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MELPHALAN-INDUCED CYTOTOXICITY IN THE BONE MARROW OF RATS BY FLOW CYTOMETRY MEASUREMENTS

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Background. *Bone marrow (BM) that contains hematopoietic cells of various lineages is a sensitive target for a number of cytotoxic agents including chemotherapy drugs.*

Objective. *Flow cytometry (FCM) was chosen to test cytotoxicity in BM of rats, that received melphalan either intravenously (i.v.) or intraperitoneally (i.p.).*

Methods. *One group of rats received melphalan i.v. (3 mg/kg) followed by the BM examination on the 3rd and 7th day after drug administration, whereas another group of animals received this drug i.p. in total doses of 9 and 15 mg/kg followed by the BM examination on the next day after the 3rd and 5th injection of the drug. BM cells were stained with acridine orange and analyzed by FCM. Cytotoxicity was assessed by determining the percentage of total nucleated cells (TNC%) among the whole BM cell population and by determining the percentage of polychromatic erythrocytes (PCE%) among the whole population of enucleated erythrocytes.*

Results. *Regardless of the dose and regimen of melphalan administration, either i.v. or i.p. administered drug caused a significant reduction of TNC%. On the average, the i.p. administered drug resulted in about 2.0-fold decrease of TNC% (P<0.05), while the i.v. administered drug resulted in about 1.3-fold decrease of TNC% (P<0.05). As for enucleated erythrocytes, the i.p. administered drug resulted in about 1.4-fold decrease of PCE% (P<0.05), whereas the i.v. administered drug did not cause any changes in the PCE%.*

Conclusions. *Under these experimental conditions, i.p. administrated melphalan is considerably more cytotoxic than i.v. administered melphalan. This cytotoxic effect is preferentially due to impaired erythropoiesis.*

KEY WORDS: **bone marrow; melphalan; cytotoxicity; flow cytometry; polychromatic erythrocytes; total nucleated cells.**

Introduction

The anti-tumor effect of alkylating chemotherapeutics primarily attributes to their ability to covalently bind DNA via alkyl groups causing intra- and inter-strand crosslinks [1]. Any alkylating drug by induction of DNA lesions can affect the replication of actively proliferating cells [2]. Moreover, an impaired replication or repair of crosslinked DNA is likely to lead to cell death [3]. Although alkylating drugs can specifically target proliferating cells, they are not cell cycle phase-specific, and for this reason, cell death is believed to directly correlate with the dose of the drug [4]. An alkylating agent melphalan (known as interstrand DNA-crosslinker [1]), which is mainly used for treatment of multiple myeloma, ovarian carcinoma, breast cancer, childhood neuroblastoma, and *polycythaemia vera*, however, may cause complications, particularly acute myeloid leukemia in the decade after therapy [5]. Melphalan-treated individuals with an increased level of chromosomal aberrations in the peripheral blood lymphocytes are at risk of developing cancer later in life [5]. In the experimental animals, melphalan induces cancer of various localizations [5], and regardless of the route of administration, it is apparently genotoxic [6-10].

In the present work, flow cytometry (FCM) has been chosen to examine cytotoxicity in the bone marrow (BM) of rats that received melphalan either intravenously (i.v.) or intraperitoneally (i.p.). BM that contains hematopoietic cells of various lineages is a sensitive object of cytotoxic studies. As for the FCM, this technique is indispensable in many areas of biology and medicine not only because of its high-speed analysis, but also because of its ability to accurately discriminate cells of various types. The FCM usually discriminates cells based on their size, intracellular granularity and selective/specific fluorescence labeling [11]. This unique

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advantage of FCM can be applied for the study of cytotoxic effects in BM cells of different lineages and maturation stages. Here we use a simple and reliable FCM approach for the analysis of BM cells stained with acridine orange (AO), a metachromatic dye that simultaneously interacts with DNA and RNA producing at λ=488 nm the dual emission spectra with peaks at 530 nm and 640 nm, respectively [12]. This proposed approach by Criswell et al. [13] allows assessing cytotoxicity particularly in erythropoietic cells based on detection of differences in AO uptake between polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE). Redistribution of erythrocytes towards NCE is indicative of cytotoxic effect. Suzuki et al. [14] suggested that the reduced PCE/NCE ratio is most likely caused by elevation of NCE population as a result of mutagen-induced rapid differentiation and multiplication or enucleation of erythroblasts which remained in the BM instead of entering the peripheral blood steam. On the other hand, Von Lebedur and Shcmid [15] claimed that as a result of mutagen-induced partial depletion of the marrow cavities of nucleated blood cell precursors the newly formed erythrocytes can be retained along with inundation with peripheral blood.

Methods

Experimental animals and administration of melphalan

Adult outbred female rats (140-160 g) were taken from the animal house of R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of the National Academy of Sciences of Ukraine (Kyiv, Ukraine). Immediately before the i.v. or i.p. injections, 0.6 mg/ml solution of melphalan (Alkeran® produced by GlaxoSmithKline, UK) was prepared by diluting 20 mg/ml solution of this drug dissolved in acidified ethanol (96% ethanol and 12 N HCl mixed in the ratio of 150:1) with saline. One group of animals (n=3) was subjected to i.v. injection (via tail vein) of melphalan of a single dose of 3 mg/kg followed by the BM examination on the 3rd and 7th day after drug administration. Another group of animals (n=3) was subjected to i.p. injections of melphalan every other day with single doses of 3 mg/kg followed by the BM examination on the next day after 3rd and 5th injection of the drug (total doses were 9 and 15 mg/kg, respectively). Since melphalan after i.p. administration is assumed to be less readily delivered to the target tissue than after i.v. administration due to its gradual absorption into blood, this prompted us to increase the total dose of melphalan for delivery via i.p. route. There was also a group of animals that did not receive the drug at all (the intact control, n=3). The study with animals was performed according to the regulations of the Ethics Committee.

BM isolation, specimen processing and fixation

The femur removal and BM isolation procedures were in general performed as proposed [13]. The BM cells were thoroughly flushed from the femur with 4 ml of RPMI-1640 (PharmBiotek, Ukraine) and immediately placed in a refrigerator (+4-6 °C). At this temperature, the BM cells were kept no longer than 1.5 hour before they were resuspended by vortexing and centrifuged at 300× g for 5 min. Specimen processing and fixation procedures were mainly performed according to the protocol [13]. The supernatant was discarded with further washing the cells in 5 ml of PBS using centrifugation at 300× g for 5 min. The supernatant was discarded with further resuspension of cells in 2 ml of PBS by vortexing. Cell aggregates were dissociated by gentle syringing of the suspension through a 21-gauge needle. While vigorous vortexing, 0.2 ml of cell suspension was added to 5 ml of fixative solution: 1% glutaraldehyde (v/v) in PBS with 30 μg/ml of SDS (Merck, Germany). The cells were fixed for 5 min and then centrifuged at 300× g for 5 min. The supernatant was removed with further resuspension of cells in 0.5 ml of PBS.

Fluorescence staining

This procedure with slight modification was performed in accordance with the protocol [13]. Solution A was prepared by dissolving of the following components in 100 ml (final volume) of distilled H_2O: 0.1 ml Triton X-100 (Loba Chemie, Austria), 8 ml 1.0 N HCl, and 0.877 g NaCl. Solution B was prepared by mixing of 37 ml 0.1 M citric acid with 63 ml 0.2 M Na_2HPO_4 (pH 6.0) and adding 0.877 g NaCl, 34 mg EDTA disodium salt (Sigma, USA), and 0.6 ml of acridine orange (AO; Sigma) stock solution (1 mg/ml). The fixed cells (0.2 ml of cell suspension) were added to the mixture of Solutions A and B (0.4 and 1.2 ml, respectively) that was chilled on ice in a 12×75 mm centrifuge tubes. While shaking, the cells were stained on ice for 30 min in the dark. Then they were centrifuged at 300× g for 5 min. After the supernatant was carefully removed, 1 ml of PBS was added to resuspend the cells. Before FCM, cell suspension

was gently syringed through a 21-gauge needle to obtain a suspension of single cells.

FCM analysis

The samples were analyzed using an EPICS XL flow cytometer (Beckman Coulter, USA) equipped with a 15-mW argon-ion laser (488 nm). The forward light scatter (FSC; related to cell size) and side (90°) light scatter (SSC; related to intracellular granularity) signals were collected in a linear mode. The fluorescence signals of DNA- and RNA-bound AO were collected respectively in the green fluorescence channel (FL1) through a 525/10-nm band-pass filter and in the far-red fluorescence channel (FL4) through a 675/10-nm band-pass filter using a logarithmic amplification [13]. The acquisition rate was 500-1000 cells per second. At least 1.0×10^5 events were collected for each sample. The analysis of the data was performed by publicly available software "WinMDI" developed by Dr. J. Trotter (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm). The cells were gated on FSC-Height vs. SSC-Height histograms to eliminate debris and aggregates from analysis (not presented here), although microscopic observation showed that their numbers were extremely low. The parameters that were examined are as follows: 1) percentage of total nucleated cells (TNC) of all BM cells, including enucleated cells such as PCE and NCE (this parameter is further denoted as TNC%); 2) percentage of PCE of all enucleated erythrocytes (denoted as PCE%). The reason why $TNC_{\%}$ was also examined is based on the fact that the nucleated erythroid cells are most numerous in the BM, and accordingly, suppressed erythropoiesis may affect TNC%. Thus, the decreased TNC% and PCE% (particularly PCE%) can be indicative of inhibited division and maturation of nucleated erythroid cells, the fact that has been previously reported [13-16]. The populations of TNC, PCE and NCE that demonstrate significant differences in AO uptake were determined on a FL1-Height vs. FL4-Height histogram (Fig. 1A).

Statistical analysis

Probability values with $p<0.05$ were considered statistically significant. The distribution of indices was estimated by using the Shapiro-Wilk Normality Test. The statistical significance of the differences between the means was assessed by the Mann-Whitney-test and ANOVA-test using Origin 7.5 software (OriginLab Corporation, USA).

Results

Regardless of the dose and regimen of melphalan administration, i.v. delivered melphalan did not cause any significant changes in the PCE%, whereas i.p. delivered drug on the average resulted in about 1.4-fold decrease of PCE% ($p<0.05$, compared with the control; Fig. 2).

As for TNC, melphalan administered either i.v. or i.p. resulted in a significant decrease of

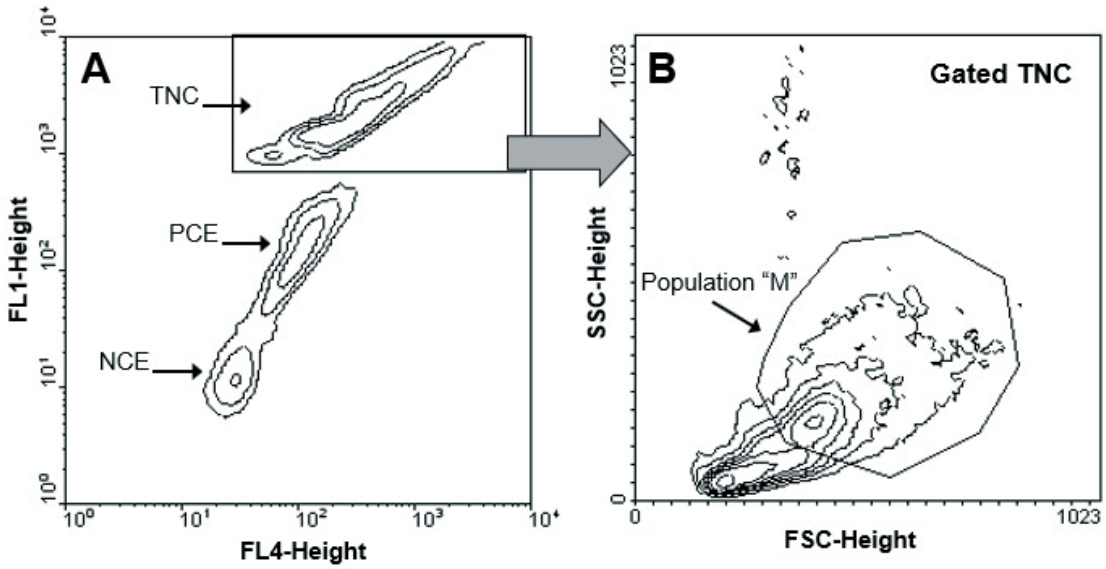

Fig. 1A. Example of FCM determination of TNC, PCE, NCE populations in AO-stained unfractionated BM cells (BM cells were isolated from the femur of the control intact rat).
Fig. 1B. Population of TNC gated on a FL1-Height vs. FL4-Height histogram (framed by the rectangular window; panel A) is shown on a FSC-Height vs. SSC-Height histogram to analyze the population 'M' comprised of the vast majority of myeloid cells.

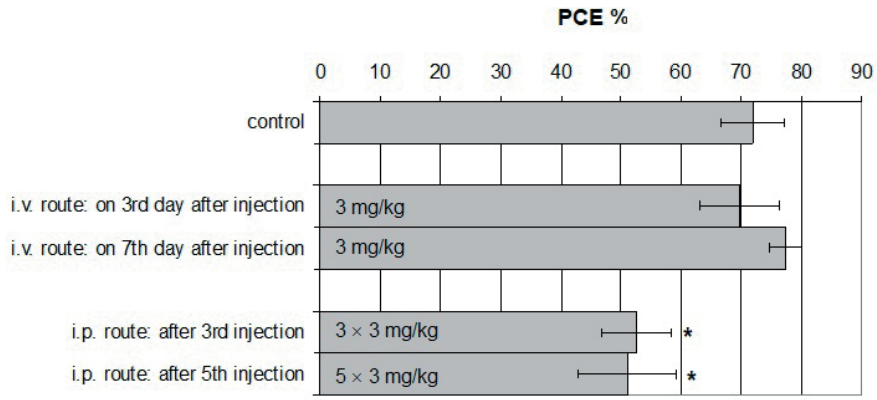

Fig. 2. Effect of variously administered melphalan on the PCE%. The data presented are the mean ± standard error of the mean. Each group consists of three animals. Asterisks (*) show significant differences if compared with the control ($p<0.05$).

TNC% ($p<0.05$, compared with the control Fig. 3). On the average, after i.p. and i.v. drug delivery there was a 2.0-fold and 1.3-fold decrease of TNC%, respectively (Fig. 3). Obviously, melphalan after several i.p. administrations (3×3 mg/kg or 5×3 mg/kg) was more cytotoxic than after a single i.v. administration (3 mg/kg). However, increasing the dose of i.p. injected melphalan up to 5×3 mg/kg did not result in more significant cytotoxic effect. Perhaps, at lower dose of this drug (3×3 mg/kg) the maximal effect could be reached. As for the i.v. delivery of melphalan, we did expect that this route of drug administration would be more efficient in terms of causing cytotoxicity in the BM. That is why a single minimal dose of melphalan (3 mg/kg) was chosen for this route of delivery.

Although the aforementioned findings seem to be indicative of suppressed proliferation of erythroid cells, particularly in case of i.p. administered melphalan, it cannot be certainly claimed that proliferation of myeloid cells remains unaffected. Since myeloid cells as well as erythroid cells are numerous in the BM [17], one can expect that the drug-induced suppression in proliferation of these cells may also contribute to significant fluctuations of TNC%. To address this issue, we were able to identify within TNC the population of cells (population 'M'; Fig. 1B), the vast majority of which are likely to be myeloid. This assumption is simply based on the evidence that they are generally large with a specific intracellular granularity [17-19]. In FCM, cell size usually correlates with the FSC, while intracellular granularity correlates with the SSC [11]. The percentage of population 'M' of the whole TNC population (denoted as population 'M'%) was similar to that revealed microscopically by the classic morphology-based evaluation (date not shown). Notably, i.p. administered melphalan on the average caused a 1.3-fold increase of the portion of population "M" (Fig. 4) with a concordant decrease of PCE% (Fig. 2). However, together with the fact that the i.p. delivered drug resulted in a 2-fold decrease of TNC% (Fig. 3) one can assume that granulopoiesis is likely be affected but certainly to a lesser extent then erythropoiesis. As for the i.v. delivered

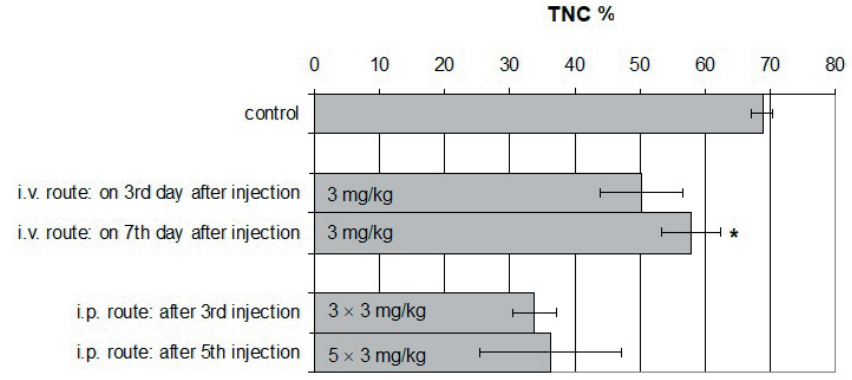

Fig. 3. Effect of variously administered melphalan on the TNC%. The data presented are the mean ± standard error of the mean. Each group consists of three animals. Asterisk (*) shows significant difference if compared with the control ($p<0.05$).

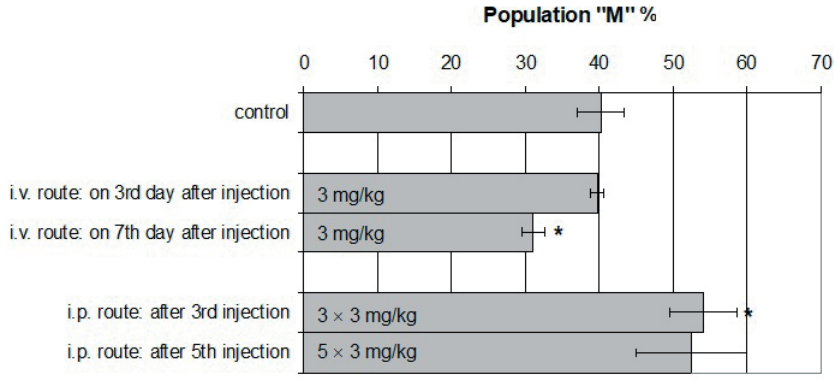

Fig. 4. Effect of variously administered melphalan on population 'M'%. The data presented are the mean ± standard error of the mean. Each group consists of three animals. Asterisks (*) show significant differences if compared with the control ($p<0.05$).

drug, there was a 1.3-fold decrease of population "M"%, but only on the 7th day of its injection ($p<0.05$, compared with the control; Fig. 4). This decrease of population 'M'% was accompanied by a slight increase of PCE% on the same day after i.v. drug administration (Fig. 2). Perhaps, on this day (the 7th day) after i.v. drug administration the erythroid cells recovered faster than myeloid cells.

Discussion

Regrettably, there is lack of reports concerning cytotoxic or genotoxic effects in the BM of rats caused by melphalan delivered either i.v. or i.p. Instead, it was reported that the rats that intramuscularly received this alkylating agent of a single dose of 1 mg/kg developed a transient but significant increase of chromosomal aberrations in BM cells peaking on the next day after the drug administration (but there were almost no aberrations on the second day after the drug administration, similar to the control), while increasing the dose up to 10 mg/kg led to the absence of mitotic figures, which is indicative of significant BM suppression [6].

Thus based on the results of our research, one can assume that erythropoiesis is more readily affected by melphalan than granulopoiesis was, but erythropoiesis seemed to be recovered faster than granulopoiesis. Apparent erythropoietic cytotoxicity in the BM of rats was shown to be caused by another alkylating drug cyclophosphamide that at the range of doses 5-40 mg/kg resulted in a significant increase of myeloid/erythroid ratios on the second day after i.p. drug delivery [20, 21].

Erythroid cells were shown to be very sensitive with respect to ionizing radiation (IR), DNA damaging agent of a physical origin [22, 23]. For example, as a result of a whole-body irradiation of rats with the dose of X-rays about 7.0 Gy (LD_{50}) the erythroid cells appeared to be significantly more sensitive than the myeloid cells; however, erythropoiesis began recovering much earlier than granulopoiesis (obvious regeneration was first clearly observed on the 12th day after irradiation as evidenced by areas of erythropoiesis) [21]. Notably, the cytotoxic effect of any alkylating agent is referred to as 'radiomimetic' because IR and alkylating agents are similar in terms of inducing cell death mechanisms (both of them induce the mitotic catastrophe) [24, 25]. Interestingly, as for melphalan, its dose-response relationship resembles that for IR as evidenced by the shape of the survival curve [24]. A series of studies has been initiated towards tackling melphalan-induced BM suppression [26, 27], and, in this regard, monitoring of BM recovery by FCM could be helpful as well.

Conclusions

Under the present experimental conditions, i.p. administrated melphalan is considerably more cytotoxic than i.v. administered melphalan, and this effect is preferentially due to impaired erythropoiesis. Granulopoiesis is less readily affected by the melphalan than erythropoiesis, but on the other hand, granulopoiesis, if affected, is slower recovering than erythropoiesis. It is expected that the FCM findings of this study could be helpful for experimental oncologists, who design experiments on antitumor effects of melphalan with less side effects.

ОЦІНКА МЕЛФАЛАН-ІНДУКОВАНОЇ ЦИТОТОКСИЧНОСТІ НА КЛІТИНИ КІСТКОВОГО МОЗКУ ЩУРІВ ЗА ДАНИМИ ПРОТОЧНОЇ ЦИТОМЕТРІЇ

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Вступ. *Кістковий мозок, як основний орган гемопоезу, особливо чутливий до впливу цілого ряду цитотоксичних протипухлинних лікарських засобів.*

Мета роботи: *за допомогою проточної цитометрії встановити прояви цитотоксичності на клітини кісткового мозку щурів за умов довенного та інтраперитонеального введення мелфалану.*

Методи. *Кістковий мозок щурів досліджували на 3-ю та 7-у доби після довенного введення мелфалану в дозі 3 мг/кг; а також на наступний день після 3-ї та 5-ї ін'єкцій препарату при його інтраперитонеальному застосуванні (при досягненні кумулятивної дози 9 та 15 мг/кг). Клітини кісткового мозку забарвлювали акридиновим помаранчевим та аналізували за допомогою проточної цитометрії. Цитотоксичність оцінювали за відсотком загальної кількості ядерних клітин (ЯК%), а також за відсотком поліхроматофільних еритроцитів (ПХЕ%) у складі всіх без'ядерних еритроцитів.*

Результати. *Незважаючи на шлях введення та обрану дозу, мелфалан викликав достовірне зниження ЯК%. В середньому, показник ЯК% знижувався у 2 рази при інтраперитонеальному введенні ($p<0.05$) та в 1.3 рази – при довенному ($p<0.05$). Стосовно без'ядерних еритроцитів, ПХЕ% знижувався в 1.4 рази при інтраперитонеальнему введенні ($p<0.05$), тоді як при довенному введенні цей показник залишався без змін.*

Висновки. *В даних експериментальних умовах, на відміну від довенного застосування, інтраперитонеально введений мелфалан виявився більш цитотоксичним, переважно за рахунок порушень еритропоезу.*

КЛЮЧОВІ СЛОВА: **кістковий мозок; мелфалан; цитотоксичність; проточна цитометрія; поліхроматофільні еритроцити; ядерні клітини.**

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