.00	2.97
120'	19.47±0.88 ↑, ^a	16.03	11.54	57.69	2.02±0.36 ↑	0.76	0.00	22.93	0.31±0.05 ↑	0.11	0.00	2.79
CONTROL SAMPLE												
0'	12.63±0.13 ^b	12.18	10.90	17.95	0.55±0.08	0.25	0.00	3.77	0.07±0.01	0.03	0.00	0.44
30'	14.74±0.22	14.10	12.18	28.20	0.64±0.09	0.38	0.00	6.32	0.09±0.01	0.05	0.00	0.81
60'	15.71±0.24	15.38	12.18	24.36	0.58±0.12	0.13	0.00	9.17	0.08±0.02	0.02	0.00	1.29
90'	15.50±0.26	15.06	12.18	27.56	0.50±0.08	0.18	0.00	4.77	0.07±0.01	0.03	0.00	0.73
120'	14.72±0.21	14.42	10.90	19.87	0.76±0.10	0.31	0.00	6.51	0.10±0.01	0.04	0.00	0.79

Data are presented as mean values of 100 comet measurements per sample per each experimental point. ↑ - significantly increased as compared to control sample analyzed at the same time; a - significantly increased as compared to samples 0', 30' and 90'; b- significantly lower as compared to samples 30', 60', 90' and 120'; (p < 0.05; analysis of variance, post-hoc Scheffé test).

Table 3.

RESULTS OF THE NEUTRAL COMET ASSAY ON ISOLATED PERIPHERAL BLOOD LYMPHOCYTES TREATED IN VITRO WITH IRINOTECAN.

Time after treatment	NEUTRAL COMET ASSAY											
	DNA migration – tail length (µm)				Tail intensity (DNA %)				Tail moment			
	Mean ± S.E.	Med.	Min.	Max.	Mean ± S.E.	Med.	Min.	Max.	Mean ± S.E.	Med.	Min.	Max.
IRINOTECAN 350 mg/m ² (in vitro treatment for 2 h)												
0'	18.49±0.46 ↑	17.31	12.82	35.90	1.86±0.29 ↑	0.71	0.00	16.82	0.30±0.05 ↑	0.11	0.00	2.26
30'	16.13±0.50 ^a	14.74	10.90	46.79	1.56±0.22 ↑	0.56	0.00	9.12	0.24±0.04 ↑	0.09	0.00	2.40
60'	16.78±0.35 ↑	16.03	11.54	37.18	0.83±0.12	0.38	0.00	9.08	0.12±0.02	0.06	0.00	1.11
90'	17.58±0.44	16.35	12.18	39.10	1.13±0.24	0.16	0.00	16.58	0.18±0.04	0.02	0.00	3.83
120'	18.51±0.34 ↑, ^b	17.63	12.82	30.13	1.37±0.24 ↑	0.59	0.00	14.01	0.22±0.04 ↑	0.09	0.00	2.60
CONTROL SAMPLE												
0'	15.69±0.21	15.38	12.18	21.15	0.90±0.13	0.38	0.00	7.05	0.13±0.02	0.06	0.00	1.18
30'	15.46±0.20	15.38	11.54	19.87	1.38±0.23	0.40	0.00	10.94	0.18±0.03	0.06	0.00	1.40
60'	14.94±0.26	14.10	10.90	24.36	1.68±0.28	0.50	0.00	12.09	0.23±0.04	0.07	0.00	1.70
90'	17.54±0.25 ^{abc}	17.31	12.82	25.00	1.24±0.16	0.68	0.00	6.48	0.19±0.02	0.10	0.00	1.10
120'	17.40±0.28 ^{abc}	17.31	12.18	24.36	0.78±0.13	0.19	0.00	6.57	0.12±0.02	0.03	0.00	0.88

Data are presented as mean values of 100 comet measurements per sample per each experimental point. ↑ - significantly increased as compared to control sample analyzed at the same time; a - significantly different as compared to sample 0'; b - significantly different as compared to sample 30'; c - significantly different as compared to sample 60'; (p < 0.05; analysis of variance, post-hoc Scheffé test).

shown in Figure 2. It should be emphasized that alkaline denaturation allowed deliberation of short DNA fragments that move freely toward anode during electrophoresis. Therefore in highly damaged cells nuclei had a shape of a really comet. After neutral denaturation large fragments of DNA often remained attached to nucleus and therefore irinotecan-treated lympho-

cytes usually had an appearance of large 'clouds'. In both modifications of the assay control lymphocytes remained their almost round shape, without or with very short tails.

SCE

The frequencies of SCEs observed in second-division metaphases in irinotecan-treated

Table 4.

RESULTS OF THE SCE ASSAY AND LYMPHOCYTE PROLIFERATION KINETICS ON ISOLATED PERIPHERAL BLOOD LYMPHOCYTES

Sample	SCE / cell			Mitotic activity			
	Mean ± S.E.	Median	Range	M ₁ (%)	M ₂ (%)	M ₃ (%)	PRI
IRINOTECAN	20.70 ± 0.64	20	9-38	28.0	61.5	10.5	1.83*
CONTROL	3.11 ± 0.12	3	0-6	14.5	69.5	16.0	2.02

Data on SCE are presented as mean values obtained by analyzing of 100 second metaphases; lymphocyte proliferation kinetics was evaluated by analyzing 200 cells per sample per each experimental point.

M₁, M₂, M₃ corresponded to the frequencies of cells in first, second and third *in vitro* division; PRI (proliferation rate index) was determined with the formula: $1M_1+2M_2+3M_3/200$; ↑ - significantly increased as compared to control sample; ($p < 0.01$; analysis of variance); * - significantly different as compared to control sample ($p < 0.05$, ± 2 test).

and control sample are listed in Table 4. In control sample the mean SCE frequency was 3.11 ± 0.12 SCEs / metaphase (median 3 SCEs / metaphase; range 0-6 SCEs / metaphase). Observed value was within the control range normally observed in our laboratory.

Irinotecan caused almost seven-fold increase of the basal SCE frequency: mean SCE frequency in treated lymphocytes was 20.70 ± 0.64 SCEs / metaphase (median 20 SCEs / metaphase; range 9-38 SCEs / metaphase) (Table 4). Observed difference was highly significant ($p < 0.01$, analysis of variance).

SCEs in second-division lymphocyte metaphase following 2-h exposure to irinotecan (350 mg/m^2) are shown in Figure 3.

Lymphocyte proliferation kinetics

The majority of cells in control sample were at the time of analysis in second *in vitro* division



Figure 3. SCEs in second-division lymphocyte metaphase following 2-h exposure to irinotecan (350 mg/m^2).

(M₂); moreover, the proportion of M₃ cells was slightly higher than the proportion of M₁ cells. The value of the proliferation rate index (PRI) was 2.02 (Table 4).

In vitro administration of irinotecan significantly disturbed lymphocyte proliferation kinetics. An increase in the relative proportion of M₁ indicate a delay in lymphocyte cell cycle, accompanied with decreases in the proportions of M₂ and M₃ cells and significant lowering of the PRI value ($p < 0.05$, χ^2 test; Table 4).

DISCUSSION

The present study reports the results of an *in vitro* evaluation of cyto- and genotoxicity of irinotecan on human peripheral lymphocytes, employed as non-target surrogate cells. Using several well-established biomarkers, we were able to show that irinotecan, administered in its therapeutic concentration (350 mg/m^2), induced significant primary and residual DNA damage in peripheral blood lymphocytes, as well as apoptosis, necrosis and disturbances of proliferation kinetics in treated cells.

Our investigation presents a first report of the application of comet assay on irinotecan-treated human lymphocytes, using alkaline and neutral modifications of the method. Similar study was performed earlier by Godard et al. (28) who investigated DNA strand breaks and apoptosis induced by related drugs, camptothecin and topotecan, on the animal cell line CHO using the alkaline comet assay. According to their observations, alkaline modification of the assay sensitively detected the DNA damage produced by

both drugs after 1 h treatment, as well as its disappearance 24 h after drug removal.

In the study presented here, we were able to compare the results of comet assay with the results obtained by simultaneous identification of apoptotic and necrotic cells performed on the same samples. This is important because the mechanism of irinotecan cytotoxicity involves both the induction of DNA strand breaks and massive DNA fragmentation leading to apoptosis. Therefore, using the alkaline and neutral comet assay we obtained complementary results that were to some extent also affected by apoptosis in treated samples. Moreover, the comet assay was also advantageous method since it enables the evaluation of DNA damage in single cells, but at the same time also indicates the heterogeneity in response to the treatment.

As reported previously, alkaline modification of the comet assay enables sensitive detection of single strand breaks and alkali labile sites that are converted into single strand breaks during alkaline denaturation (34); double strand breaks represent less than 5% of the total damage detected using this modification of the assay (35). On the other hand, neutral modification is specifically designed to detect double strand breaks in DNA (16, 19, 35). It was estimated earlier that single strand breaks in DNA occur more often than double strand breaks. Although double strand breaks occur 25 to 40 times less frequently than single strand breaks, they are more cytotoxic lesions and considered as major source of stable chromosome aberrations and rearrangements (17, 35, 36).

Positive results obtained using both modifications of the comet assay indicate that in lymphocyte DNA following treatment with irinotecan a lot of single and double strand breaks are induced. The amount of single strand breaks gradually increased reaching its first peak on 60' after treatment. This amount of breaks temporarily decreased due to the repair processes, but since repair itself generates additional breaks, and also due to the oxidative damage of DNA, in later time points we detected a second increase (peak) of DNA damage. It was reported earlier that repair of irinotecan-induced DNA damage is coupled with RNA transcription (12). According to this model, the collision between the elonga-

tion RNA polymerase complex and the Topo-I cleavable complex (on the template strand) results in transcription arrest and the formation of a Topo I linked single-strand break.

The highest amount of double strand breaks was recorded immediately after treatment and it gradually decreased after cells were allowed to recover in the culture medium free of irinotecan. Dynamics of damage infliction as observed both in alkaline and neutral modification of the comet assay clearly reflects the 'poisoning' of the topoisomerase I, reported as the main mechanism of the irinotecan cytotoxicity (8). Topoisomerase I binds to single-strand DNA breaks, and the reversible Topo I-irinotecan-DNA cleavable complex is not lethal to the cells by itself. However, upon their collisions with the advancing replication forks, the formation of a double strand DNA break occurs, leading to irreversible arrest of the replication fork and cell death (Liu et al. 2000).

The amount of double strand breaks caused by the treatment is of special interest because their repair is much slower and more complicated as compared with single strand breaks that are successfully repaired in a few minutes (4-15 min) (37) to a couple of hours (2-3 h) (17, 18) after infliction. In our study, the abundance of double strand breaks increased in later time-points, when it was also in positive correlation with apoptosis.

Cells undergoing programmed death or apoptosis demonstrated highly fragmented DNA (35). Irinotecan was established earlier as an effective inducer of apoptosis (38). Similar was also confirmed in our study. We observed that the percentage of apoptotic cells was higher as compared to necrotic ones in all time-points when irinotecan-treated samples were analyzed. Moreover, in earlier time-points, we also observed gradual decrease of percentage of apoptotic cells with intact membrane and corresponding increase of apoptotic cells with damaged membrane. Critical point was 60' when their proportions were the same. Later on, the proportion of apoptotic cells with intact membrane continued to decrease again. Although the percentage of necrotic cells gradually increased during first 120 minutes after the treatment, it should be stressed that the mortality of irino-

tecantreated lymphocytes was primarily caused by apoptosis. It was reported earlier that at higher concentrations of irinotecan, non-S-phase cells can also be killed. The mechanism of non-S-phase cell killing appears to be related to transcriptionally mediated DNA damage, and through the mechanism of apoptosis (13).

Our results indicate that irinotecan inflicted a lot of primary DNA damage in treated lymphocytes. That was also confirmed when the levels of residual DNA damage were studied using SCE analysis. The principal difference between these two methods is that the comet assay reflects DNA damage in resting cells, whereas SCE is assessed on replicating lymphocytes and in this case DNA lesions could be subjected to more efficient repair (39). It is necessary for cells to pass through the S phase before DNA damage results in the formation of sister chromatid exchanges. If induced lesions are completely repaired before cells enter S phase, then these cells show no increase in SCE frequency. On the contrary, if there is no repair of induced lesions within the consecutive 2 cell cycles, those lesions can contribute to the formation of SCE (40). After treatment with irinotecan we observed an almost 7-fold increase of SCE frequency in exposed as compared to untreated lymphocytes. It was obviously caused by topoisomerase poisoning in S-phase. Positive correlation between the comet assay and SCE results, as observed in our research, also suggest that the comet assay responses provide a good prediction of cytogenetic damage. Therefore, an increased level of DNA damage recorded in surrogate cells following a treatment with antineoplastic drug indicate the possibility of mutations in treated patients that could be sources for genome instability and chromosome rearrangements possibly leading to secondary malignancies.

Lymphocyte proliferation kinetics is often studied in parallel with the SCE assay, as it was also in our study. Considering the results obtained we can conclude that irinotecan caused a delay of *in vitro* cell proliferation in first mitotic cycle. It was reported earlier that the mutagens that induce increased SCE frequencies also cause longer delays and the retardation of the cell cycle (41), as well as that the correlation exists between higher value of PRI and lower SCE frequency.

The delays in cell cycle progression are exploited by the cell to perform DNA repair (33). Our observations also confirmed this statement. It was obvious that, due to the topoisomerase I poisoning, irinotecan caused a delay in M₁ since topoisomerase I has a lot of intracellular functions, among them also the involvement in the processes of chromosome segregation (11) as well as in cell transition from G₀ to G₁ phase and in DNA replication (42).

Despite their limitations, the results of our study indicate that irinotecan in its therapeutic concentrations is able to cause significant amount of primary and residual DNA damage in human peripheral blood lymphocytes. Although peripheral blood lymphocytes are predominantly in G₀ phase with lower rates of metabolism, transcription and translation than in active cells (43), their amounts of DNA damage were significantly increased. We could assume that the actual levels of DNA damage produced in actively divided cells of patients treated with irinotecan are much higher as compared to those estimated *in vitro*, since DNA damaging potential of irinotecan *in vivo* is up to one thousand times higher due to effectively conversion to its more potent metabolite SN-38 (8).

Although repair mechanisms tend to remove DNA damage inflicted by antineoplastic drugs in non-target cells, it should be stressed that the restoration of primary DNA sequence is not always ideal, and that the residual levels of DNA damage are strong determinants for the secondary cancer risk.

Better knowledge of the irinotecan mechanisms can be used to identify modulation strategies aimed at increasing the antitumor activity and reducing the toxicity of this unique agent. Clinical trials of irinotecan should incorporate, whenever possible, analyses of potential markers of clinical activity and toxicity. Moreover, some of the results of *in vitro* studies could be also taken into account when planning chemotherapy strategies. With the increasing understanding of irinotecan intracellular interactions and the intracellular mechanisms mediating resistance to this agent, the potential to prospectively identify patients with tumors especially susceptible to irinotecan therapy warrants careful attention. In this view, a multi-biomarker approach to study

the level of DNA damage in non-target cells of cancer patients after successful chemotherapy could be also a good predictive factor to detect sensitive subpopulations of patients with genome instability that have an increased risk for developing of secondary malignancies.

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