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COMPARISON OF DNA STRAND BREAK INDUCTION IN CHO CELLS MEASURED BY ALKALINE ELUTION AND BY FLUOROMETRIC ANALYSIS OF DNA UNWINDING (FADU)

CHRISTA BAUMSTARK-KHAN, UTA GRIESENBACH and HERMANN RINK

Radiologische Universitätsklinik, Experimentelle Radiologie und Strahlenbiologie, Sigmund-Freud-Str. 15, D-5300 Bonn 1, Germany

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DNA damage in X-irradiated CHO cells was measured by alkaline filter elution and compared to fluorometric analysis of DNA unwinding (FADU). The FADU method proved to be as sensitive as the alkaline filter elution technique in detecting X-ray induced DNA breaks. Strand break induction was also measured after treatment with four radical generating chemicals (hydrogen peroxide, bleomycin, mitomycin C and methyl viologen) using the FADU technique.

KEY WORDS: CHO cells, alkaline elution technique, fluorometric analysis of DNA unwinding, X-irradiation, hydrogen peroxide, bleomycin, mitomycin C, methyl viologen

1. INTRODUCTION

There is a growing number of chemicals producing reactive oxygen species (hydrogen peroxide, superoxide and hydroxyl free radicals) which have been shown to induce DNA damage *in vitro*.[1,2] DNA strand breaks are considered the most important lesions, primarily responsible for induction of mutations, chromosome aberrations and cell death.[3] To asses the role of this type of lesion after exposure to enviromental carcinogens, X-ray treatment of chemotherapy of tumors, it is necessary to have methods at hand which measure DNA strand breaks quickly and reproducibly.

Several methods for quantitative measurements of DNA strand breaks in bacteria, yeasts and mammalian cells have been described. All these methods are based on the fact that double-stranded DNA is denatured in alkaline solutions. The extent of denaturation depends on the experimental conditions: (a) under stringent alkaline conditions, DNA unwinding is complete and the reduction of molecular length reflects the amount of all induced DNA strand breaks, (b) under moderately alkaline conditions DNA unwinding is a time-dependent process starting from free DNA ends (DNA single- or double-strand breaks) which are enumerated in cells having been exposed to DNA-reactive agents.

Complete DNA denaturation is required for velocity sedimentation after lysing protoplasts directly on top of an alkaline sucrose gradient[4] as well as for the alkaline elution assay.[5] With both methods the decrease in DNA length as a result from chemical or radiation exposure can be estimated. The time-dependent transformation of duplex DNA to the single-stranded form under moderately alkaline conditions is

the base for DNA unwinding.[6-10] Quantification of double-stranded DNA can either be performed by fluorescence measurements (FADU) or by scintillation counting of ^{3}H-thymidine in fractions of double-stranded and single-stranded DNA after hydroxyapatite chromatography. Ahnström and coworkers[6,7] showed comparable results for DNA unwinding followed by hydroxyapatite chromatography and for velocity sedimentation of X-irradiated V 79 cells. Kanter and Schwartz[10] compared results obtained by hydroxyapatite chromatography and by FADU measurements in L1210 cells.

In the present study we induced DNA strand breaks in DNA of CHO cells by X-irradiation and compared the results obtained by alkaline filter elution with the results from fluorometric analysis of DNA unwinding. Additionally DNA strand breaks in CHO cells exposed to hydrogen peroxide, bleomycin, mitomycin C and methyl viologen (1,1′-dimethyl-4-4′-bipyridinium dichloride, paraquat) were measured by fluorometric analysis of DNA unwinding.

2. MATERIALS AND METHODS

2.1. *Cell Line and Growth Conditions*

Chinese hamster ovary cells (CHO) were cultured under a standard set of conditions in Eagle's Minimal Essential Medium (MEM, Boehringer, 209996) supplemented with 15% foetal calf serum (FCS, Biochrom KG, S-0115), glutamine (0.292 μg ml^{-1}) and pyruvate (0.11 μg ml^{-1}) buffered with sodium hydrogencarbonate. Cultures were maintained in 95% air, 5% CO_2 at 37°C.

Cells were harvested from plateau phase cultures by trypsinization (0.5 mg ml^{-1}) at 37°C. After counting, cells were inoculated with a density of 1.0×10^4 cells per cm^2 in 5 ml complete medium (MEM + 15% FCS) in 25 cm^2 flasks or petri dishes (60 mm). Medium was changed after two days and confluent cell layers were obtained within four days.

2.2. *Irradiation Conditions and Chemical Treatments*

Confluent cell cultures were used. For X-irradiation, cells were exposed on ice to X-rays generated by a X-ray unit (Müller) operated at 200 kV, 20 mA and a 0.5 mm copper filter yielding a dose rate of 2 Gy min^{-1} (focus-object distance: 312 mm). Chemical treatment was done by direct addition of concentrated drug solutions into the medium. After an exposure period of 30 min on ice the plates were washed twice with PBS. The test substances were obtained from Mack (Illertissen, Germany: bleomycin), Sigma (Deisenhofen, Germany: mitomycin C, methyl viologen) and Merck (Darmstadt, Germany: hydrogen peroxide).

2.3. *Detection of DNA Strand Breaks by Alakline Elution*

DNA strand breaks were measured by a modification of the alkaline elution method.[5] Exponentially growing cells were labelled with 1.85×10^4 Bq ml^{-1} [methyl-^{3}H] thymidine (Amersham TRK 120, 185 GBq mmol^{-1}). After 24 h the radioactive medium was discarded, cells were washed twice with PBS and incubated for an additional period of 12 h in non-radioactive medium.

Cells were layered onto 25 mm (0.22 μm pore size) polyvinylchloride filters (Millipore

GVLP 25) immediately after X-irradiation, washed twice with ice-cold phosphate buffered saline, and lysed on the filters at room temperature for 60 min with 2.5 ml of lysis buffer (40 mmol l^{-1} H_4EDTA, 2 mol l^{-1} NaCl, 7 mmol l^{-1} N-lauroylsarcosine, pH 10.0), followed by washing with 5 ml of 0.02 mol l^{-1} Na_2EDTA, pH 10.0. Alkaline elution was performed afterwards with continuous pumping (flow rate of 0.05 ml min^{-1} with fractions collected at 30-min intervals) using elution buffer (0.02 mol l^{-1} H_4EDTA, adjusted to pH 12.4 with tetrapropylammonium hydroxide). Elution was finished after collection of 10 fractions per column. After neutralization the radioactivities of all samples and of the removed filters were measured by addition of 10 ml scintillation cocktail (Quickszint 2000, Zinsser) in a LS 7500 (Beckman) liquid scintillation counter, programmed for automatic quench correction.

The amounts of DNA retained on the filter were calculated for each fraction from radioactivities in the samples and the filter. With these values elution profile curves were established for all columns. The fractions of non-elutable DNA (F), which are used for calculating dose effect curves correspond to DNA retained on the filters for fraction 5 (after 7.5 ml elution).

2.4 *Detection of DNA Strand Breaks by Fluorometric Analysis of DNA Unwinding (FADU)*

To quantify DNA strand breaks by the fluorometric procedure, cells were removed from the culture plates by cold trypsinization (trypsine 0.2 g l^{-1}, NaCl 135 mmol l^{-1}, KCl 3 mmol l^{-1}, Na_2HPO_5 5 mmol l^{-1}, KH_2PO_4 1 mmol l^{-1}, trishydroxyaminomethane 5 mmol l^{-1}, Na_2EDTA 0.45 mmol l^{-1}). Suspended cells (750 μl) were transferred into ice-cold plastic tubes (triplicate samples). The samples were grouped in three categories, which were treated in different ways:

- samples B (background): the contribution to fluorescence by components other than double-stranded DNA (including unbound dye) is estimated from these samples, in which the cell extract is sonicated under alkaline conditions in order to cause complete DNA unwinding.
- samples T (total): the contribution to fluorescence by double-stranded DNA plus contaminations (including unbound dye) is estimated from these samples, in which the cell extract is sonicated under neutral conditions in order to prevent unwinding of the DNA. The difference (T − B) provides an estimate of the amount of double-stranded DNA in the cell extracts.
- Samples P (partial denaturation): these samples which received drug treatment or X-irradiation are used to estimate the unwinding of damaged DNA under alkaline conditions. The fluorescence (P − B) provides an estimate of the amount of DNA remaining double-stranded and thus the amount of induced DNA damage.

Suspended cells (750 μl) of all sample categories (B, T and P) were lysed by the addition of 750 μl of NaOH (0.1 mol l^{-1}). Samples T were neutralized immediately by addition of 750 μl of HCl (0.1 mol l^{-1}). Samples B were sonicated immediately (Braun Labsonic 1510, Melsungen, Germany, 100 W, 15 s). All samples (T, B and P were incubated at 20°C (30 min, if not indicated otherwise). During this unwinding period the tubes must be kept vibration-free in the dark in order to avoid artifically induced strand breaks. After DNA unwinding, the samples B and P were also neutralized by addition of 750 μl HCl (0.1 mol l^{-1}). Samples T and P were also sonicated in order to

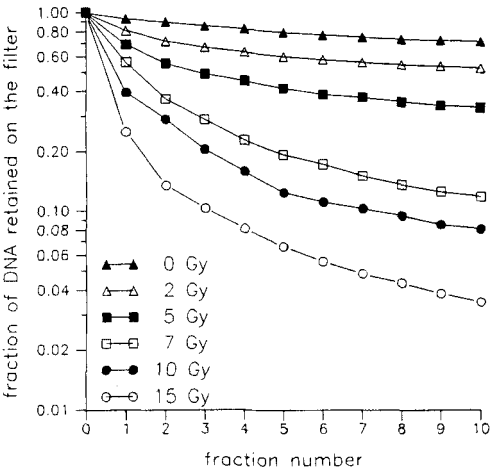

FIGURE 1 Alkaline elution of DNA from polyvinylchloride filters. CHO cells prelabelled with [^{3}H]dTd were lysed on the filters and eluted immediately after X-irradiation with varying doses. 1.5 ml fractions were collected at 30 min intervals. The fractions of DNA remaining on the filter at the time of fraction collection was calculated as described in materials and methods.

prevent renaturation. 750 μl fluorochrome containing buffer (1.25 μmol l^{-1} bisbenzamide in 0.15 mol l^{-1} phosphate buffer, pH 7.2) was added to all samples. After mixing, relative fluorescence intensities were read in a spectrofluorimeter (Hitachi) operating at 355 nm (excitation) and 450 nm (emission).

After alkali treatment the fraction of double-stranded DNA (F) was calculated as $F = (P - B)/(T - B)$, where T, P and B are fluorescence intensities of T, P and B samples, respectively.

2.5. *Calculations of Dose-effect Curves*

Data from the fractions of non-elutable DNA (alkaline elution) or fractions of double-stranded DNA (FADU) were used for calculations after standardization ($F_D/F_{D=0}$, where F_D and $F_{D=0}$ were the DNA fractions of treated and untreated cells, respectively). Resulting data are expressed as strand scission factor (SSF) which is defined as $-\ln F_D/F_{D=0}$. The dose effect curves were fitted by least squares linear regression analysis of SSF versus X-ray dose or versus drug concentration.

3. RESULTS

The alkaline elution assay for the detection of DNA strand breaks is based on the rate at which single-stranded DNA elutes through an inert filter membrane under stringent denaturing conditions. Figure 1 shows selected elution profiles for X-irradiated samples. The elution rate decreases exponentially with elution time (fraction number) as described in Ref. 5.

The FADU technique is based on time-dependent alkaline denaturation of DNA under moderate denaturing conditions. Aliquots of cells (1×10^6 cells) were lysed for different time periods in alkaline solution (pH 12.4). During this period unwinding

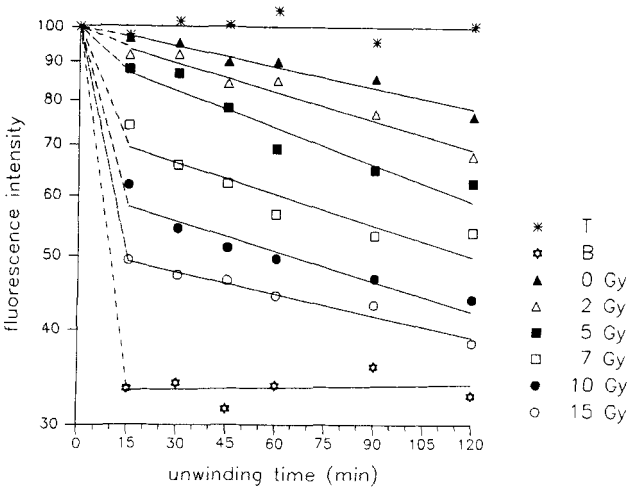

FIGURE 2 Kinetics of DNA unwinding in alkaline solution. Cells were lysed immediately after X-irradiation with varying doses. Fluorescence intensities of bisbenzamide/DNA were measured after different unwinding times for B-, T- and P-samples.

started at the ends of strands or unwinding units. This process was stopped by addition of HCl (final pH of 7.1–7.4) and renaturation was prevented by sonication. The fluorescent dye was added (pH 7.0–7.2). The fluoresence intensities measured for samples B which received sonication before alkali treatment were extremely low and kept constant during the unwinding period. Because of extensive DNA fragmentation conversion of native DNA to single-stranded DNA was completed during the initial time period ($F = 0$). The DNA in samples T, which received no alkali treatment, remained in duplex form and showed no reduction in fluorescence ($F = 1$). The P-samples contain DNA in partly denatured form, accordingly the amount of double-stranded DNA is a function of dose and unwinding time ($0 < F < 1$). As shown in Figure 2 DNA unwinding shows an initial fast component during the first minutes, followed by a slow progression in fluorescence reduction. During the initial exposure to alkali the unwinding rate is much slower for control cells than for irradiated cells, the dose-dependency is obvious. The dose-dependent difference is observed as soon as after 15 min at 20°C, the curves remain approximately parallel up to 120 min afterwards. The use of 30 min unwinding time as reported here has proven to be the most reliable procedure especially for assessing huge numbers of samples (30–60 per experiment).

The fluorescence intensity is a function of double-stranded DNA, and thus depends not only on dose but also on cell numbers. Figure 3 shows data from experiments with varying numbers of CHO cells which were subjected to X-irradiation followed by fluorometric analysis of DNA unwinding. As indicated in Figure 3 the minimal number of cells to be used is limited by the difference between T- and B-value (T should be about twice of B). For experiments using cell numbers from 1×10^5 to 1×10^6 cells per sample an exponential relationship between fluorescence intensity and dose could be obtained. Expressed as strand scission factors, there are no significant differences ($2p = 0.05$) between the assays containing different cell numbers.

From the elution profiles (Figure 1) or from the unwinding kinetics (Figure 2) dose

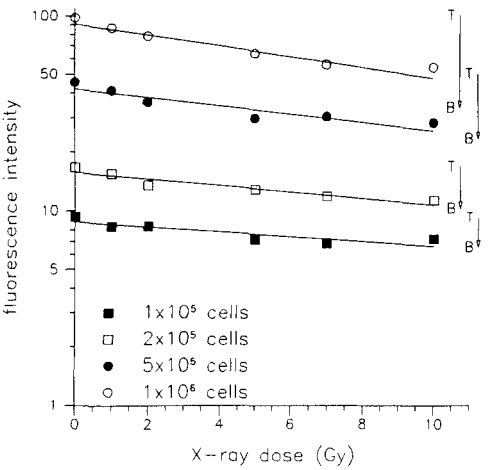

FIGURE 3 Dose-dependent DNA unwinding in alkaline solution. Varying numbers of CHO cells were subjected to X-irradiation, DNA unwinding for 30 min was recorded using the FADU method. Between the two corresponding extremes (maximal fluorescence: T, minimal fluorescence: B) an exponential relationship between fluorescence intensity and dose could be obtained. Strand scission factors show linear dose-relationships (1×10^5 cells: SSF/Gy = 0.101, 2×10^5 cells: SSF/Gy = 0.101, 5×10^5 cells: SSF/Gy = 0.097, 1×10^6 cells: SSF/G = 0.089).

response relationships were established. As shown in Figure 4, no difference was found in the dose response curve for strand break induction by X-irradiation between alkaline elution and fluorometric analysis of DNA unwinding (FADU). Thus in subsequent experiments with radical generating chemicals the fluorometric assay was used to determine DNA damage.

Four chemicals which are known to produce free radicals and therefore have the potential to interfere with cellular DNA were tested for strand break induction during short-term exposure. The selected agents (bleomycin, mitomycin C, methyl viologen and hydrogen peroxide) were added directly to pre-cooled cells for 30 min. Dose-response relationships (SSF versus dose) were established and are presented in Figure 5. Hydrogen peroxide and bleomycin show a high efficiency in strand break induction which increases with concentration. For hydrogen peroxide a concentration of 50 μmol l^{-1} (30 min) makes the same amount of strand breaks as 10 Gy X-irradiation. Mitomycin C and methyl viologen show only weak DNA breaking capacities.

4. DISCUSSION

The FADU method was orginally introduced into the field of DNA strand break analysis by Birnboim and Jevcak[9] and was improved by Kanter and Schwartz.[10] Though it has a lot of advantages over other methods (i.e. it does not require radioactively labelled cells), it is not yet frequently in use. A possible explanation may be the fact that results obtained by FADU have not yet been compared directly to those obtained by the most frequently used technique of alkaline elution, using identical cell material. We have, therefore, measured DNA strand breaks in X-irradiated CHO cells with both methods and compared the results.

The FADU method makes use of differences in DNA unwinding dependent on the

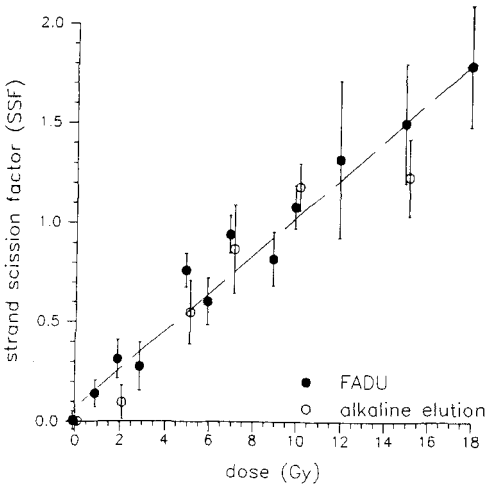

FIGURE 4 Comparison of results obtained by alkaline elution (○) and fluorometric analysis of DNA unwinding (■) after X-irradiation with varying doses. Strand scission factors ($-\ln F_D/F_{D=0}$) were compared using Student's t-test (significance level was $2p \leqslant 0.05$). SSF/Gy were 0.0981 and 0.0923 for FADU and for alkaline elution, respectively. Data obtained by the two techniques are nearly identical ($2p \geqslant 0.9$).

number of DNA strand breaks. Double stranded portions were identified using the fluorochrome bisbenzamide which complexes with ds-DNA resulting in fluorescence.

A generally accepted explanation for the detection of DNA strands breaks by DNA unwinding is that denaturation starts at each break site produced by physical or chemical noxae. Kinetics of DNA unwinding under moderate alkaline conditions as

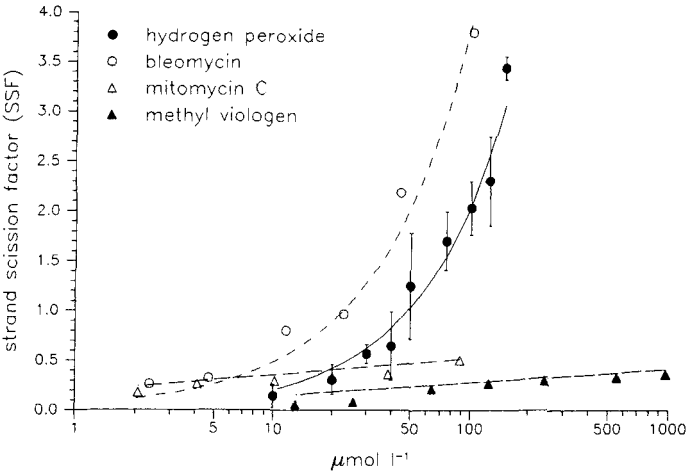

FIGURE 5 Comparison of results obtained by fluorometric analysis of DNA unwinding after treatment with radical generating chemicals. Strand scission factors ($-\ln F$) were calculated for hydrogen peroxide (SSF/μmol = 0.0204), bleomycin (SSF/μmol = 0.03835), mitomycin C (SSF/μmol = 0.004) and methyl viologen (SSF/μmol = 0.0004).

applied in the irradiation experiments reported here are of biphasic type. The initial slope (fast unwinding) is obviously related to the amount of strand breaks present and represents the immediate unwinding starting from DNA breaks. With growing single-strands the degree of free movement is reduced, thus unwinding continues more slowly. Assuming that X-irradiation induces randomly distributed strand breaks along the DNA molecule and that the slow denaturation process proceeds at a constant rate, a severely damaged DNA with only short duplex portions would separate completely under alkaline conditions. This would result in a wrong estimation of strand break induction at high doses especially when long unwinding times are used. We have therefore chosen a relatively short period of alkali treatment. Under these conditions (20°C, 30 min) also severely damaged DNA will not completely unwind, thus even relatively close spaced DNA strand breaks may be detected as single events. It is of interest to note that the kinetics of unwinding, as monitored here by FADU are very similar to unwinding as determined by hydroxyapatite chromatography.[8,11]

Under the alkaline unwinding conditions specified by Birnboim and Jevcak[9] large numbers of cells (5×10^6–$1 \times 10^7\,ml^{-1}$) are required for reproducible results. By simple modification of Birnboim's FADU procedure Kanter and Schwartz (10) have been able to use the same assay with as less as 5×10^5 cells per 2 ml. The experiments reported here shown reliable results for cell numbers as low as 1×10^5 per 3 ml test volumes, if fluorescence intensities are read in a cuvette spectrophotometer. Cell numbers as low as 5×10^4–1×10^5 cells per 400 μl can be used, if fluorescence intensities are measured using a Fluoroskan II 96-well microtitre plate reader (results not shown).

The most frequently used method for strand break determination is alkaline elution which was developed by Kohn and Grimek-Ewig.[5] In contrast to FADU, alkaline elution is based on complete removal of proteins and complete unwinding of DNA under stringent alkaline conditions before being sucked through an inert filter. As elution volumes tend to reach high values (15–30 ml), cells have to be labelled radioactively ([methyl-^{3}H] thymidine) for accurate DNA measurements. Radioactive labelling is expensive (label, scintillation fluids and vials, radioactive disposal) and troublesome to perform (need for security laboratory). Therefore, it should be avoided if adequate non-radioactive methods are available. In the experiments reported here, results for DNA breaks in X-irradiated cells determined by FADU and by alkaline elution are compared. Results are expressed as strand scission factors ($SSF = -\ln F_D/F_{D=0}$), they show no differences between both methods ($2p \geqslant 0.9$). The FADU technique, as applied in our experiments, detects DNA damage in the same dose range (0–18 Gy) as alkaline elution (0–15 Gy). Reliable measurements of double-stranded DNA are possible in the range from 10 to 100 per cent of DNA from untreated cells (corresponding to $3 < SSF < 0$). It is interesting to note that Kanter and Schwartz[10] reported no differences in their results obtained by FADU and hydroxyapatite chromatography for human leukemia L1210 cellular DNA and Ahnström and Erixon[6] have shown the same for hydroxyapatite chromatography and velocity sedimentation of DNA from chinese hamster V 79 cells.

In this report, we have shown that the FADU technique compares well to the alkaline elution technique which measures DNA strand breaks. Because FADU is simple, rapid and sensitive, it was also applied in a series of experiments with radical generating chemicals. Bleomycin and hydrogen peroxide require traces of metal ions (especially ferrous ions) to form reactive radicals via Fenton-type reactions[12] near to

the DNA in order to induce strand breaks. Both substances were shown to be effective in strand break induction. Methyl viologen, known to generate superoxide anion radicals[13] via mitochondrial pathways, is only poorly effective in strand break induction, possibly due to the long distance which radicals have to diffuse from the site of their origin to the site of DNA. Mitomycin C is also poorly effective because it affects DNA only when reduced to the semiquinone radical. This acts as a bifunctional alkylating agent crosslinking DNA interstrands rather than inducing strand breaks.[14]

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