Measurement of DNA Cross-linking in Patients on Ifosfamide Therapy Using the Single Cell Gel Electrophoresis (Comet) Assay

Janet M. Hartley, Victoria J. Spanswick, Marc Gander, Giusi Giacomini, Jeremy Whelan, Robert L. Souhami, and John A. Hartley

CRC Drug-DNA Interactions Research Group, Department of Oncology, UCL Medical School, London W1P 8BT, United Kingdom [J. M. H., V. J. S., M. G., R. L. S., J. A. H.], and The Meyerstein Institute of Oncology, Middlesex Hospital, London W1N 8AA, United Kingdom [G. G., J. W.]

ABSTRACT

The single cell gel electrophoresis comet assay has become established as a sensitive technique for measuring DNA strand breaks. The technique has been modified to allow the sensitive detection and quantitation of DNA interstrand cross-linking at the single cell level. Cells are irradiated immediately before analysis to deliver a fixed level of random strand breakage. After embedding of cells in agarose and lysis, the presence of cross-links retards the electrophoretic mobility of the alkaline denatured cellular DNA. Cross-links are, therefore, quantitated as the decrease in the comet tail moment compared with irradiated controls. Using this method, a linear response of cross-linking versus dose of chlorambucil over a wide dose range was demonstrated in human lymphocytes after drug treatment ex vivo. The method was also sensitive enough to determine cross-linking in clinical samples after chemotherapy. For example, cross-linking was observed in the lymphocytes of patients receiving ifosfamide (3 g/m²/day) as a continuous infusion for 3–5 days or as a 3-h infusion daily for 3 days. Cross-links were detected in all patients within 3 h, with no evidence of DNA single strand break formation. In patients receiving continuous infusion, a plateau of cross-linking was reached after 24 h. In the patients receiving ifosfamide over 3 h, a clear decrease in the peak level of cross-linking was observed before subsequent infusions.

INTRODUCTION

Measuring DNA damage and its repair directly in clinical samples after drug treatment has, up to this time, been difficult. Using atomic absorption spectroscopy, it has been possible to explore the relationship between exposure to cisplatin, DNA adduct formation in leukocytes, and tumor response in patients with solid tumors (1). Similarly, the availability of antibodies to DNA adducts produced by platinum drugs or melphalan has allowed DNA adduct formation in leukocytes and tumor material to be studied (2-4). For many DNA damaging drugs, however, it has not been possible to relate drug pharmacokinetics or clinical outcome directly to levels of critical lesions, such as the DNA interstrand cross-links produced by bifunctional alkylating agents and platinum-based drugs (5, 6). Although sensitive methods exist for the detection of cross-links in vitro (e.g., alkaline elution (7), these methods are not generally applicable to the clinical situation. Although DNA cross-links have been detected in peripheral mononuclear cells of ovarian cancer patients during chemotherapy with cyclophosphamide/carboplatin (8) and in chronic lymphocytic leukemia cells after cyclophosphamide therapy (9) using alkaline elution, the standard technique required modification to allow fluorometric quantitation of DNA, thereby limiting sensitivity. The large number of cells required and the complexity of the procedure means that the method is not suitable for studies requiring multiple sampling.

The single cell gel electrophoresis (comet) assay has become established as a highly sensitive technique for measuring DNA strand breaks (10). The technique can be easily modified, however, to allow the sensitive detection and quantitation of DNA cross-linking at the single cell level (11). The principle of the technique is similar to that for alkaline elution. Cells are irradiated immediately before analysis to deliver a fixed level of random DNA strand breakage. After embedding of cells in agarose and lysis, the presence of cross-links retards the electrophoretic mobility of alkaline denatured cellular DNA. Cross-links are, therefore, quantitated as the decrease in comet tail moment compared with irradiated controls. The method is more sensitive than alkaline elution, requires fewer cells, and has the advantage that analysis can be made at the single cell level, allowing heterogeneity of response to be studied. In the present study, the use of the technique in the clinical situation is demonstrated by the sensitive detection of DNA cross-links in lymphocytes from patients after treatment with ifosfamide, a drug that requires metabolic activation in vivo to produce an active DNA cross-linking agent.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma Chemical Co. (Poole, United Kingdom). Chlorambucil was dis-
solved in DMSO and further diluted in RPMI 1640 so that the final solvent concentration added was not >0.01%.

**Patient Population.** All patients studied had Ewing’s sarcoma and had received previous chemotherapy at least 3 weeks before the treatment. When studied, patients received single agent ifosfamide as a continuous 3- or 1-h infusion at a dose of 3 g/m²/day for 3–5 days and mesna at an equivalent dose as uroprotector. Blood samples were collected before and at timed intervals throughout treatment, with informed consent. The protocol had the approval of the local ethics committee.

**Preparation of Lymphocytes.** Blood samples were collected from patients and healthy volunteers into 8-ml Vacutainers CPT tubes containing sodium citrate as anticoagulant (Becton Dickinson). After collection, the tubes were immediately centrifuged at 1800 × g, 24°C for 20 min, and the lymphocytes were harvested, washed, resuspended in RPMI 1640 with 10% DMSO and 10% FCS at ~2–3 × 10⁷/ml and frozen in 1-ml aliquots at -80°C until assayed.

**Treatment of Lymphocytes Ex Vivo.** Lymphocytes from healthy volunteers were diluted to 2–3 × 10⁷/ml with RPMI 1640 containing 10% FCS and 2 mm glutamine, then incubated with chlorambucil for 1 h at 37°C in a humidified atmosphere with 5% carbon dioxide. After drug treatment, lymphocytes were washed by centrifugation at 200 × g for 5 min and resuspended in drug-free medium. Lymphocytes were then incubated for an additional 3 h, under the conditions described above.

**Determination of DNA Interstrand Cross-linking.** All of the procedures were carried out on ice and in subdued lighting. Cells were diluted to give 2–3 × 10⁷/ml in RPMI 1640

![Fig. 1 Typical comet images from lymphocytes treated ex vivo with chlorambucil. Drug treatment was for 1 h, and cells were incubated for an additional 3 h in drug-free medium. a, no drug, no irradiation. b, no drug, 10Gy irradiation. c, 90 μM chlorambucil, no irradiation. d, 90 μM chlorambucil, 10Gy irradiation.](image-url)
and aliquoted into two tubes for each time or dose point. One tube was irradiated with 10Gy using an X-ray source (dose rate, 2.35 Gy/min) and the other tube was unirradiated. Cell suspension (0.5 ml) was mixed with 1 ml of 1% low gelling temperature agarose (type VII) at 40°C. This was then pipetted onto a precoated microscope slide and covered with a coverslip (24 × 30 mm) to give a uniform gel. [Precoated slides used in the assay were prepared a day in advance by pipetting 1 ml of 1% agarose (type 1-A) in distilled water onto a single frosted glass slide, placing a coverslip on top, and removing it when the agarose had set]. Duplicate slides were prepared for each treatment. When the low gelling temperature agarose had set, the coverslips were removed and the slides submerged in ice-cold lysis buffer [2.5 M NaCl, 100 mM EDTA, and 10 mM Tris HCl (pH 10.5–11.5) containing 1% TritonX-100, added just before use] for 1 h. After lysis, the slides were washed for 1 h using four changes of distilled water. Slides were arranged lengthwise in an electrophoresis tank and submerged in alkali buffer [50 mM NaOH and 1 mM EDTA (pH 12–12.5)] for 45 min.

Slides were then electrophoresed for 25 min at 18V (0.6V/cm), 250 mA. The slides were removed and washed with neutralization buffer [0.5 M Tris HCl (pH 7.5)] for 10 min, followed by a PBS wash. The slides were then left to air dry overnight at room temperature. To stain the slides, they were first rehydrated with distilled water for 30 min, then flooded with 2.5 µg/ml propidium iodide for 20–30 min and destained for an additional 30 min in water. Slides were dried and stored until visualization. To view the slides, a drop of distilled water was placed on the slide and covered with a coverslip. Images were visualized using a NIKON inverted microscope DIAPHOT model TMD, with high pressure mercury light source and a 580 nm dichromic mirror, 510–560 nm excitation filter, and 590 nm barrier filter at ×20 magnification. Images were captured using an on-line CCD camera and analyzed using Komet assay software (Kinetic Imaging). For each duplicate slide, 25 images were analyzed. The tail moment for each image was defined as the product of the percentage in the comet tail, and the distance between the means of the head and tail distributions, based on the definition by Olive et al. (12). Cross-linking was expressed as the percentage decrease in tail moment calculated by the formula:

\[
\% \text{ decrease in tail moment} = \left[ 1 - \frac{\text{TM}_{\text{di}} - \text{TM}_{\text{cu}}}{\text{TM}_{\text{ci}} - \text{TM}_{\text{cu}}} \right] \times 100
\]

where \(\text{TM}_{\text{di}}\) = tail moment of drug–treated irradiated sample.
\(\text{TM}_{\text{cu}}\) = tail moment of untreated, unirradiated control.
\(\text{TM}_{\text{ci}}\) = tail moment of untreated, irradiated control.
The method has recently been described in detail (11).

RESULTS

The sensitivity and reproducibility of the comet assay for quantitating DNA interstrand cross-linking in human lymphocytes was initially determined \textit{ex vivo}. For these studies, the nitrogen mustard chlorambucil was used because it is widely used clinically and does not require metabolic activation. Isolated lymphocytes were exposed to chlorambucil for 1 h and analyzed after an additional 3-h incubation in drug-free medium to allow time for peak cross-link formation. Typical comet images are shown in Fig. 1. After irradiation of nondonor drug-treated control lymphocytes (10Gy), distinct comets were observed (Fig. 1b) compared with unirradiated controls (Fig. 1a). After treatment with 90 µM chlorambucil, no DNA single strand breaks were observed in unirradiated samples (Fig. 1c). After irradiation of drug-treated cells, a comet tail was visible (Fig. 1d), but this was decreased in length and intensity compared with the control irradiated cells (Fig. 1b). A linear decrease in tail moment with increasing dose of chlorambucil is observed in a lymphocyte population from a single individual, as a result of...
cross-link formation (Fig. 2a). The variability of cross-linking response in lymphocytes from three healthy volunteers was small (Fig. 2b). To make comparisons between different individuals, the data are expressed as the percentage decrease in tail moment compared with controls from the same individual.

DNA cross-linking was measured in patients receiving the bifunctional alkylating agent ifosfamide as a single agent. Before chemotherapy, lymphocytes from the patients gave typical comet images without irradiation (Fig. 3a) and after irradiation (Fig. 3b). After ifosfamide treatment, no significant level of DNA single strand breakage was observed in unirradiated lymphocyte samples (Fig. 3c). Clear evidence of DNA cross-linking was observed in irradiated samples during ifosfamide therapy (Fig. 3d). Detailed profiles of DNA interstrand cross-link formation were obtained from several patients. Data are expressed as percentage decrease in tail moment calculated from matched samples from the same patient before treatment. Three examples are shown in Fig. 4. In each example, patients received the drug at 3 g/m²/day. This was either as a continuous infusion over 5 days (Fig. 4a), over 3 days (Fig. 4b), or over 3 h on 3 consecutive days (Fig. 4c). In 10 patients thus far studied, extensive DNA cross-linking has been observed in the absence of significant DNA strand breakage. Cross-links were detectable within 3 h, and in patients receiving the drug as a continuous infusion a peak level of cross-link formation is reached within 24 h, which persists throughout the remainder of the infusion (Fig. 4, a and b). At a dose of 3 g/m²/day, the extent of cross-linking results in >50% decrease in comet tail moment. In patients receiving drug over 3 h (Fig. 4c), a clear decrease in the peak level of cross-linking is observed before subsequent treatments.

Fig. 3 Typical comet images from lymphocytes taken from a patient on ifosfamide therapy. a, before therapy, no irradiation. b, before therapy, 10Gy irradiation. c, 3 g/m² ifosfamide, 1-h infusion, sample at 15 h, no irradiation; d, 3 g/m² ifosfamide, 1-h infusion, sample at 15 h, 10Gy irradiation. The percentage decrease in tail moment for this sample was 70%.
DISCUSSION

The single cell comet assay, after irradiation of cells, allows the sensitive detection of DNA interstrand cross-linking in human lymphocytes after treatment with bifunctional chemotherapeutic agents. The sensitivity of the comet assay for quantitating DNA interstrand cross-linking was initially determined using human leukemic K562 cells in culture by direct comparison with the well established method of alkaline elution (7). After exposure to chlorambucil for 1 h, the peak of cross-linking was found by both assays to occur after an additional 3-h incubation in drug-free medium, and the cross-links were repaired within 48 h (data not shown). Using the alkaline elution method, cross-links were only detectable at doses at or above the IC50 value (30 μM), as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Using the comet assay, cross-linking was clearly evident at doses one-tenth of the IC50. In addition, the comet assay required far fewer cells for analysis than alkaline elution. It can be seen in the present study that the assay is reproducible and can measure DNA interstrand cross-linking in lymphocytes at pharmacologically relevant doses. Because of the small number of cells required, and the sensitivity and reproducibility of the technique, detailed time courses of the formation and removal of cross-links can be obtained easily.

In 10 patients treated with ifosfamide, studied to date, cross-linking has been easily detected in lymphocytes. Because ifosfamide requires metabolic activation to an active cross-linking agent (13), significant levels of active metabolite must be present in the circulation. Indeed, treatment of lymphocytes ex vivo produced no detectable cross-linking (data not shown). Cross-links were detected, however, when lymphocytes were treated ex vivo with isophosphoramide mustard, the ultimate alkylating species produced by the metabolism of ifosfamide (data not shown). Treatment with 4-hydroxyifosfamide produced a high level of DNA strand breakage, presumably as a result of its breakdown to produce acrolein, in addition to isophosphoramide mustard. Acrolein has been shown previously to produce DNA single strand breaks (14). Strand breaks interfere with the accurate measurement of DNA cross-linking in the comet assay. Strand breaks were, however, not detectable in the lymphocytes of ifosfamide-treated patients, presumably due to the coadministration of the uroprotector Mesna, which inactivates the acrolein produced (15).

In two patients receiving ifosfamide as a continuous infusion, levels of DNA cross-links reached a plateau within 24 h, which remained throughout the rest of the infusion (Fig. 4, a and b). Previous pharmacokinetic studies have shown that metabolites reach a plateau at around 24 h and remain near this level for the duration of the treatment (16, 17). In contrast, after a short infusion, metabolite levels reach a peak, but then decrease before subsequent infusions (17, 18). In the present study, DNA cross-linking levels followed a similar pattern in a patient who received ifosfamide as a 3-h infusion daily (Fig. 4c) and has also been observed after 1-h infusions daily (data not shown).

The single cell gel electrophoresis (comet) assay, adapted to measure DNA cross-linking, provides for the first time a powerful technique for the routine detection of critical lesions produced by bifunctional chemotherapeutic agents in the clinical setting. The small sample size makes the assay amenable for multiple sampling analysis of DNA damage in conjunction with pharmacokinetic studies and to measure the formation of the critical DNA lesion in Phase I/II studies of novel DNA cross-
linking agents. The method is applicable to any tissue from which a single cell population can be obtained. Clearly analysis of DNA cross-linking directly in tumor material would be of most relevance in the clinical setting. Measurement of DNA strand breaks using the comet assay has been achieved in animal tumor models (19). The method, adapted to measure DNA cross-linking, is presently being evaluated for the analysis of human tumor material.

REFERENCES


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