DNA TOPOISOMERASE ACTIVITIES IN CHINESE HAMSTER RADIOSENSITIVE MUTANTS AFTER X-RAY TREATMENT

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Received 5 July 2001; revised 3 January 2002; accepted 20 March 2002

In the last years the attractive hypothesis of a possible involvement of mammalian topoisomerases in DNA repair has been proposed, given their molecular mechanism of action. So far, using asynchronous cultures a lot of controversial results have been reported, without taking into account the frequently dramatic fluctuations of topoisomerase activities depending upon the cell cycle stage and proliferation rate (mainly for topoisomerase II).

We have addressed this question making use of G1 synchronous cultures of the Chinese hamster radiosensitive mutants \textit{xrs} 5 (defective in DNA double strand breaks rejoining) and \textit{irs} 2 (which shows radioresistant DNA synthesis), as well as their parental lines CHO K1 and V79 respectively, which show a normal radiosensitivity. Cells were irradiated with 5 Gy of X-rays and the activities of topoisomerases I and II in nuclear extracts were studied for comparison with non-irradiated controls in both the mutants and parental cell lines.

Our results clearly show a modulation of the topoisomerase activities after irradiation, that varies depending upon the mutation that the different lines bear.

While this hypothesis needs further testing, an interesting idea is that DNA topoisomerases might be involved in the cellular response to radiation damage, either through a direct participation in the repair mechanisms or in a preparative step to allow repair to proceed.

KEYWORDS: cell stress; ionizing radiation; radiosensitive mammalian cell lines; topoisomerase activity.

INTRODUCTION

DNA topoisomerases (topos) are ubiquitous enzymes that regulate the topology of DNA for a correct DNA metabolism, and their specific activity is required during DNA replication, transcription (mainly topo I) and homologous recombination (Wang, 1985, 1991; Wang \textit{et al.}, 1990). Besides, a specific role for topo II in segregation of daughter chromatids after DNA replication as well as for chromatin condensation has been proposed (Di Nardo \textit{et al.}, 1984; Uemura \textit{et al.}, 1987). Given their molecular mechanism of action, through concerted breakage and rejoining of just one (topo I) or both (topo II) DNA strands (Wang, 1985), and since their malfunction has been implicated in mutation (Overbye \textit{et al.}, 1982), sister chromatid exchange (Pommier \textit{et al.}, 1985), illegitimate recombination (Bae \textit{et al.}, 1988), fragmentation of DNA (Jaxel \textit{et al.}, 1988) and tumor promotion (Kaneko and Hirikoshi, 1987), a possible key role of topoisomerases in DNA repair has been proposed (Downes and Johnson, 1988; Friedberg \textit{et al.}, 1995).

Nevertheless, the question of the possible role of topoisomerases in DNA repair, either through a direct or indirect mechanism is currently a controversial one. While some authors (Wilkins, 1983; Synder, 1987) propose that these nuclear enzymes should play a direct role, others, based on inhibitor studies and other observations (Overbye \textit{et al.}, 1982; Sternglanz \textit{et al.}, 1981; Webster and Bhattacharya, 1995) rule out such a role in the
repair of radiation- or chemical mutagen-induced DNA damage.

A number of mammalian mutant cell lines showing DNA repair abnormalities have been isolated in the last 20 years and, focusing on ionizing radiation-sensitive mutants, there is a series of them exhibiting different degrees of repair ability and hypersensitivity to X-ray or γ-ray damage to DNA (Zdzienicka, 1995). A common, but not universal, feature in radiation sensitive mutants is a defect in the efficiency of DNA strand-break repair (Collins, 1993), though there are some radiation-sensitive mutants, for example _irs_ 2, which do not show a deficiency of DNA strand-break repair (Collins, 1993; Zdzienicka, 1995).

Since using asynchronous cultures, as in the studies reported above, without taking into account the sometimes dramatic fluctuations of topoisomerase amounts and activities depending upon cell cycle stage and proliferation rate (mainly for topo II) (Duguet _et al._, 1983) may lead to wrong conclusions, we have recently addressed the question of the possible role of topoisomerasers in radiation repair using synchronous cultures of the radiosensitive CHO mutant EM9 and its parental line AA8, which shows a normal sensitivity to ionizing radiation (Thompson _et al._, 1982). An interesting observation was that, while both topo I and II activities clearly increased after X-ray treatment in the parental AA8, the radiosensitive mutant EM9, which is defective in the repair of DNA strand breaks (Thompson _et al._, 1982), appears as non-responsive to irradiation as regards to topoisomerase activities (Pastor _et al._, 1999).

In the present report, we have carried out a comparative study of topoisomerase I and II activities in the two parental cultured hamster cell lines CHO-K1 and V79 and their corresponding radiation-sensitive mutants (_xrs_ 5 and _irs_ 2, respectively) irradiated in G1 after synchronization. While _xrs_ 5 is defective in double-strand breaks repair, _irs_ 2 shows a radioresistant DNA synthesis (Collins, 1993; Zdzienicka, 1995), but it is not abnormal in its proficiency to repair double-strand breaks. Our results point to differences between the non-sensitive cell lines and their respective mutants in the response to X-rays treatment concerning topoisomerase activities.

**MATERIALS AND METHODS**

**Cell culture**

The CHO-K1 and _xrs_ 5 Chinese hamster cell lines were kindly provided by Dr F. Darroudi (Leiden, The Netherlands), while V79 and _irs_ 2 strains were a generous gift of Dr P. Bryant (St Andrews, Scotland, U.K.).

The mutant _xrs_ 5 and its normal parental CHO-K1 cell lines were grown as monolayers in McCoy’s 5A medium, whereas _irs_ 2 and its normal parental V79 were maintained routinely in monolayer culture in Eagle’s MEM. Both media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and the antibiotics penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were cultured in a dark environment at 37°C in an atmosphere containing 5% CO₂.

**Irradiation**

G1 synchronized cells (5 × 10⁵/ml) were X-irradiated to a total dose of 5 Gy delivered at 1.6 cGy/s at room temperature using an X-ray Philips MG 103/2.25 system.

**Cell cycle distribution analysis**

Since our four cell lines show differences concerning their adherence to the surface of the flasks, we have designed two different methods to obtain synchronous G1 cells. On the one hand, CHO-K1 and _xrs_ 5 cells were synchronized by mitotic ‘shake-off’ and accordingly, in order to obtain a high number of mitotic cells, exponentially growing CHO-K1 and _xrs_ 5 cells, cultured in 175 cm² flasks (Nunc), were incubated for 3 h in the presence of Colcemid (2 × 10⁻⁷ M), before they were collected. Mitotic cells were then washed two times with fresh medium in order to overcome the negative effect of Colcemid on microtubule assembly as observed, and seeded in several culture flasks (25 cm²) which were subsequently sampled by trypsinization at various times for cell cycle analysis by flow cytometry.

On the other hand, whereas V79 and _irs_ 2 mitotic cells show a high degree of attachment to their substrate and cannot be collected by standard ‘shake off’ as described above for the other two cell lines, these cell lines were synchronized in G1 by allowing them to reach confluence for 4 days.

Before the cytometric analysis, samples were first washed with PBS and centrifuged at 1200 rpm for 5 min. The pellet was resuspended by the dropwise addition of ice-cold 70% (v/v) ethanol and incubated for at least 30 min at 4°C. Afterwards, cells were washed once with PBS, thoroughly resuspended in 1 ml of 1 mg/ml fresh made RNase A (Boehringer) and incubated for 30 min at 37°C. A minimum of 15,000 cells were then analyzed per
sample for DNA content after staining with 40 μl of 0.1 mg/ml propidium iodide (PI) using a FACScan (Becton Dickinson) flow cytometer. Cell-cycle distribution was determined using Cell Fit/LYSYS® II software.

Preparation of nuclear extracts

The CHO-K1 and xrs 5 synchronous mitotic cells were seeded in 75 cm² cell culture flasks and irradiated with 5 Gy of X-rays when the synchronous cells reached G1 phase, that is, approximately 4 h after having seeded the mitotic cells, according to the results obtained by flow cytometry. V79 and irs 2 cells synchronized by confluence in G1 were trypsinized, seeded in several flasks and irradiated shortly after their attachment to the substrate. Fifteen min and 1 h after irradiation, the cells were processed to obtain extracts of nuclear proteins, while control unirradiated cells were also sampled in parallel for comparison. The procedure followed was basically that described by Heartlein et al. (1987). Approximately 1 × 10⁷ cells were suspended in 1 ml of 0.32 M sucrose, 0.01 M Tris-HCl pH 7.5, 0.05 M MgCl₂, 1% Triton X-100 and thoroughly washed in 1 ml of 0.32 M sucrose, 0.01 M Tris-HCl pH 7.5, 10 mM potassium phosphate buffer, and 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM β-mercaptoethanol, and 0.5 mM dithiothreitol (DTT). The nuclei were then pelleted as described above and resuspended in 50 μl of nucleus wash buffer, and 50 μl of 4 M EDTA was added. Following incubation at 0°C for 15 min, the nuclei were lysed by adding 100 μl of 2 M NaCl, 20 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol, 1 mM PMSF. Following a 15 min incubation at 0°C, 50 μl of 18% polyethylene glycol (PEG-6000) in 1 M NaCl, 50 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol, and 1 mM PMSF was added. The suspension was incubated for a further 40 min period at 0°C. The supernatant from a 30 min centrifugation at 12,500 rpm at 4°C was then collected. Total protein concentration in each extract (Bradford, 1976) was determined in a Beckman DU-64 spectrophotometer by the Bradford protein assay and extracts were kept at −80°C for no longer than a month.

Topoisomerase I and II activities in nuclear extracts

Topo I and II activities in nuclear extracts were assayed using TopoGen (Columbus, OH, U.S.A.) assay kits based upon relaxation of supercoiled plasmid DNA and decatenation of kinetoplast DNA (kDNA), respectively. The amount of nuclear extract protein from the different cell lines used in each assay was 100 ng. Reaction products were resolved using agarose gel electrophoresis of DNA. After incubation (40 min at 37°C for topo II; 30 min for topo I) the samples were loaded onto 1% agarose gels and subjected to electrophoresis for either 2.5 h at 100 V (topo II assay) or 3.5 h at 50 V (topo I). Finally, gels were stained with 0.5 μg/ml ethidium bromide, destained (30 min) in distilled water and photographed using a standard photodyne set.

Western blot

Control and treated cells were harvested 15 min and 1 h after X-irradiation (5 Gy) and lysed in a buffer consisting of 5 mM Tris-HCl, 50 mM EDTA, 0.5% Triton X-100, pH 8 in the presence of protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin and 5 mg/ml leupeptin, all from Sigma). The protein content for each sample was quantified and 30 μg from each sample were loaded on 7.5% SDS-polyacrylamide gels. After separation, the proteins were transferred onto PVDF strips and blocked with 5% nonfat milk for 1 h at room temperature. PVDF strips were incubated with primary antibody anti-topo I or anti-topo II α (Topogen, Columbus, U.S.A.) for 1 h at 37°C. Finally, the peroxidase-conjugated anti-human Ig G (for topo I, from Sigma) or anti-rabbit Ig G (for topo II, from Amersham) antibody was incubated with the blots for 1 h at 37°C and detected by the enhanced chemiluminescence method (Amersham). Densitometry was finally used for quantification of the western blots (PCBAS 2.08).

RESULTS

Cell synchronization analysis

Two different protocols were followed in order to obtain synchronous cultures, due to differences in the adherence of the various fibroblast cell lines to their corresponding substrates. While the hamster ovarian fibroblast parental line CHO-K1 and its radiosensitive mutant xrs 5 were easily synchronized by mitotic ‘shake off’, the lung fibroblast strains V79 and irs 2 did not, and they were synchronized by confluence in G1 instead (Fig. 1). According to our observations of cell cycle histograms, to make sure that we treated G1 cells, we
carried out irradiation with 5 Gy of X-rays 4 h after harvesting mitotic CHO-K1 and \( xrs \) 5 cells by 'washing off' (Fig. 1(A)), and shortly after collecting V79 and \( irs \) 2 confluent cells (Fig. 1(B)), just allowing them to attach to their substrate before irradiation.

**Topoisomerase activities after irradiation in G1**

Topo I activity was measured as the ability of nuclear extracts to relax supercoiled pRYG plasmid DNA as assessed by DNA gel electrophoresis, while topo II activity was tested using as a substrate catenated double-stranded circular DNA (kinetoplast or kDNA), which topo II converts into decatenated double-stranded DNA minicircles (Cortés et al., 1993).

The experimental protocol consisted of irradiation of the different cell lines (parental CHO-K1 and V79, and their radiosensitive mutants \( xrs \) 5, and \( irs \) 2) with 5 Gy of X-rays when
cells were in G1, as confirmed previously by flow cytometry (Fig. 1). Nuclear extracts were prepared for the analysis of topo I and topo II activities, at two different times, 15 min and 1 h after X-rays treatment. Non-irradiated control cells were sampled in parallel for comparison.

As can be seen in Figure 2(A), nuclear extracts from irradiated \textit{xrs} 5 (radiosensitive mutant) showed a lower topo I activity than that observed in unirradiated mutant cells or in parental CHO-K1 cells in general (both irradiated and unirradiated parental cells were proficient as regards to topo I activity in nuclear extracts).

As stated above, in the presence of ATP and Mg\textsuperscript{2+}, topo II present in nuclear extracts is able to produce double-strand breaks in such a way that yields decatenated circular DNA from catenated kDNA (Fig. 2(B)). Regarding topo II in the parental CHO-K1, there was a moderately increased activity in nuclear extracts from cells sampled 1 h after irradiation, in good agreement with that previously reported by us in AA8 cells (Pastor \textit{et al.}, 1999). Contrasting with this observation, the \textit{xrs} 5 mutant showed a comparatively higher topo II activity, but a dramatic inhibition 1 h after irradiation was observed (Fig. 2(B)).

Figure 3 shows the topoisomerase activities in nuclear extracts from the other cell lines, i.e. the parental V79 and the radiosensitive mutant \textit{irs} 2. As can be seen (Fig. 3(A)) topo I activity was clearly enhanced for both V79 and \textit{irs} 2, 1 h after treatment with 5 Gy of X-rays. Topo II activity, on the other hand, also appeared as moderately increased after irradiation at both 15 min and 1 h after irradiation in V79, and at 1 h post-treatment in \textit{irs} 2 (Fig. 3(B)).

**Western blot**

Possible changes in topoisomerase protein expression in the different cell lines as a result of irradiation were assessed by Western blotting using specific antibodies against topoisomerases I and II. Contrasting with changes in activity observed (Figs 2 and 3), the relative amount of enzyme did not seem to change significantly shortly after irradiation as compared with non-irradiated controls (Fig. 4) as assessed densitometrically. This latter observation seems to rule out any change in expression of the enzymes shortly after irradiation. Accordingly, the above reported differences in DNA topoisomerase catalytic activities as a result of X-ray treatment should be most likely attributable to enzyme posttranslational modifications.

**DISCUSSION**

Regarding ionizing radiation damage to DNA, it is well known that a set of genes and their corresponding products are induced, including DNA repair enzymes (Herrlich \textit{et al.}, 1992; Keyse, 1993).
The attractive hypothesis of a possible involvement of topoisomerases in repair has drawn in the last years a lot of attention and controversy as well. Increased activity of topo I after irradiation of human lymphoid cells has been reported (Johnstone and McNerney, 1985), and a significant positive correlation between the degree of activation of topo II and radioresistance in human skin fibroblasts has also been well established (Cunningham et al., 1991). Besides, an altered topo I has been found to be present in a radiation-sensitive strain of mouse lymphoma cells (Kowalska-Loth et al., 1993) while it has also been reported that lowering the levels of topo II renders cells sensitive to DNA-damaging agents (Froelich-Ammon and Osheroff, 1995).

Contrasting with these reports that, taken as a whole, support a putative role of topoisomerases in the repair of radiation damage, Warters et al. (1989) didn’t find any increase in topo I after irradiation of mammalian cells, while Boothman (Boothman et al., 1994) even reported on a marked decrease in topo I in irradiated Chinese hamster embryo fibroblasts.

A common drawback of the studies carried out so far on the possible involvement of topoisomerases in DNA repair is that asynchronous cultures have been commonly used, without taking into account the sometimes dramatic fluctuations of topoisomerase levels and activities depending upon the proliferation rate and cell cycle stage (mainly for topo II) (Duguet et al., 1983).

We have recently addressed the above mentioned controversial question using synchronous cultures (by mitotic ‘shake off’) of the CHO radiosensitive mutant EM9 which, at the molecular level, shows a defect in the rate of rejoining DNA-strand breaks after treatment with X-rays or alkylating agents (Thompson et al., 1982) and its parental line AA8, which shows a normal behaviour as regards to radiation damage. Our results on the catalytic activities and levels of topoisomerases in G1- and S- synchronous cells showed a dramatic increase in the parental AA8 cells after X-rays (5 Gy) treatment, while the radiosensitive mutant EM9 cells appeared as refractory to any change in either activity or expression of topoisomerases as a consequence of irradiation (Pastor et al., 1999).

In the present report we have extended our previous studies making use of synchronous cultures of another two radiosensitive Chinese hamster cell lines (xrs 5 and irs 2) and their corresponding parental cells (CHO-K1 and V79, respectively) and compared their response to X-ray treatment while in G1 of the cell cycle regarding topoisomerase activities (topo I and topo II). In agreement with our previous findings (Pastor et al., 1999) we have observed differences between the parental, non-radiosensitive cell lines and their radiosensitive counterparts.

Nuclear extracts from G1 xrs 5 cells, which are about 10 times more radiosensitive than the parental CHO-K1 cells and, at the molecular level,
are defective in DNA strand-break rejoining (Jeggo and Kemp, 1983), showed a decreased ability to relax supercoiled DNA (as a measure of topo I activity) after irradiation with 5 Gy of X-rays. As for topo II activity, CHO-K1 cells showed a moderate increase 1 h after irradiation (higher yield of decatenated DNA), while in the radiosensitive xrs 5 the observation was just the opposite, i.e. a total lack of decatenating effect (measure of topo II activity) in nuclear extracts from cells sampled 1 h after X-ray treatment.

At present, any explanation about these differences between ionizing radiation repair proficient cells and radiosensitive ones reported here and in previous papers (Johnstone and McNerney, 1985; Pastor et al., 1999) must be necessarily highly speculative, given the still poorly understood mechanisms of DNA processing after ionizing radiation damage in order to accomplish at least a partial repair in mammalian cells (Friedberg et al., 1995).

Anyway, the observation that seems consistent is that differences do exist between mutant cell lines which are defective in radiation repair (as is the case for EM9 and xrs 5) and their respective repair-proficient counterparts (parental lines AA8 and CHO-K1, the present report) in what concerns to topoisomerase activities after irradiation.

In our opinion, on the other hand, the results obtained in the lung fibroblast strains V79 (parental) and the radiosensitive mutant irs 2 deserve special consideration, given the different repair phenotype of this latter cell line. While the former mutants discussed above are defective to different degrees in DNA-strand repair, irs 2 strain repairs normally, and its defect is that it doesn’t stop replication as commonly observed in normal mammalian cells as a response to irradiation (the so-called radioresistant DNA synthesis in irs 2). As a consequence, replication proceeds without allowing repair enzymes to deal with lesions in DNA and this has been proposed as the origin of the radiosensitivity observed in irs 2 cells (Jones et al., 1987, 1990). Nevertheless, irs 2 cells are also very sensitive in the G2 phase of the cell cycle where there is no replication (Cheong et al., 1992).

In both V79 and irs 2, topo I activity increases 1 h after treatment with 5 Gy of X-rays, i.e. no difference is found between parental and mutant strains. For topo II, irradiation moderately increases the activity of this nuclear enzyme in the parental V79 cells as well as in the mutant irs 2, in this latter mainly 1 h after irradiation. In our opinion, this latter observation seems to be in good agreement with the radioresistant DNA synthesis of irs 2.

Given the great deal of uncertainties still existing concerning the molecular mechanisms of DNA repair operating in mammalian cells, further studies are necessary to ascertain whether the changes observed by us and others (Pastor et al., 1999, the present report) are related either directly or indirectly with DNA repair or simply are a consequence of a more general response to cell stress.

**Fig. 4.** Assessment of topoisomerase expression in unirradiated controls for comparison with irradiated cells (Western blotting). For each cell line analyzed, lanes 1 and 4 show the control topoisomerase expression for the parental and the radiosensitive mutant, respectively. Lanes 2 and 5 correspond to the amount of either topo I or topo II 15 min. After irradiation with 5 Gy of X-rays. Finally, lanes 3 and 6 correspond to the protein expression 1 h after irradiation.
ACKNOWLEDGEMENTS

The excellent technical assistance of M. A. Ledesma is very much appreciated. We are grateful to Dr J. C. Mateos (Department of Physiology, Faculty of Medicine, Seville, Spain) and Dr M. de Miguel (Department of Normal and Pathologic Cytology and Histology, Faculty of Medicine, Seville, Spain) for their invaluable help with irradiation and flow cytometry, respectively. This work was carried out under a Contract between the University of Seville (Spain) and the European Union (Nuclear Fission Safety Programme; FI4PCT950001).

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