Detection and Quantification of Melphalan-DNA Adducts at the Single Cell Level in Hematopoietic Tumor Cells

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Bifunctional alkylating agents, such as melphalan, are widely used in the treatment of hematological malignancies. The effects of these drugs on particular types of hematological cells and the causes of treatment failure are poorly understood. The aim of this work was to establish an ability to measure the extent to which melphalan reacts with the DNA of individual tumor cells, thereby creating new possibilities for molecular pharmacological studies on clinical samples. A novel approach for staining drug-DNA adducts is described in which cells were embedded in agarose and then lysed. The DNA from each cell remained in an ideal state for quantitative immunofluorescent staining using a previously described monoclonal antibody. Immunofluorescence and DNA-Hoechst dye fluorescence were quantified using a cooled slow scan charge coupled device camera and image analysis procedures. Immunofluorescence of drug-treated cells from a human leukemia cell line was partially correlated with DNA content. Mean integrated immunofluorescence of 50 to 100 cells was dependent on drug concentration and was linearly related to adduct levels. In these cells and in chronic lymphocytic leukemia cells obtained from patients, there was considerable intercell heterogeneity in apparent adduct levels. This was also seen in peripheral blood mononuclear cells isolated from a patient after melphalan therapy. © 1996 by The American Society of Hematology.

MATERIALS AND METHODS

Isolation of PB lymphocytes. Tumor cells from two patients with chronic lymphocytic leukemia (CLI) and normal PB mononuclear cells (PBMC) from a patient with non-Hodgkins lymphoma undergoing high-dose melphalan therapy were isolated from heparinized blood using lymphoprep (Nycomed, Birmingham, UK). The cells were washed twice with Dulbecco’s phosphate-buffered saline solution (PBS) and resuspended in RPMI medium at 5 x 10^5/mL. Cell culture. CCRF-CEM (human T-lymphoblastic cell line) cells were maintained as an asynchronously growing culture in hepes buffered RPMI 1640 medium (GIBCO, Paisley, UK) supplemented with glutamine (300 mg/L), penicillin (50 U/mL), streptomycin (50 mg/mL), neomycin (100 mg/mL) (Sigma, Poole, UK) and 10% fetal bovine serum. Cells were trypsinized and stained using monoclonal antibodies directed against DNA adducts. A novel approach for staining drug-DNA adducts is described in which cells were embedded in agarose and then lysed. The DNA from each cell remained in an ideal state for quantitative immunofluorescent staining using a previously described monoclonal antibody. Immunofluorescence and DNA-Hoechst dye fluorescence were quantified using a cooled slow scan charge coupled device camera and image analysis procedures. Immunofluorescence of drug-treated cells from a human leukemia cell line was partially correlated with DNA content. Mean integrated immunofluorescence of 50 to 100 cells was dependent on drug concentration and was linearly related to adduct levels. In these cells and in chronic lymphocytic leukemia cells obtained from patients, there was considerable intercell heterogeneity in apparent adduct levels. This was also seen in peripheral blood mononuclear cells isolated from a patient after melphalan therapy. © 1996 by The American Society of Hematology.

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calf serum (GIBCO) at 37°C 5% CO₂. CLL cells were maintained in the same medium and conditions.

*Exposure to melphalan.* Melphalan (Sigma) was initially dissolved (10 mg/mL) in acid ethanol (ethanol: 5M HCL 49:1 vol/vol). Cells were treated with drug at the appropriate concentration in complete medium for 1 hour at 37°C. Flasks were then put on ice to minimize cellular DNA repair and further drug interaction with DNA. Cells were washed three times by centrifugation with PBS (4°C) before use for microscopy. In certain experiments, aliquots of drug-treated cells were removed for analysis of adduct levels on DNA by competitive ELISA.

*DNA extraction and ELISA measurements of DNA adducts.* The DNA extraction and the competitive ELISA methods have been described elsewhere.5, 6, 8 MoAb MP5/735 was used in all the experiments.

**Immunofluorescent detection of melphalan-DNA adducts.** The method has been outlined very briefly elsewhere. A solution of low melting point agarose (Seaprep, FMC, Rockland, USA; 0.5% wt/vol in water) was spread thinly onto clean microscope slides (Menzel-Glaser from Weston Lab Services, Aldershot, UK) and dried at 37°C to form an adherent coating. An aliquot of cell suspension was briefly equilibrated to 37°C, mixed with an equal volume of agarose solution (Seaprep, FMC, 2% wt/vol in PBS; preheated to 37°C) and then spread on the precoated slides. These were placed onto a refrigerated surface to solidify the agarose and minimize evaporation of water. The slides were placed in lysis solution (1% Sarcosyl, 80 mmol/L potassium phosphate, 10 mmol/L EDTA, pH 6.8) for 15 minutes and then washed three times for 5 minutes in PBS, treated with alkalai to denature the DNA (0.1 mmol/L NaOH in water, 5 minutes; this was the optimum treatment time—data not presented) and washed again in PBS.

**MP5/73 rat MoAb (hybridoma culture supernatant diluted ×100 in PBS containing Tween 20 at 0.1% vol/vol [PBSTw] containing 1% bovine serum albumin [BSA]) was applied to each slide and incubated for 1 hour at room temperature. Slides were then washed three times with PBSTw. Rabbit anti-rat-IgG-fluorescein isothiocyanate (FITC) conjugate (Fab2) preparation from Serotec (Oxford, UK), diluted ×100 in PBSTw 1% BSA) was applied for 1 hour in the dark. Then, after washing three times for 20 minutes in PBSTw, the DNA on each slide was stained by mounting slides in Hoechst dye 33258 (10 pmoVL in PBS). Coverslips were placed carefully and then spread on the precoated slides. These were placed onto a refrigerated surface to solidify the agarose and minimize evaporation of water.

**DNA extraction and EUSA measurements of DNA adducts.** DNA extraction and EUSA measurements of DNA adducts was subtracted from the sample image. All images of samples were also subjected to a shading correction to compensate for variation in intensity of illumination and for non-uniformities in light transmission. This required uniformly fluorescent specimens, which consisted of solutions of fluorescein (3 μmol/L) or 4-methylumbelliferone (200 μmol/L) in a 20-μm deep observation chamber. The images obtained from these solutions were subjected to background correction as described above before being used. The intensity of each pixel in each of the shade-correction images was divided by the mean intensity of all the pixels in that image. The resulting ratios were used to correct the corresponding pixels in the sample images.

The corrected images of Hoechst fluorescence were used to define the areas occupied by DNA from each cell. A binary image (Fig IC) was created using a gray-scale threshold as low as was consistent with the demarcation of distinct objects. This was the only subjective part of the analysis, and the very low background staining levels made this straightforward. The final integrated fluorescence intensity values were not significantly affected by small variations in the threshold (data not presented). Each binary image was first refined by erosion and reconstruction to remove specks of fluorescence between the cells (binary image 2, Fig ID). The background level of fluorescence for each image was calculated as the mean fluorescence intensity of all the pixels not lying in the object areas that were defined in binary image 2. This value was subtracted from each pixel in the corrected gray-scale image. Integrated fluorescence intensities for blue and green images were calculated as the sums of the gray-scale values of all the pixels within each valid object. Valid objects were those defined in binary image 2 that did not touch the edge of the image.

**Preparation of CLL cells for flow cytometric analysis.** Flow cytometric analysis of DNA content was performed using cycleTEST kit (Becton Dickinson, San Jose, CA). Briefly, after an overnight incubation at 37°C, PBMCs from two CLL patients were treated with trypsin and RNAse before staining with ice cold propidium iodide for 10 minutes in the dark as described in the manufacturer’s protocol. The sample was analyzed on a FACScan flow cytometer using CellFIT software (Becton Dickinson). Coefficient of variation (CV) values were calculated for the combined populations of G1, S, and G2 peaks.

**RESULTS**

CCRF-CEM cells were treated with a range of concentrations of melphalan and prepared for immunostaining as described in Materials and Methods. After lysis, the cells were no longer visible by phase contrast illumination, but the DNA from each cell remained trapped in the agarose and could be visualized through the blue Hoechst dye fluorescence (Figs 1A and B). Using the CCD camera, two images were captured for each field of view, to record blue and green fluorescence separately. Melphalan–adduct specific green fluorescein immunostaining was observed in drug-treated cells and was colocalized with the blue DNA fluorescence.
Staining intensity was dependent on drug dose, and control cells showed no detectable immunofluorescence (Fig 2A-H). The gradation in fluorescence intensities is not shown optimally in the printed images, but is demonstrated clearly by plotting pixel intensities of blue and green fluorescence along cross-sections through the objects (Figs 2C, F, and I). Staining of adducts with MP5/73 was completely dependent on pretreatment of the embedded DNA with alkali (Fig 2G, K, and L). Cells that had been treated with 100 µg/mL melphalan showed no detectable staining when the MoAb was omitted. Exposure of CCRF-CEM cells to mechlorethamine (nitrogen mustard) (20 and 40 µg/mL, 1 hour) resulted in no detectable staining. Exposure to chlorambucil (100 and 200 µg/mL, 1 hour) resulted in a barely detectable level of fluorescence. These concentrations of drugs were chosen because they have been reported to produce levels of DNA damage at least as great as 100 µg/mL melphalan, the highest concentration used in this work.

Quantification of DNA and adduct immunofluorescence was achieved by the development of an image analysis procedure as described in Materials and Methods. Experiments using CCRF-CEM cells that had been exposed to melphalan in vitro (1 hour), embedded in agarose, lysed, and stained with MP5/73 MoAb demonstrated that there was considerable intercell heterogeneity in DNA-Hoechst blue fluorescence, as expected for an asynchronously growing culture. Heterogeneity was also observed in adduct content as reflected in green immunofluorescence (Fig 3A). This was partly correlated with DNA content. (Fig 3A). Similar results were obtained at other drug concentrations (data not shown). The binding of adduct-specific antibody to DNA, as determined by the mean integrated green light intensities of 50 to 100 cells, showed a clear dependency on drug concentration (Fig 4B). The integrated blue DNA fluorescence was not significantly affected by drug exposure (Fig 4A).

To assess the accuracy of the quantification of fluorescence by the present techniques, CLL cells were used because of their narrow distribution of DNA contents. As expected, DNA content of CLL cells showed a much smaller variation (Fig 3B) than did DNA content of the CCRF-CEM cells. However, the adduct immunofluorescence of the CLL cells was heterogenous (Fig 3B). The accuracy of quantification of fluorescence by the agarose-embedding/image analysis technique was shown by the similarity of CV values observed for the DNA contents of CLL cells measured by
this method and by a standard flow cytometry procedure. At least 50 PB cells from each of two patients were cultured overnight, processed, stained, and analyzed. Mean integrated blue fluorescence values obtained by fluorescence microscopy and image analysis were 688,775 and 662,274 (arbitrary units) with CV values of 6% and 7%, respectively. The corresponding CV values from flow cytometric analysis of the same samples were 8% and 9%, respectively.

The green immunofluorescence, but not blue DNA fluorescence of the CLL cells, was approximately linearly dependent on drug concentration (Fig 5), as was observed for the CCRF-CEM cells (Fig 4). In the CLL cells, however, no significant correlation was observed between immunofluorescence and DNA content (Fig 3B).

Measurements of absolute levels of melphalan-DNA adducts in CCRF-CEM cells treated with a range of melphalan doses was made using the established competitive ELISA method. A linear relationship between absolute adduct measurements and quantitative immunofluorescence measurements of adducts in single cells from the same culture was demonstrated (Fig 6).

Immunofluorescence detection of melphalan-DNA adducts was demonstrated in individual PBMCs isolated from a patient 1 hour after intravenous (IV) administration of high-dose melphalan (2.8 mg/kg). Using the ×10 objective, the green adduct immunofluorescence was weaker than that observed following a 10-μg/mL exposure in the above in vitro experiments, but was clearly detected using a ×40 objective. Graphs in Fig 7 represent fluorescence intensities through areas of DNA. Adduct immunofluorescence showed marked variations between individual cells (Fig 7D, E, F, and G), as observed in the in vitro experiments. PB cells isolated from the same patient before administration of melphalan showed no detectable immunofluorescence when observed with the ×40 objective (Fig 7A, B, and C).

DISCUSSION

The ability to reliably quantify the extent to which a drug such as melphalan reacts with its molecular target in individual cells will permit a number of investigations that were previously impractical because the number of cells available were too small, those involving clinical samples being of particular interest. Single cell gel electrophoresis permits detection of DNA strand-breaks but, following treatment with alkylating agents, these are only formed as a result of events secondary to the initial drug-target interaction. Immunological staining methods currently offer the only prospect of accurate measurement of adducts in individual cells. However, such methods have not previously been described for the measurement of DNA adducts formed by any of the bifunctional alkylating agents.

Immunological quantification of drug-induced modifications of DNA in individual cells requires reliable access of antibody to the epitopes on DNA and also optimum DNA
MELPHALAN-DNA ADDUCTS IN SINGLE CELLS

The cells were then exposed for 1 hour to melphalan, from two patients with CLL were cultured overnight. The cells were then exposed for 1 hour to melphalan, embedded in agarose, processed, stained, and analyzed as described in the text. (A) Blue DNA-Hoechst dye fluorescence Patient 1 (A), Patient 2 (B). Green immunofluorescence Patient 1 (A), Patient 2 (B). Each point represents mean integrated fluorescence of 50 to 100 cells (±SE).

conformation.

Furthermore, because of the low levels of modifications at clinically relevant drug exposures, a high detection sensitivity is essential. The development of the agarose embedding immunostaining technique appears to overcome these potential problems. During the lysis step, DNA is released so as to occupy a volume larger than that occupied by the nucleus in a living or fixed cell, and most cellular proteins are removed. This, plus the lack of fixed proteins, results in the DNA being highly accessible to antibody. The lack of fixed protein also reduces the nonspecific binding of immunological reagents to an undetectable level. This makes it possible to take full advantage of the high sensitivity and photometric accuracy of fluorescence detection that is possible with the cooled slow-scan CCD camera. Lysis of living cells trapped in agarose forms the basis of electrophoretic assays of DNA damage in individual cells, but has not previously been used as a basis for immunological staining of DNA modifications. We have successfully applied this method to the detection of DNA adducts formed by platinum drugs (unpublished data, March 1993) and it should be applicable to immunological detection of other types of DNA modifications.

Using ELISA techniques, antibody MPS/73 has been shown to recognize a melphalan-DNA adduct involving guanine, and the ELISA results were shown to be directly proportional to the absolute level of melphalan-DNA adducts determined using radioactively labeled drug. Analysis of nucleotides indicated that the antibody recognized cross-linked guanine adducts much better than monofunctional adducts. However, recent data has established that monofunctional adducts are detected with high sensitivity in polymeric DNA. As expected, this antibody appears to exhibit the same specificity when used in the present fluorescent staining technique, as was seen with ELISA. Staining was dependent on denaturation of the DNA, comparable to ELISA data and fluorescence was directly proportional to ELISA data (Fig 6). Chlorambucil is structurally similar to melphalan in that in addition to the basic mustard moiety, both molecules contain an aromatic group. Preliminary ELISA data shows that, unlike antibody Amp4/42, the MPS/73 does not recognize chlorambucil-DNA adducts (unpublished data, February 1992). The possible low level of staining seen in cells treated with relatively high concentrations of chlorambucil could have been due to a low level of crossreactivity with chlorambucil adducts. This is currently being further investigated using radioactively labelled drug. The complete absence of detectable staining of cells treated with mechloethamine is consistent with the much greater difference in chemical structure between this drug and melphalan.

The possibility that the immunological reagents were binding "nonspecifically" to sites of DNA damage can be excluded because of the clear dependence of fluorescent staining on the presence of specific types of DNA adducts, as discussed above. Also, in the absence of primary antibody, the second antibody showed no staining of cells treated with the highest melphalan concentration.

The DNA adducts recognized by antibody MPS/73 were shown to be alkali-labile, however, more recently we have...
observed that at 0.1 mol/L NaOH, they are sufficiently stable to permit denaturation of the DNA to occur. Denaturation was previously shown to increase immunorecognition of the adducts on DNA that had been reacted with melphalan in pure solution. The present data indicate that, for DNA present in chromosomal sized fragments, immunorecognition is much more dependent on denaturation than was observed for DNA of lower molecular weight. This is consistent with our previous suggestion that recognition of adducts in purified native DNA was due to adducts in regions such as DNA strand ends that denature spontaneously. The sensitivity of immunofluorescence detection of melphalan-DNA adducts using antibody MPS73 was much higher than was previously reported for antibody Amp4/42, which recognizes an alkali-stabilized adduct.

An important part of this work was the development of an appropriate image analysis procedure incorporating background and shading corrections to correct images for: (1) stray and background light, (2) spatial variation in illumination intensity, and (3) spatial variation in efficiencies of light transmission and detection. To ensure that errors in fluorescence were not caused by fluorochrome bleaching, it was established that no significant fading of blue or green fluorescence occurred during the exposure times used for the quantitative data reported here (data not shown). CCRF-CEM cells treated with melphalan and stored at -80°C are now being used as standards by which the staining and fluorescence detection can be compared from day to day.

Results obtained using both the hematological tumor cell line CCRF-CEM and CLL cells from patients show a high degree of intercell heterogeneity in melphalan-DNA adduct immunofluorescence. This was also observed on cells obtained from a patient undergoing high-dose melphalan therapy. Heterogeneity in adduct staining has been reported for antibody Amp4/42, which recognizes an alkali-stabilized adduct.

The heterogeneity reported here was seen despite the DNA being free of fixed proteins, and in the cell line, it was only partly explained by variation in DNA content. Data in Fig 3B, comparisons with flow cytometry analysis, and other results (not presented) indicate that photometric errors contributed only a small proportion of the heterogeneity. Variation in adduct levels between cells could result from variation in drug uptake, intracellular drug inactivation, access of drug to DNA, or DNA repair processes. In the case of the normal PB cells, heterogeneity in these factors could be due to the presence of different cell types. PBMCs obtained from the patient receiving melphalan treatment showed a wide range of adduct levels. It will be of interest to determine the generality of this finding and whether or not adduct level is related to cell type.

The distribution of DNA fluorescence values in Fig 4A is consistent with a variation in DNA content per cell due to different positions in the cell cycle, but the numbers of cells analyzed is too small to see convincing G1 and G2 populations. The linearity of the relationship between immunofluorescence and DNA content suggests that the level of adducts expressed as adducts/g DNA does not vary through the cell cycle, although, the total number of adducts per cell is higher in G2 cells because of their greater DNA content.

Quantification of drug-DNA adducts in individual cells enables investigations into the molecular pharmacology of melphalan and other DNA damaging drugs in clinical samples, which will enhance the understanding of drug resistance mechanisms. The principles of the immunofluorescence staining technique described here could be applied to quantifying the molecular effects of related drugs such as cyclophosphamide, ifosphamide, and chlorambucil. We have described antibodies for the detection of adducts formed by cisplatin. Other antibodies that recognize alkali-stabilized melphalan-DNA adducts also recognize chlorambucil adducts (unpublished data, September 1995). Melphalan has been a convenient drug for initial study because of the extent of knowledge of the nature of its DNA adducts and because metabolic activation is not necessary for its cytotoxic activity. A number of mechanisms conferring resistance to drugs, such as melphalan, have been identified in experimental studies. However, the relevance of these to clinical tumors is unclear. Several of these mechanisms involve the reduction of access of drug to its DNA target, and it will be possible to identify the operation of these in clinical samples by measurement of drug-DNA adduct formation. A knowl-
edge of adduct levels is also of importance in interpretation of results obtained from investigations into events secondary to DNA damage, such as the changes that lead to the induction of apoptosis. The ability to assess adduct levels in single cells has two implications. First, it is now possible to study cells only obtainable in small numbers. Second, in addition to determining the average adduct level in the cell population, it will be possible to identify any subpopulations with markedly different adduct levels. The latter will be relevant to studies of CD34 positive cells, of which stem cells comprise only a subpopulation. It seems that the new opportunities to investigate the molecular pharmacology of melphalan in clinical samples that are now available should be exploited, but, as with any new technique, the interpretation of the results will be dependent on an understanding of the nature of the cells being analyzed. Therefore, it will be of considerable interest to compare the ease of melphalan-DNA adduct formation in various hematological cell types, particularly hematopoietic stem cells. Assessments will be possible on purified CD34 positive cell populations from normal marrow and peripheral stem cell harvests in addition to disease states, such as acute myeloid leukemia and the myelodysplastic syndromes. Of particular interest for our group with a program of high-dose melphalan therapy and autologous transplantation in adult acute lymphoblastic leukemia will be the experiments that will become possible to compare in vitro adduct formation and DNA repair in samples of acute lymphoblastic leukemia blasts at presentation with the view to relating these properties to long-term therapeutic outcome.

We are at present studying the formation of melphalan-DNA adducts, after ex vivo drug treatment, in bone marrow plasma cells from patients with multiple myeloma where the use of melphalan in certain patients as a single agent greatly simplifies interpretation of laboratory and clinical observations.

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