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Yield of SCEs and translocations produced by 3 aminobenzamide in cultured Chinese hamster cells

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Abstract

Different concentrations of 3-aminobenzamide (3AB), a strong inhibitor of poly(ADP-ribose) polymerase (PARP), were used to study their effect on the BrdU-substituted DNA of the Chinese hamster AA8 cell line. The frequencies of sister chromatid exchanges (SCEs) and translocations were determined using the fluorescence plus Giemsa (FPG) and fluorescence in situ hybridization (FISH) techniques, respectively. The results indicate that 3AB effectively induced a dose-dependent increase in the frequency of SCEs, but this enhancement in the yield of SCEs was not paralleled by an increase in translocations. These results are discussed in terms of the as yet poorly understood molecular mechanisms of action of the enzyme PARP. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Poly(ADP-ribose) polymerase; 3-Aminobenzamide; Sister chromatid exchanges; Translocations; DNA damage

1. Introduction

The nuclear enzyme poly(ADP-ribose) polymerase (PARP) is an eucaryotic DNA-binding protein that specifically recognizes DNA strand breaks produced by several damaging agents. PARP binds to strand interruptions in DNA and undergoes a rapid automodification with the synthesis of poly(ADP-ribose). This polymer also modifies a large number of cellular target proteins, which makes its role in cellular recovery difficult to clarify [1,2].

There is evidence that PARP plays an important role in cellular events, such as DNA replication and DNA repair in which breaking and rejoining of DNA strand breaks may occur [3–5]. Although the precise physiological role of PARP is still a matter of discussion, recent experimental approaches [6–11] have clearly revealed the involvement of PARP in the maintenance of genomic integrity due to its role during base excision repair (BER).

The molecular mechanisms leading to stable aberrations (translocations), sister chromatid exchanges (SCEs) and unstable aberrations (dicentrics) have not yet been clarified. The CHO mutant EM9 is especially useful for studying the mechanism of formation of SCEs. This cell line, whose DNA ligase activity is greatly reduced, has an extremely high

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frequency of SCEs compared to its parental line AA8 [12], and its defect is fully corrected by the human *XRCC1* gene [13]. The availability of chromosome-specific libraries for Chinese hamster cell [14,15] makes possible to analyse stable chromosomal aberrations (translocations) in derived cell lines.

Recent results using EM9 cell line have shown a parallel increase in the frequencies of SCEs and translocations induced by BrdU. These results have been attributed to a possible relationship between the mechanisms involved in the formation of SCEs and translocations [16].

To provide additional information concerning the molecular mechanism involved in the formation of SCEs and translocations, we have focused on the possible role of PARP since, to our knowledge, it is the only nuclear enzyme whose catalytic inhibition results in a high increase in SCEs [17]. It appears that PARP acts as a nick sensor rapidly recruiting different enzymes as XRCC1 and ligase III [18]. The use of inhibitors of PARP can produce an indirect inhibition of ligase III activity at the site of the lesion.

In order to see if SCEs and translocations were affected in AA8 in a similar fashion as in EM9 treated with BrdU [16], the PARP was inhibited.

In this study, the frequencies of SCEs and translocations have been analysed in the AA8 cell line treated with 5-bromodeoxyuridine (BrdU) and different doses of the PARP inhibitor 3-aminobenzamide (3AB), one of the more efficient inhibitors of poly(ADP-ribose) synthesis. The results indicate that, in agreement with others reports, 3AB effectively induced SCEs, but unexpectedly, in contrast to our earlier observations in EM9 cells [16], did not induce a parallel increase in the frequency of translocations. These results are discussed in terms of the molecular mechanism of action of PARP.

2. Materials and methods

2.1. Culture conditions

The CHO line AA8 was used . The cells were grown as monolayers in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and the antibiotics penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were grown in the dark at 37°C in a 5% CO_2 atmosphere.

Exponentially growing cells were cultured in a mixture made up of 1 μM 5-fluorodeoxyuridine (FdU, Sigma), 100 μM deoxycytidine (dC, Sigma) and 10 μM BrdU. 3AB (Sigma) was added to the cultures after one round of replication (13 h for AA8) at concentrations of 0, 1, 3 and 10 mM. After another round of replication, i.e., at 26 h, colcemid (2×10^{-7} M) was added for 3 h to produce metaphase arrest.

Parallel cultures, which were treated with 3AB at the above-mentioned concentrations but did not receive the BrdU treatment, served as controls.

The flasks were shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 0.075 M KCl for 2 min, fixed in methanol/acetic acid (3:1) and dropped onto clean glass microscope slides. One set of slides was used for FPG and another one was stored at −20°C until its use for in situ hybridization. As for control slides, only the fluorescence in situ hybridization (FISH) technique was done. Two independent experiments were carried out.

2.2. Analysis of SCEs

Differential staining of BrdU-substituted sister chromatids was obtained by the fluorescence-plus-Giemsa (FPG) method of Perry and Wolff [19] modified by Morgan et al. [20]. At least 100 metaphases were scored.

2.3. In situ hybridization

Slides were thawed and baked at 55°C overnight prior to FISH. They were then incubated with pepsin (0.005%) in 10 mM HCl for 10 min at 37°C. After washing with PBS/50 mM $MgCl_2$, the slides were post-fixed with 1% formaldehyde in PBS/$MgCl_2$ for 10 min at room temperature, washed with PBS and dehydrated.

The probes were kindly provided by Professor A.T. Natarajan (Leiden University, The Netherlands) and they were developed by the linker-adaptor method as described previously [14]. The probes

were labelled by nick-translation. For each treatment, two different cocktails of biotin-labelled probes were used. In one set of slides, the cocktail contained the probes for chromosomes 2 and 8, and for another set of slides, it contained the probes for chromosomes 5 and the long arm of chromosome X (X_1).

The amount of nick-translated DNA for the individual chromosomes in the cocktails was 200 ng. The labelled probes and 2 μg of hamster Cot I DNA were diluted in hybridization mixture (50% deionized formamide, $2 \times$ SSC, 50 mM phosphate buffer (pH 7.0) and 10% dextran sulphate) to a total volume of 20 μl and denatured at 70°C for 10 min. The probes were then immediately put on ice-water for 5 min and competition was carried out for 3 h at 37°C. About 30 min before the end of probe competition, cells were denatured with 70% formamide/$2 \times$ SSC under 24×50 mm coverslip at 80°C for 2.5 min. The slides were then put in cold 70% ethanol for 5 min and dehydrated. When the probe competition was finished, the slides were incubated with the probes overnight in a moist chamber with 60% formamide/$2 \times$ SSC pH 7.0 at 37°C. After hybridization, the slides were washed four times for 5 min each in 50% formamide at 42°C, three times for 5 min each in $0.1 \times$ SSC at 60°C and once for 5 min in $4 \times$ SSC/0.05% Tween 20 at room temperature. Thereafter, the slides were incubated with 5% non-fat dry milk/$2 \times$ SSC for 15 min in a moist chamber. Visualization of the biotin-labelled DNA probes was performed by means of an avidin-FITC conjugate (Sigma) and two rounds of amplification by biotinylated anti-avidin (Pierce). Antibodies were diluted in 5% non-fat dry milk/$2 \times$ SSC and incubated at room temperature for 30 min. After each incubation, the slides were washed three times in $4 \times$ SSC/0.05% Tween 20. Finally, slides were dehydrated and embedded with Vectashield mounting medium (Vector Labs) containing 1.5 μg/ml DAPI (Boehringer) as blue counterstain.

2.4. Scoring of aberrations

Fluorescence microscopy was performed on an Olympus (Vanox AHBT3) microscope equipped with filters for observation of DAPI (Blue) and FITC (green). Aberrations were scored in second division metaphases after BrdU treatment. Translocations (reciprocal, terminal and interstitial) involving the painted chromosomes were scored. The relative length of the chromosomes was calculated in our previous paper [16], and the values in AA8 were: 2, 15%; 8, 5.6%; X_1, 4.01%; and 5, 7.94%. The genomic frequencies of translocations were calculated on the basis of this measurements, assuming that DNA contents of these chromosomes are directly proportional to the length of the chromosomes. The conversion to genome equivalence was made using the formula suggested by Lucas et al. [21].

A total of 300 metaphases were analysed from two independent experiments for each cocktail and treatment.

3. Results

The results on the frequencies of SCEs and translocations induced by BrdU and different doses of 3AB in the AA8 cell line are shown in Table 1. Data from Table 1 have been replotted in Fig. 1 to clearly compare the frequencies of both endpoints. In order to check if most of the cells had completed two rounds of replication, the percentage of second mitosis was also analysed. The results show that for all the treatments, more than 90% of the cells were in their second division.

SCEs were scored in at least 100 metaphases. The results clearly show, in agreement with earlier results obtained in other laboratories [17,22], that 3AB effectively induced a dose-dependent increase in the frequency of SCEs in cells treated with BrdU.

Translocations were scored in about 300 second division cells per cocktail (i.e., about 600 per treatment) using the FISH technique. The chromosome probes used for it represented about 32% of the total genome. The presented data on the frequencies of translocations are the average of the values observed for the two chromosome cocktails corrected for the whole genome. As can be seen in Table 1 and in Fig. 1, cells treated with BrdU plus different doses of 3AB showed no dose-dependent increase in the frequency of translocations, contrasting with what we observed for SCEs. The same doses of 3AB without BrdU did not produce any significant increase in the frequency of translocations either. Higher doses of

Table 1
Frequencies of chromosomal aberrations and sister chromatid exchanges in the Chinese hamster fibroblast cell line AA8 induced by BrdU and/or different doses of 3AB

[BrdU], μM	[3AB], mM	Translocations per 100 metaphases	Aberrations per 100 metaphases	SCEs per metaphase
–	–	0.80 ± 0.07	0.65 ± 0.06	–
10	–	11.68 ± 0.27	15.93 ± 0.32	9.22 ± 0.60
10	1	13.35 ± 0.29	10.83 ± 0.26	14.68 ± 0.75
10	3	7.48 ± 0.22	11.00 ± 0.26	22.04 ± 0.92
10	10	10.61 ± 0.26	17.70 ± 0.34	38.10 ± 1.20
–	1	10.45 ± 0.26	2.80 ± 0.13	–
–	3	7.15 ± 0.21	1.65 ± 0.10	–
–	10	5.11 ± 0.18	0.50 ± 0.05	–

The frequencies of translocations are the average of the values (from two independent experiments) corrected for the whole genome obtained from each cocktail of chromosomes.

3AB did not induce a significant increase in the frequency of SCEs as compared with 10 mM 3AB and, because of that, they were not used to check their possible effect on translocations.

Chromosome aberrations were also analysed in all the treatments. In general terms, the percentage of total chromosome aberrations in the combined treatments did not show any significant difference as compared with the controls treated with BrdU alone, according to previous results [23]. Cells treated with different doses of 3AB alone presented a very low frequency of aberrations [23].

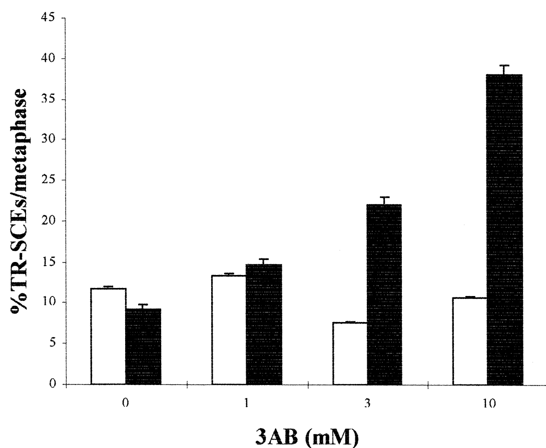

Fig. 1. Comparison between the frequencies of SCEs (■) and translocations (□) induced on BrdU-substituted DNA by different doses of 3AB in the Chinese hamster AA8 cell line (data replotted from Table 1). Bars represent the standard errors from two independent experiments.

4. Discussion

The molecular pathways, leading to the production of SCEs and translocations, remain to be fully elucidated. A recent report has indicated that these two endpoints could share, at least in part, a common mechanism [16]. Our aim to carry out the present investigation was that the study of the effect of 3AB on the frequencies of SCEs and translocations could add information about to what extent some possible pathways should be shared.

Our results indicate that contrasting with those previously reported by us for the CHO mutant EM9 [16], the increase in the yield of SCEs induced by 3AB on BrdU-substituted DNA is not paralleled by an enhancement in the production of translocations. In our opinion, however, these results could be explained, taking into account the complex molecular mechanism of action of PARP.

Unmodified PARP binds to DNA ends generated in the initial stages of DNA repair/replication, which blocks DNA strand ends. Modified PARP carries chains of negatively charged ADP-ribose polymers, which cause the enzyme to lose affinity for DNA [24]. The enzyme will consequently dissociate from DNA ends, allowing access to DNA repair enzymes [25]. It has been reported that ribosylation occurs on

several enzymes, including histones [26,27]. Ribosylated histones have low affinity for DNA, which will cause relaxation of chromatin superstructure [28,29]. It has been speculated that this change in the chromatin conformation could also contribute to increase the accessibility of repair enzymes to the lesion [30].

3AB is a strong inhibitor of poly(ADP-ribose) synthesis. The dose-dependent increase in the frequency of SCEs induced by 3AB, has been previously reported [17,22,23]. In the presence of 3AB, the enzyme PARP binds and blocks DNA strand ends, but the polymer is not synthesised. Inhibition of ribosylation could complicate the accessibility of the repair enzymes to the lesions, because PARP will not dissociate and chromatin will keep its conformation. Since the accessibility of repair enzymes to the lesions is decreased, the first consequence is that lesions would remain open longer: that will cause an increase in the production of SCEs [17]. Nevertheless, while PARP acts on single strand breaks and its inhibition should increase the number of single strand breaks, there is no contrasted evidence that will show that this specific lesion leads to SCE. Anyway, the second outcome, as a result of PARP inhibition, is expected to be that DNA superstructure will be maintained and nucleases will have a more difficult accessibility to DNA. The result is that no additional breaks will be produced, which could give rise to double strand breaks. In our opinion, this could explain why we have not found an increase in the number of translocations when 3AB was used.

EM9 CHO mutant cell line presents a defect in the rate of rejoining DNA single-strand breaks [12] and a greatly reduced DNA ligase III activity. The XRCC1 is a single-strand break-binding protein that interacts with ligase III [31] and is required for normal levels of this enzyme activity [18]. Some recent results indicate that EM9 has an enhanced spontaneous and BrdU-induced frequency of both chromosome translocations and SCEs [16]. In those experiments, PARP was not inhibited so that it is likely to have been automodified by synthesis of poly(ADP-ribose) after its binding to DNA strand breaks. PARP will, consequently, dissociate from DNA, allowing accessibility for repair enzymes. Since this cell line has low levels of DNA ligase III activity, the repair of the lesions would not be as efficient as it should. The lesions would, consequently, remain open longer, giving rise to an increase in the yield of SCEs. Histones might be ribosylated as well, causing chromatin superstructure release. The exposure to nucleases' attack would render additional breaks, which, in turn, can produce double strand breaks and an increased frequency of translocations.

While the above discussion is highly speculative, the still poorly understood mechanisms of DNA repair, as well as the influence of chromatin on such a mechanism in mammalian cells, make it difficult to reach any final conclusion and, while investigations in the line of the present report add new information, further studies are needed for a more complete knowledge.

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