Lack of interference of DNA single-strand breaks with the measurement of double-strand breaks in mammalian cells using the neutral filter elution assay

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ABSTRACT

In this study, the effect of DNA single strand breaks (ssb) on the neutral (pH 9.6) filter elution of DNA from Chinese hamster ovary (CHO K1) cells containing DNA double strand breaks (dsb) was investigated. Protein associated ssb were induced by the inhibition of DNA topoisomerase I with camptothecin (cpt). Protein associated dsb were introduced by treating cells with the DNA topoisomerase II poison; etoposide (VP-16). Protein associated ssb and dsb were converted to ssb and dsb by proteinase K present in the lysis solution. In some experiments dsb were generated by the restriction endonuclease *Pvu* II. It was found that elution of DNA in the presence and absence of ssb was similar under neutral conditions. This finding is consistent with the view that the fast component of the bi-phasic repair kinetics observed in irradiated mammalian cells with the neutral filter elution technique is not attributable to the interference of ssb with the measurement of dsb, and thus suggests that the two components of repair observed with the neutral filter elution technique may represent two different types of dsb or modes of repair of dsb.

INTRODUCTION

The frequency of double-strand breaks (dsb) induced in the DNA of mammalian cells by a given dose of ionizing radiation can be measured by the neutral velocity sedimentation (NVS) technique which gives an absolute estimate of the frequency per unit mass of DNA (1). However, more recently, many investigators have used the neutral filter elution (NFE) technique (2), which offers technical advantages and simplicity of data analysis, but gives only a relative measure of dsb frequency. Unfortunately, the two methods can yield different results (3). Whereas the NVS technique shows that dsb in mammalian cells are induced linearly with dose (1) NFE usually shows a curvilinear dose response relationship, with an initial threshold region (4) although there are exceptions to this (5). In addition, the kinetics of repair of dsb with the two techniques are sometimes found to be different. With NVS a first-order disappearance of breaks with time after radiation exposure has been reported (6, 7) whereas the kinetics of dsb repair measured by the NFE method show a rapid initial component with a repair half-time

($t_{1/2}$) of 5–15 minutes and a slower component with a $t_{1/2}$ of 1–4 Hrs. (8, 9, 10). The question arises as to whether these biphasic repair kinetics result from differences in the types of dsb measured (as discussed by Hutchinson (1989) (3) and Prise et. al. (1987) (11)), or from the interference of a second type of DNA lesion, such as the single-strand break (ssb) with the elution of DNA containing dsb. It seems likely that the non-linear kinetics of dsb induction could result from a technical artefact since it has been shown that the size of the 'threshold' on the NFE dose-response curve is strongly dependant on lysis conditions (12). Dsb repair kinetics measured by neutral elution are reminiscent of ssb repair, and therefore it, has been suggested (13) that the kinetics of dsb repair measured by NFE could be influenced by the presence of repair of ssb. There are two possible ways in which ssb could interfere with the NFE assay of dsb. The first is that during elution the DNA could rupture at sites of ssb, converting them into dsb; and the second is that the presence of ssb in DNA would render the molecules more flexible and therefore more easily eluted through holes in the filter (a snake versus a stick might be a useful analogy to describe the properties of unrepaired and repaired DNA molecules respectively). That the elution of DNA, measured by the NFE technique, results from the presence of dsb in the DNA is clear from original experiments of Bradley and Kohn (1979) (2) in which they treated DNA on filters with restriction endonucleases. Some evidence also already exists which suggests that ssb do not contribute to the elution of DNA from filters at pH 9.6. DNA from cells treated with hydrogen peroxide (which induces principally ssb in DNA) did not elute at a significantly higher rate at pH 9.6 than DNA from untreated cells (2, 14).

We have further examined this question by specifically inducing dsb or ssb, separately or together, using the mammalian topoisomerase inhibitors camptothecin (cpt) and etoposide (VP-16). Topoisomerases are enzymes involved in the alteration of the conformation and topology of cellular DNA by the production of transient protein-bridged breaks in either one (type I topoisomerase) or both DNA strands (type II topoisomerase). Topoisomerase I cleaves DNA, primarily, on the transcribed strand in transcribed regions (15), whereas topoisomerase II cleaves DNA at nuclear matrix attachment sites which are predominantly non-transcribed and has a major role in replication of DNA (16). Camptothecin is a specific topoisomerase-I inhibitor, generating protein associated ssb. VP-16 specifically inhibits topoisomerase-II, generating protein

associated staggered (four base pairs) dsb (17). These protein associated strand breaks can be converted to apparent strand breaks by enzymic proteolysis or denaturation by detergents. Dsb were also introduced into DNA following cell lysis on the filters using the restriction endonuclease *Pvu* II to give blunt dsb. We examined the extent of elution produced by these agents using the NFE technique at pH 9.6 and by cpt alone using the alkaline elution technique (pH. 12.1).

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary (CHO K1) cells were used for all experiments. Cells were grown as mono-layers on plastic tissue-culture flasks in Eagle's minimal essential medium (supplemented with 10 per cent calf serum containing 100 μ M FeCl₃ to saturate transferrin, penicillin (50 units/ml) and streptomycin (50 mg/ml). All incubations were at 37°C, in an atmosphere of 5 per cent CO₂ and 95 per cent air except where stated. Exponentially growing cells (doubling time 12 hours) were obtained by growing 5×10^5 cells in 75 cm² tissue culture flasks (Sterilin) in 15 ml of medium for 48 hours to give approximately 4.5 to 5.0×10^6 cells per flask.

DNA labelling

Two days prior to the experiment, flasks containing approximately 5×10^5 cells were labelled with 3.7 kBq/ml [methyl-³H]-thymidine (1.59 TBq/mmol). 1 μ M cold thymidine was also added to produce even labelling of DNA.

Drug treatment

Camptothecin (Sigma) was dissolved in dimethylsulphoxide (DMSO) to produce a 1 mM stock solution, which was stored at -20°C in 0.5 ml aliquots until immediately before use. VP-16 (Bristol-Myers) was received as a gift from Dr. K. Caldecott at the National Institute for Medical Research, Mill Hill, London. It was dissolved in DMSO at a concentration of 34mM (20mg/ml) and for the purposes of the experiments further diluted to 1mM in DMSO and stored at -20°C. The drugs were added to between 0.3 and 100 μ M for CPT and between 2.5 and 20 μ M for VP-16. The maximum concentration of DMSO in medium that cells were exposed to was 10% (v/v), with the usual range <1% (v/v). Treatment of cells with CPT and VP-16 was as follows.

0.1 ml of medium containing appropriate amounts of CPT and/or VP-16 was placed in 2 ml Eppendorf tubes, the tubes gassed and sealed. 5×10^5 cells were added to the Eppendorfs containing various drug dilutions and the volume made up to 1 ml. The contents of the Eppendorfs were then immediately transferred to Swinnex filter units (Millipore Corporation), containing polycarbonate filters (Nucleopore Corporation, 45 mm diameter and 2 μ m pore size) pre-moistened with phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1mM KH₂PO₄) and incubated 20 minutes. The entire filter units were briefly gassed with CO₂-air mixture, sealed and incubated for 1 hour at 37°C.

Filter elution

The filter elution procedure was essentially that described by Bradley and Kohn (2) for neutral elution, and by Kohn et. al. (18) for alkaline elution.

After incubation the filter units were connected up to 50ml syringe barrels, acting as reservoirs, and a peristaltic pump. 20 ml of ice-cold PBS was added to the reservoirs and the pump was run until all air bubbles were removed from the system and flow could continue under gravity. All of the PBS in the reservoirs was then allowed to drain out.

Lysis and deproteinisation

The lysis procedure followed the protocol of Okayasu and Iliakis (19). Once all PBS had drained from the system under gravity, the Swinnex filter units were clamped off and disconnected from the pump and the reservoirs. 1 ml of lysis solution comprising 0.025 M Na₂EDTA, 0.1 M glycine, 0.068 M sodium N-lauroylsarcosine (Na-NLS) adjusted to pH 9.6 with 10N NaOH and containing 0.5 mg/ml proteinase K (Sigma) added freshly, was pipetted into the filter unit ensuring the whole surface of the filter was covered. Filter units were then capped to prevent evaporation and the units incubated at 60°C for 1 hour.

Restriction enzyme digestion

In some experiments, following cell lysis, DNA was cleaved using the restriction enzyme *Pvu* II (Northumbria Biologicals) using a method adapted from Bradley and Kohn (2). After lysis, the filter units were reconnected to the pump and reservoirs. The filters were washed with 10 ml of washing buffer A (20 mM tris-HCl, 20 mM Na₂EDTA adjusted to pH 8.0 using 5N HCl.), followed by 5 ml of washing buffer B (10 mM tris-HCl, 60 mM KCl, 10 mM MgCl₂ adjusted to pH 8.0 with 5N HCl). Just before the last of the washing buffer B ran out of the reservoir 1 ml of reaction buffer (6 mM tris-HCl, pH 7.5, 60 mM KCl, 6 mM MgCl₂, 6 mM β -mercaptoethanol) containing various dilutions of *Pvu* II was placed in the reservoir and allowed to

Figure 1. Dose response curves for CHO K1 cells incubated with various concentrations of camptothecin for one hour at 37°C and the released DNA eluted under neutral (pH 9.6) or alkaline (pH 12.1) conditions. Plotted as the fraction of radioactivity (representing DNA) eluted from the filter after 16 hours elution with increasing concentration of camptothecin. Points were adjusted for background elution levels. Error bars represent SEM of 2-5 experiments. Data for neutral filter elution were taken from a single representative experiment.

flow onto the filter under gravity. When all of the restriction enzyme solution was on the filter the units were clamped off, disconnected from the pump and the reservoirs, capped, and were incubated at 37°C for 20 minutes.

After restriction enzyme digestion and/or lysis and proteinase K digestion, the filter units were reconnected to the pump and to the 50 ml reservoirs and 40 ml of elution buffer ('neutral'; 20 mM Na₂EDTA adjusted to pH 9.6 using 20% tetra-propyl ammonium hydroxide(TPAH), 'alkaline'; 20 mM Na₂EDTA, 0.1% sodium dodecyl sulphate adjusted to pH 12.1 using TPAH) added to the reservoir. The filter units were unclamped and the pump run at 2.6 ml/hr for 15–16 hours. Fractions were collected in 20 ml plastic scintillation vials (Packard). The first eluted fraction was collected over a period of one hour (2.6 ml) except in the restriction enzyme experiments. This initial fraction was not included in the results, since this fraction contains the cell lysate containing any free tritiated thymidine. For restriction enzyme treatments the cellular lysate was already removed during the washing procedure. Five fractions were subsequently collected for three hours each (7.8 ml).

Scintillation counting

Fractions were counted in a scintillation counter using a quench correcting programme, after mixing with Optiphase MP (LKB). 5 ml was added to the 1 hour fractions, 10 ml to the 3 hour fractions. The filters were removed from the units and placed in plastic scintillation vials. 5 ml of filter count (Packard) was added and the vials thoroughly vortexed for 1 minute before counting. Normalisation of results from separate experiments was accomplished by summing the radioactivity eluted from the filter after each time period and calculating the total fraction eluted.

Figure 2. Neutral filter elution dose response curves for CHO K1 cells incubated for one hour at 37°C with VP-16 in the presence or absence of 0.5µM camptothecin. Results are plotted as the fraction of radioactivity (representing DNA) eluted from the filter after 16 hours elution with increasing concentration of VP-16. Points were adjusted for background elution levels. Error bars, where given, show SEM from 2–3 independent experiments. Data points with no error bars represent results from a single experiment.

RESULTS AND DISCUSSION

The dose response curves for CHO cells treated for one hour at 37°C with varying doses of camptothecin and eluted under 'alkaline' (pH 12.1) or 'neutral' (pH 9.6) conditions are shown in figure 1. Under alkaline conditions extensive elution was recorded, as opposed to neutral conditions where very little occurred. For further experiments the dose of cpt applied to the cells was kept at 0.5µM (X-ray dose equivalent approximately 5 Gy, data not shown).

In figure 2 the results are shown of experiments in which cpt (0.5µM) was added to cells with VP-16 for 1 hour and the DNA eluted under 'neutral' (pH 9.6) conditions. The results show that cpt (inducing ssb) had no significant effect on the elution of DNA damaged by VP-16 (inducing dsb). Alkaline filter elution indicated that cpt action was only partially inhibited by the presence of VP-16 (data not shown). In figure 3 we show results of treatment of cells with cpt followed by treatment of released DNA on the filter with the restriction endonuclease *Pvu* II. Again there was no significant difference in the elution of DNA following *Pvu* II treatment between cells treated with or without cpt. This again verifies that the presence of ssb in the DNA does not affect the rate nor the extent of elution of DNA containing dsb from the filter under non-denaturing conditions. This work supports that of Prise et al (14) who found that ssb induced by H₂O₂ did not affect the elution of DNA from filters at pH 9.6.

The lack of an effect of ssb induced by 0.5 µM camptothecin on elution of DNA from cells treated simultaneously with VP-16, or on the subsequent elution of DNA treated on filters with *Pvu* II, suggests that the neutral elution technique may genuinely measure dsb and that the fast component of dsb repair measured by neutral filter elution can not be attributed to ssb repair.

On this basis it seems possible that the biphasic repair kinetics observed by neutral elution in irradiated cells represents the repair

Figure 3. Neutral filter elution dose response curves for CHO K1 cells treated with or without 0.5µM camptothecin for one hour at 37°C and subsequent treatment of released DNA on the filter for one hour at 37°C with *Pvu* II. Results are plotted as the fraction of radioactivity (representing DNA) eluted from the filter after 15 hours elution with increasing concentration of *Pvu* II. Points were adjusted for background levels. Error bars show the SEM for 2–6 independent experiments.

of more than one type of dsb. It is known that x-irradiation leads to the formation of a spectrum of lesions including sugar-phosphate bond cleavage (as in the lesions produced in this study), which might be considered as ligatable lesions, and more complex sugar and base lesions which may require excision or recombinogenic repair processes (8). An additional possibility is that the fast repairing component represents dsb with cohesive termini. We have already suggested (20, 21) that this type of dsb is repaired by a single strand repair mechanism; i.e. the two ssb separated by four bases comprising the dsb would be repaired sequentially. Such a rapid repair of cohesive ended dsb would explain the very low efficiency of restriction endonucleases such as *Bam* H1 and *Eco* R1 in inducing chromosomal aberrations (20, 22, 23). We have moreover shown (24) that whereas dsb induced by Pvu-II (blunt-ended) accumulate with time in electroporated CHO cells, presumably due to a competition between incision and repair, dsb induced in cells by *Eco* R1 and *Bam* H1 (25) do not accumulate, presumably because they are repaired very rapidly.

However, the main conclusions from this study are that the presence of ssb in the DNA during filter elution do not lead to additional dsb by shear at the pore site, nor changes in flexibility of the DNA such that the rate of elution from the filter is enhanced. We conclude from our results that neutral filter elution is a valid method for the measurement of dsb in DNA of cells treated with clastogenic agents.

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