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CORRELATION BETWEEN THE CYTOTOXICITY OF MELPHALAN AND DNA CROSSLINKS AS DETECTED BY THE ETHIDIUM BROMIDE FLUORESCENCE ASSAY IN THE F_1 VARIANT OF B_{16} MELANOMA CELLS

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Abstract—The relationship between DNA crosslinks and cell death as a result of exposure to melphalan (MLN) was studied in the F_1 variant of B_{16} melanoma cells. The formation of DNA crosslinks is believed to represent the lethal lesion following exposure of cells to bifunctional alkylating agents. The production of DNA crosslinks by MLN was determined by the recently described ethidium bromide fluorescence assay [De Jong *et al., Int. J. Cancer* 37, 557 (1986)]. A direct correlation between the percentage of DNA crosslinks (C_l) and cytotoxicity of melphalan has not been previously reported utilizing the fluorescence assay. The cytotoxicity of MLN and the production of DNA crosslinks by this drug were determined following a 1-hr incubation at 37°. The concentrations of MLN necessary to reduce colony growth to 37% of control and 10% of control were 6.7 μM (EC_{37}) and 26 μM (EC_{10}) respectively. Utilizing the ethidium bromide fluorescence assay (EFA), the relationship between MLN concentration (x axis) and DNA crosslinks expressed as C_l (y axis) was best described by a power curve ($y = 0.28\,x^{0.81}$; $r = 0.985$). The respective C_l values at the EC_{37} and EC_{10} of MLN were 1.3 and 3.8%. It appears that the sensitivity of the EFA is similar to the alkaline elution assay and, in addition, that the EFA is less technically difficult to employ with tumor cells obtained from patients.

To study the mechanisms through which DNA is damaged by alkylating agents, methods have been developed to detect and quantify the formation of interstrand crosslinks. There are several methods which measure the formation of DNA crosslinks in cells, such as: (a) the renaturation method [1, 2], (b) the alkaline elution assay (AEA) [3, 4], (c) the alkaline sucrose sedimentation [2], (d) the density labeled hybrid DNA method [5], and (e) the ethidium bromide fluorescence assay (EFA) [6].

The alkaline elution technique is one of the most widely employed assays for the detection of DNA crosslinks. This assay can detect protein–DNA crosslinks, interstrand DNA crosslinks and also DNA strand breaks [4, 7–9]. There have been several comparisons of the drug concentrations necessary to achieve cytotoxicity compared with the dose necessary for detection of DNA crosslink formation using this method [10–14].

It is possible to detect DNA crosslink formation with concentrations of nitrogen mustard (NH_2) and melphalan (MLN) that produce 30 and 65% cell kill in L1210 cells respectively [10]. Similarly, concentrations of NH_2 and MLN that produce approximately a 30 and 60% cell kill, respectively, in human melanoma cells result in detectable crosslinking using the AEA [14]. However, in fresh human tumor cells the AEA is laborious and difficult to use because the DNA cannot be easily radiolabeled, and thus it is necessary to measure DNA fluorometrically in each fraction of the AEA [15].

The EFA is based on the fact that the fluorescence of ethidium bromide increases 20 to 25-fold (depending on the ionic strength of the medium) on binding to double-stranded DNA as compared to single-stranded DNA [16]. The production of single-strand breaks in DNA up to 750 Rad-equivalents does not interfere with the EFA [6, 17]. However, it is difficult to know if the EFA has a sensitivity similar to the AEA. Results with the EFA have been compared with AEA [6, 12]. This comparison can only be qualitative because the end points of both assays are quite different. A comparison of EFA results with cytotoxicity was done for cyclophosphazenes, but the drug incubation for cytotoxicity was 1 hr whereas a 6-hr incubation was utilized for the EFA [12]. Furthermore, to our knowledge, the EFA has not been utilized to detect crosslinks produced by nitrogen mustards.

In this report we describe the production of DNA crosslinks by MLN and compare these results to cytotoxicity with the F_1 variant of B_{16} melanoma cells. A good indication of the sensitivity of a crosslink assay is to compare the concentration of drug necessary to produce cytotoxicity to a given concentration which would yield DNA crosslinks. To determine the sensitivity of EFA, we compared the cytotoxicity of MLN to the production of DNA crosslinks by MLN in the EFA with the F_1 variant of B_{16} melanoma cells.

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MATERIALS AND METHODS

Materials. MLN was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Silver Springs, MD (NSC No. 8806). MLN solutions were prepared in ethanol containing an equimolar concentration of hydrochloric acid. Further dilutions were made in phosphate-buffered saline (PBS), pH 6, prior to its use to minimize hydrolysis, and the solution was stored at −20°.

Bovine serum albumin was obtained as serum fraction V from Miles Laboratories (Elkhart, IN). Phosphate-buffered saline (Dulbecco's modification) and preservative free heparin were purchased from GIBCO, Canada (Burlington, Ontario, Canada). Ethidium bromide and sarkosyl were purchased from the Sigma Chemical Co. (St. Louis, MO). McCoy's 5A medium, CMRL medium, fetal calf serum (FCS), horse serum, trypsin, EDTA, culture flasks and Petri dishes were obtained from GIBCO Laboratories (Grand Island, NY). Insulin was provided by Connaught Laboratories. Agarose was purchased from Bethesda Research Laboratories (Rockville, MD) and trypticase soy broth from Difco Laboratories (Detroit, MI).

Cell culture. The F_1 variant of B_{16} melanoma cells (hereafter called F_1 variant cells) were cultured in McCoy's 5A medium enriched with 10% fetal calf serum and 5 μg/ml of gentamycin (Schering, Pointe Claire, Quebec, Canada) in T-80 flasks. Cultures were maintained at 37° in a humidified atmosphere at 5% CO_2. For these experiments the cells used were at passage 9.

In vitro *exposure of tumor cells to drug.* Assays for cell growth were performed by plating 7×10^4 F_1 variant cells on 60 mm Petri dishes in the same medium as described earlier. After 3 days of incubation under the same conditions, the plates were aspirated and washed two times with McCoy's 5A without serum; then the cells were exposed to MLN at different concentrations (0–40 μM) in fresh medium and incubated at 37° for 1 hr. The drug was then removed and the plates were washed twice with PBS. The cells were harvested by 0.05% trypsin plus 0.02% EDTA and washed with McCoy's 5A plus 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES).

Human tumor cloning assay (HTCA). The culture system used in this study has been described extensively elsewhere [18–22]. The overlayer in which the tumor cells were suspended consisted of 0.3% agarose in enriched Connaught Medical Research Medium 1066 supplemented with 15% horse serum, gentamycin (8 μg/ml), glutamine (2 mM, Sigma), $CaCl_2$ (4 mM), and insulin (2 units/ml). Just before use, several substances were added to this enriched medium [0.66 mM asparagine, DEAE-dextran 0.4 mg/ml, and 0.01 mM dithiothreitol (Sigma)]. Then, 1 ml volumes were pipetted onto 2 ml feeder layers in 35 mm plastic Petri dishes. The final count of cells was 10,000 per ml at the lower concentrations of MLN and 30,000 per ml at the higher concentrations of MLN. The feeder layers used in this study were made with McCoy's 5A medium plus 15% heat-inactivated fetal calf serum and the nutrients as previously described [20]. Before use, 3% tripticase soy broth, 0.3 ml of 88 mM asparagine, and 5% DEAE-dextran were added to 40 ml of enriched underlayer medium. Agar was added to this enriched medium (final concentrations 0.5%). The underlayers were poured into 35 mm Petri dishes and cooled in the refrigerator for 8 min. Triplicates for each MLN concentration were done. The Petri dishes were then stored at 37° in a humidified atmosphere (5% CO_2) incubator.

Colonies (consisting of more than 40 cells) appeared in 21–28 days and plates were counted for drug effects, at 40× amplification. Control samples had 186 ± 30 colonies per plate.

Determination of DNA crosslinks. F_1 variant cells were suspended in McCoy's 5A medium with 10% FCS and gentamycin (5 μg/ml) at 2×10^6 cells/ml. The cells were exposed to various concentrations of MLN at 37° for 1 hr. The cells were then centrifuged and washed once with the above medium at room temperature. The cells were resuspended in drug-free medium and incubated for 4 hr at 37° to allow for the formation of maximal DNA crosslinks [3].

DNA crosslinks were determined by utilizing the ethidium bromide fluorescence assay as previously described [6]. Forty microliters of drug-exposed cells (approximately 9×10^5 cells) was added to 200 μl of lysing solution (4 M NaCl, 50 mM KH_2PO_4, 10 mM EDTA and 1% sarkosyl, pH 7.4). Twenty microliters of heat-inactivated pancreatic RNase (2 mg/ml) was added to each lysate. Following a 16-hr incubation at 37° in a shaking water bath, 25 μl of heparin (500 I.U./ml) was added to the lysates and the incubation was allowed to continue for 25 min at 37°. The total volume of 285 μl was added to 3 ml of a solution containing ethidium bromide (EB) (10 μg/ml), 20 mM K_2HPO_4 and 0.4 mM EDTA (pH 12.0).

The sample tubes were wrapped in aluminium foil and kept in the dark to prevent light-induced cleavage of DNA by EB. Fluorescence was measured in an SPF-500C SLM-Aminco spectrofluorometer with an excitation wavelength of 525 nm and an emission wavelength of 580 nm. Fluorescence was measured prior to, and following, a 5-min denaturation at 100°. The percentage of DNA crosslinks was determined by measuring the difference in fluorescence of denatured control cell lysates and denatured melphalan-treated cell lysates by the formula:

$$C_t = \frac{f_t - f_n}{1 - f_n} \times 100\%$$

where C_t = percentage of DNA crosslinks in treated cells; f_t = fluorescence after denaturation divided by fluoresence before denaturation of treated cells, and f_n = fluorescence after denaturation divided by fluorescence prior to denaturation of control cells.

We prepared dihydroxymelphalan by heating melphalan at 60° for 1 hr in NaOH; this inactive analog of melphalan was utilized as a control. There was no evidence of crosslinks in DNA, following treatment of cells with 80 and 160 μM dihydroxymelphalan.

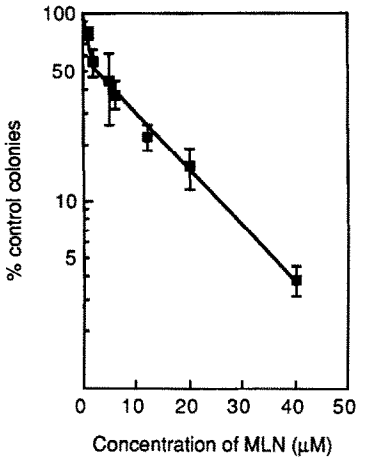

Fig. 1. Survival curve for the F_1 variant of B_{16} melanoma cells exposed to increasing concentrations of MLN for 1 hr at 37°. The results represent the mean ± SE for nine experiments, with triplicate plates in each experiment. Control samples had 186 ± 30 colonies per plate.

RESULTS

The HTCA was used as an *in vitro* assay for antitumor activity. The survival curve of tumor colony forming cells of the F_1 variant following exposure to MLN is shown in Fig. 1. The results clearly illustrate an inverse relationship between percent survival and increasing concentrations of MLN. The relationship between the dose of MLN and the fraction of surviving cells is best described exponentially ($r = 0.9963$) resulting in an EC_{37} (concentration of MLN that results in 37% of control colonies) of 6.73 μM MLN and an EC_{10} (concentration of MLN that results in 10% of control colonies) of 25.7 μM MLN.

DNA interstrand crosslinking has been shown to correlate with cytotoxicity [15, 23–26]. We measured DNA crosslinks using the EFA. The percent of DNA crosslinks (C_t) produced by various concentrations of MLN was determined in the F_1 variant cells. The results (Fig. 2) demonstrate that there is a clear dose response between increasing concentrations of MLN and C_t. The relation between C_t (y axis) and the concentration of MLN (x axis) was best described by a power curve ($y = 0.28\,x^{0.81}$; $r = 0.985$). Utilizing this curve, the EC_{37} of MLN resulted in a C_t of 1.3% while the EC_{10} of MLN resulted in a C_t of 3.8%. The C_t of 1.3% obtained with the EC_{37} of MLN respresents the limit of sensitivity of the fluorescence assay.

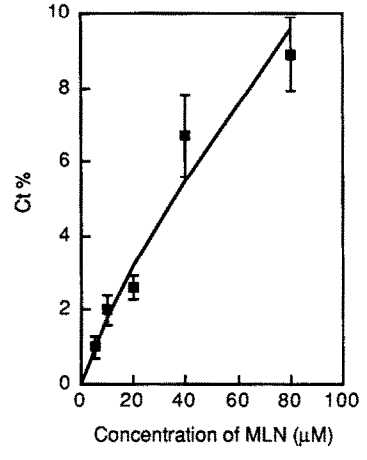

Fig. 2. Relationship between concentration of MLN and the percentage of crosslinked DNA in the F_1 variant of B_{16} melanoma cells as measured by the ethidium bromide fluorescence assay. The results represent the mean ± SE for five experiments.

DISCUSSION

These results support the proposition that DNA crosslinks as measured by the EFA can be detected at the same concentrations that produce cytotoxicity in the colony formation assay with F_1 variant cells. The EC_{37} of melphalan resulted in 1.3% DNA crosslinks using the EFA, which is the limit of sensitivity of the assay in our laboratory. These data compare favorably with previous reports in which the concentration of MLN necessary to produce detectable crosslinks in the AEA resulted in approximately 60% cytotoxicity in the colony formation assay [14]. Detection of DNA interstrand crosslinks seems to be technically easier, using the EFA. In addition, there should be no interference by DNA strand breaks up to 750 Rad-equivalents [6], and radiolabeled DNA is not required. This is important since it is possible to use this method in clinical studies. The feasibility of large scale processing of fresh clinical material (tumor cells in a single cell suspension) with the EFA could lead to a better understanding of the mechanisms of resistance to alkylating agents such as MLN in patients. We have preliminary results utilizing the EFA to determine DNA crosslinks in lymphocytes from chronic lymphocytic leukemia patients exposed to MLN *in vitro* [27].

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