L-Phenylalanine Mustard (Melphalan) Uptake and Cross-Linking in the RPMI 6410 Human Lymphoblastoid Cell Line

L. W. Brox, B. Gowans, and A. Belch

McEachern Laboratory and Cross Cancer Institute, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

ABSTRACT

L-Phenylalanine mustard (melphalan) induced a time- and concentration-dependent arrest of cycling RPMI 6410 cells in the G2 phase of the cell cycle as evidenced by flow cytometry. A melphalan exposure of 1 µg/ml for 1 hr caused a temporary G2 block which was overcome by 48 hr. Higher concentrations or longer exposures lead to irreversible blockages. Melphalan caused DNA cross-linking which was monitored by the alkaline elution method. The cross-linking was shown to be between DNA and protein. The degree of DNA cross-linking increased for approximately 4 hr after a 1-hr drug exposure of 1 µg/ml. At 36 to 48 hr after the drug exposure, the cells overcame the G2 block and were dividing. The DNA cross-links have apparently been repaired as they are no longer detected by alkaline elution. The extent of melphalan cross-linking was dependent on both drug dosage and exposure time. Using a culture medium lacking amino acids, it was shown that melphalan uptake into RPMI 6410 cells was inhibited by leucine, isoleucine, or glutamine. The increased uptake of melphalan and the increased cross-linking in amino acid-deficient media were reduced by readdition of the aforementioned amino acids.

INTRODUCTION

Melphalan (L-phenylalanine mustard), a bifunctional nitrogen mustard, has recently been shown by Ross et al. (7) to cause DNA cross-links in mouse L1210 cells. Using the method of alkaline elution, these workers demonstrated that melphalan and nitrogen mustard display different time courses for both the formation and removal of DNA cross-links. Vistica et al. (10, 11) have shown, again using mouse L1210 cells, that certain amino acids inhibit melphalan uptake and reduce toxicity. These observations were taken to indicate that melphalan is transported into L1210 cells by a high-affinity amino acid transport system of the leucine (L) type (10).

In this study, we have used a human lymphoblastic cell line, RPMI 6410, to examine the concentration and time dependence of melphalan-induced DNA cross-linking as well as the effect of leucine on this cross-linking. With the alkaline elution methodology, it was also possible to distinguish between DNA-DNA and DNA-protein cross-linking. The appearance and disappearance of this cross-linking were related to the perturbations in the cell cycle distribution as evidenced by flow cytometric analysis.

MATERIALS AND METHODS

The human lymphoblastic cell line, RPMI 6410, was obtained from Associated Biomedic Systems (Buffalo, N. Y.) and maintained in suspension cultures using Roswell Park Memorial Institute Medium 1640 supplemented with 10% dialyzed fetal calf serum (Grand Island Biological Co., Burlington, Ontario, Canada). The cells were routinely subcultured to 50,000 cells/ml, and they grew exponentially to approximately 1 million cells/ml with a doubling time of about 18 hr. Cell counts and volumes were determined with a Coulter Model ZM electronic particle counter equipped with a Coulter Channelizer II.

The relative DNA content of control and melphalan-treated cells was determined by flow microfluorometry after propidium iodide staining. Cells were collected by centrifugation and stained for 30 min at a density of 4.0 x 10^6 cells/ml with propidium iodide (0.05 mg/ml in 0.1% sodium citrate (6). The cellular fluorescence intensities were recorded with a Bio/Physics Model 4800 A cytofluorograph equipped with a Bio/Physics Model 2100 pulse height analyzer (Bio/Physics Systems, Inc., Mahopac, N. Y.). The output from the pulse height analyzer was through a standard teletype.

The DNA of the RPMI 6410 cells was labeled by a 24-hr incubation with either 1 µCi [methyl-3H]thymidine (50 Ci/mmol, 0.1 µCi/ml; Moravek Biochemicals, City of Industry, Calif.) or [2,4,6-tritiated 3H]thymidine (50 µCi/mmol, 0.01 µCi/ml; Moravek Biochemicals). The [methyl-3H]thymidine-labeled cultures were then exposed to melphalan (Burroughs Wellcome Ltd., Lachine, Que., Can.) at the indicated concentrations and times. At various times after resuspension in drug-free medium, alkaline elution experiments were carried out. The [2,4,6-3H]thymidine-labeled cells were used as the internal reference cells (7).

Alkaline elution was carried out as described by Kohn et al. (5) and Ross et al. (7). Experiments to distinguish DNA-DNA and DNA-protein cross-linking utilizing proteinase K (EM Laboratories, Elmsford, N. Y.) were carried out as described by Ewig and Kohn (2). Irradiation of the experimental culture with 300 R and the internal reference cultures with 100 or 150 R prior to the alkaline elution procedure was at a dose rate of 100 R/min utilizing a Picker 127Cs therapy unit. Radioactivity in the eluted fractions from the alkaline elution experiments was determined by liquid scintillation counting using a Triton X-100-toluene (1:2) scintillation cocktail (J. T. Baker Chemical Co., Phillipsburg, N. J.).

Studies on the uptake of melphalan by the RPMI 6410 cells and on the effect of various amino acids on this process were carried out in Dulbecco's phosphate-buffered saline containing 10% dialyzed fetal calf serum. Melphalan uptake experiments were performed as described by Goldenberg et al. (3, 4). [G-3H]Melphalan (8 Ci/mmol) was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

1 This work was supported by the National Cancer Institute of Canada.
2 To whom requests for reprints should be addressed.

Received October 29, 1979; accepted January 15, 1980.
RESULTS

The effect of melphalan at 1 μg/ml on the growth of RPMI 6410 cells is shown in Chart 1. Cell cultures exposed to melphalan for 1 hr display a growth lag from which they recover to grow at the normal rate. However, cell cultures exposed for longer times do not regain the capacity to proliferate.

An explanation of the growth lag with the 1-hr drug exposure is shown in Chart 2. Melphalan induces a temporary blockage in the movement of cells through the cell cycle with an accumulation in late-S or G2 phase. This blockage is overcome, and a normal DNA distribution is observed by 72 hr. Increasing the melphalan exposure time to 4 and 6 hr results in a S-phase blockage which is not reversible and leads to cell lysis (Chart 3). Cell lysis was evidenced by trypan blue exclusion and the appearance of debris on the Coulter Channelyzer. This is to be contrasted with the data from the 1-hr drug exposure where in excess of 94% of the cells were viable by dye exclusion, and no increased cell debris was seen on the Channelyzer. Similarly, a 1-hr exposure to melphalan concentrations in excess of 2 μg/ml results in cell cycle blockages which are not reversible.

The melphalan-induced DNA cross-linking as monitored by alkaline elution is shown in Chart 4. It is seen that the extent of the cross-linking is dependent on both the drug concentration and the exposure time. Table 1 shows that after a 1-hr exposure to melphalan (1 μg/ml), the degree of cross-linking increases for approximately 4 hr after the drug exposure and that this cross-linking persists for at least 24 hr. By 48 hr, the cross-linking has apparently been repaired as evidenced by the alkaline elution technique. The relative retention value is the fraction of the 3H-labeled DNA remaining on the filter when 40% of the internal standard DNA has been eluted. An increase in relative retention indicates slower DNA elution which is interpreted as increased cross-linking.

A bifunctional alkylating agent such as melphalan may conceivably cause cross-links between separate DNA strands or between a DNA strand and some proximal protein molecule. Both types of cross-linking would increase the effective size of the DNA aggregates and result in slower alkaline elution. We used the proteinase K method described by Ewig and Kohn to distinguish the type of cross-linking. Chart 5 shows that proteinase K treatment converts the drug-induced elution pattern to the control pattern which suggests that the observed cross-linking is DNA-protein in character.

Table 2 shows that certain amino acids inhibit melphalan uptake into RPMI 6410 cells as has been reported for mouse L1210 cells (11, 12). These inhibition experiments were carried out in Dulbecco's phosphate-buffered saline instead of culture medium as the concentrations of leucine, isoleucine, and glutamine in Roswell Park Memorial Institute Medium 1640 are

---

Chart 1. Effect of melphalan on growth of RPMI 6410 cells. Cultures of RPMI 6410 cells were exposed to melphalan (1 μg/ml) for 1 (○), 4 (■), and 6 (▲) hr. Untreated (○) and treated cultures were then resuspended in drug-free media, and the cell number was determined at indicated times.

Chart 2. Flow cytofluorometric analysis of melphalan-treated RPMI 6410 cells. Cultures were treated for 1 hr with melphalan (1 μg/ml) and resuspended in drug-free media. Cell cycle distributions were determined at indicated times by flow cytofluorometry.

Chart 3. Flow cytofluorometric analysis of melphalan-treated RPMI 6410 cells. Cultures were exposed to melphalan (1 μg/ml) for the indicated times, and the cell cycle distributions were determined after 19 hr.
Melphalan Cross-Linking in RPMI 6410 Cells

0.38, 0.38, and 2.05 mM, respectively. Chart 6 shows that a 1-hr incubation with melphalan (1 \( \mu \)g/ml) causes significantly more cross-linking when the drug exposure is carried out in Dulbecco’s phosphate-buffered saline instead of culture medium (see Chart 4). Addition of 0.4 mM leucine to the incubation mixture is seen to greatly reduce the extent of cross-linking. Similar results were obtained with isoleucine and glutamine. Cell cultures exposed for 1 hr to melphalan (1 \( \mu \)g/ml) in Dulbecco’s phosphate-buffered saline do not recover their proliferative capacity and appear to be arrested in S phase as evidenced by flow microfluorometry.

DISCUSSION

During the past 3 years, 3 significant studies on the mechanism of action of melphalan have been reported. Barlogie and Drewinko (1) described a concentration-dependent and exposure time-dependent blockage of cells in the G2 phase of the cell cycle. Ross et al. (7) described the rates of formation and removal of melphalan-induced DNA cross-links in L1210 cells. In a series of papers, Vistica et al. (10-12) have described the transport of melphalan into L1210 cells and the effect of amino acids on this process. In this study, we have examined the relationship of these 3 studies in the human lymphoblastoid RPMI 6410 cell line.

Melphalan induced a time- and concentration-dependent cross-linking of DNA in the human RPMI 6410 cells similar to that observed with L1210 (7). The degree of cross-linking increases for a few hr following the removal of melphalan after a 1-hr exposure. We have also demonstrated that the DNA cross-linking is between DNA and protein as evidenced by the experiments with proteinase K (2). The type of cross-linking was not distinguished in the mouse L1210 study (7). As seen in Table 1, cross-linking from a sublethal dose of melphalan is apparently repaired sometime between 24 and 48 hr after drug exposure. It is also at this time that the G2-phase blockage is being overcome and that the cell population is returning to normal as evidenced by flow cytofluorometry (Chart 2). This suggests that the cross-linking results in the G2-phase blockage and that the cells cannot divide until repair is effected. With drug concentrations or exposure times from which cells do not recover a normal growth rate, it was observed that the DNA distribution did not return to normal and that the cross-linking was still present when cells were lysing. These results support the conclusion that in these cells, the DNA-protein cross-linking plays a central role in the mechanism of mel-
Fraction [14C] DNA Retained

Chart 6. Effect of leucine on melphalan-induced DNA cross-linking. Cultures of RPMI 6410 cells were exposed for 1 hr to melphalan (1 µg/ml) in phosphate-buffered saline with (O) and without (■) 0.4 mM leucine.

Phalan toxicity. In contrast to these data, a recent communication has implicated melphalan alkylation of 4 to 5S RNA as being important in the melphalan mechanism of action since 85% of the polynucleotide-bound drug was found in this fraction (8). These results were in a mouse tumor system in vivo. How the DNA-protein cross-links in the RPMI 6410 cells are removed is currently unknown. During the time period when the cross-links were being removed, there was no indication of an increase in single-strand DNA breaks.

The increased melphalan toxicity observed by Vistica in the absence of certain amino acids with the L1210 system is also seen with the RPMI 6410 cells. As seen in Chart 6, this increased toxicity may be explained by an increase in the degree of cross-linking effected by a particular concentration versus time exposure over that observed when certain amino acids are present. The toxicity resulting from a melphalan exposure at 1 µg/ml for 1 hr, which was reversible when cells were exposed in culture medium-containing amino acids, is no longer reversible if the drug exposure is carried out in a balanced salt solution.

This protective effect by amino acids such as leucine, isoleucine, and glutamine may have pharmacological significance. It may be desirable to standardize melphalan administration with respect to food intake so that blood levels of amino acids may be somewhat normalized. Salmon et al. (9) have recently advocated colony assay procedures for drug sensitivity testing. Their standard procedure is to carry out drug exposures in Hanks' balanced salt solution which contains no amino acids. With melphalan, this would lead to a lower 50% lethal dose than may be observed under more physiological conditions. Furthermore, it is conceivable that the amino acid protective effect may vary from one plasma cell population to another.

REFERENCES

I-Phenylalanine Mustard (Melphalan) Uptake and Cross-Linking in the RPMI 6410 Human Lymphoblastoid Cell Line

L. W. Brox, B. Gowans and A. Belch