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

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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 *xrs* 5 (defective in DNA double strand breaks rejoining) and *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 *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 *et al*., 1984; Uemura *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 *et al*., 1982), sister chromatid exchange (Pommier *et al*., 1985), illegitimate recombination (Bae *et al*., 1988), fragmentation of DNA (Jaxel *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 *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 *et al*., 1982; Sternglanz *et al*., 1981; Webster and Bhattacharya, 1995) rule out such a role in the

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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 radiosensitive 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 topoisomerases 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% CO_2.

Irradiation

G1 synchronized cells (5×10^5/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 cm^2 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 cm^2) 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 cm^2 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 1×10^7 cells were suspended in 1 ml of 0.32 M sucrose, 0.01 M Tris-HCl pH 7.5, 0.05 M $MgCl_2$, 1% Triton X-100 and thoroughly vortexed to lyse the cells. Nuclear pellets were obtained by centrifugation at 2000 rpm (Eppendorf centrifuge), for 5 min at 4°C. Nuclei were then washed in 1 ml of nucleus wash buffer (5 mM potassium phosphate buffer, pH 7.5, 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 mM 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 blot 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

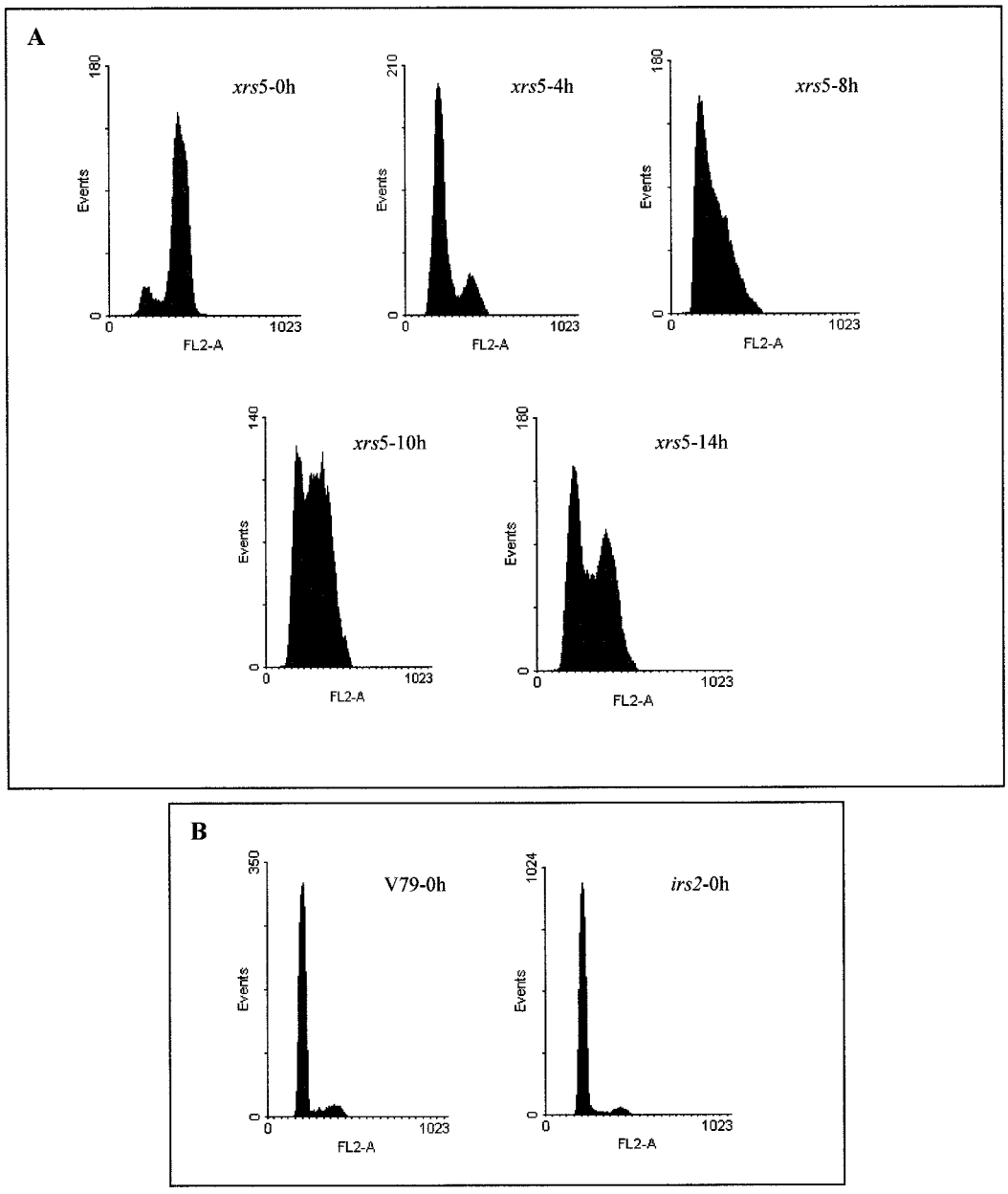

Fig. 1. Two different methods to obtain synchronous G1 cells as observed by flow cytometric analysis. (A) *xrs* 5 (radiosensitive line) synchronized by mitotic 'shake off'. Observe the peak corresponding to G2-mitosis at 0 h (just after gently shaking to disloge the less adherent cells) as well as the good synchrony achieved at 4 h, when we assayed the topoisomerase activities. Very similar histograms were observed for the parental line CHO-K1 (not shown). (B) Synchronization of V79 (parental line) and *irs* 2 (mutant line) by confluence in G1.

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

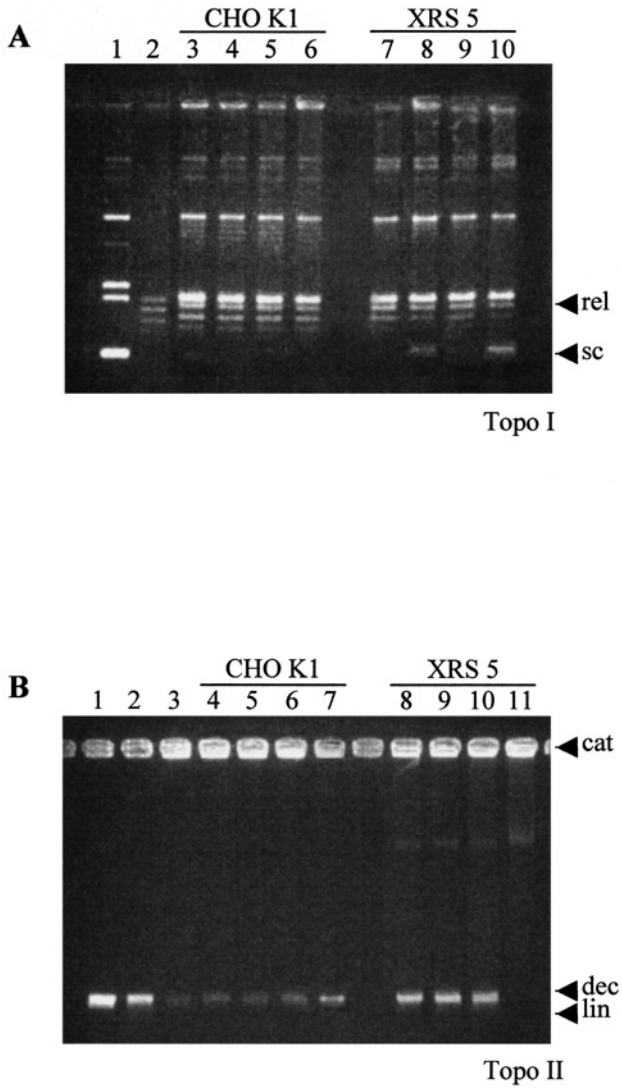

Fig. 2. Measurements of catalytic activities of topo I and topo II in nuclear extracts from G1 parental CHO-K1 and the radiosensitive mutant cell line *xrs* 5 unirradiated or after irradiation with 5 Gy of X-rays. (A) (topo I assay): *lanes 1* and *2*, marker supercoiled (sc) and relaxed (rel) pRYG plasmid DNA, respectively; *lanes 3, 5, 7* and *9*, unirradiated samples; *lanes 4* and *8*, samples harvested 15 min after irradiation; *lanes 6* and *10*, the same 1 h after irradiation. (B) (topo II assay): *lane 1* and *2*, linear (lin) and decatenated (dec) DNA markers, respectively; *lane 3*, marker catenated kinetoplast DNA (cat); *lanes 4, 6, 8* and *10* unirradiated cells; *lanes 5* and *9*, samples harvested 15 min after X-rays exposure; *lanes 7* and *11*, the same 1 h after irradiation.

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 *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^{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 *et al.*, 1999). Contrasting with this observation, the *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 *irs* 2. As can be seen (Fig. 3(A)) topo I activity was clearly enhanced for both V79 and *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 *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 *et al.*, 1992; Keyse, 1993).

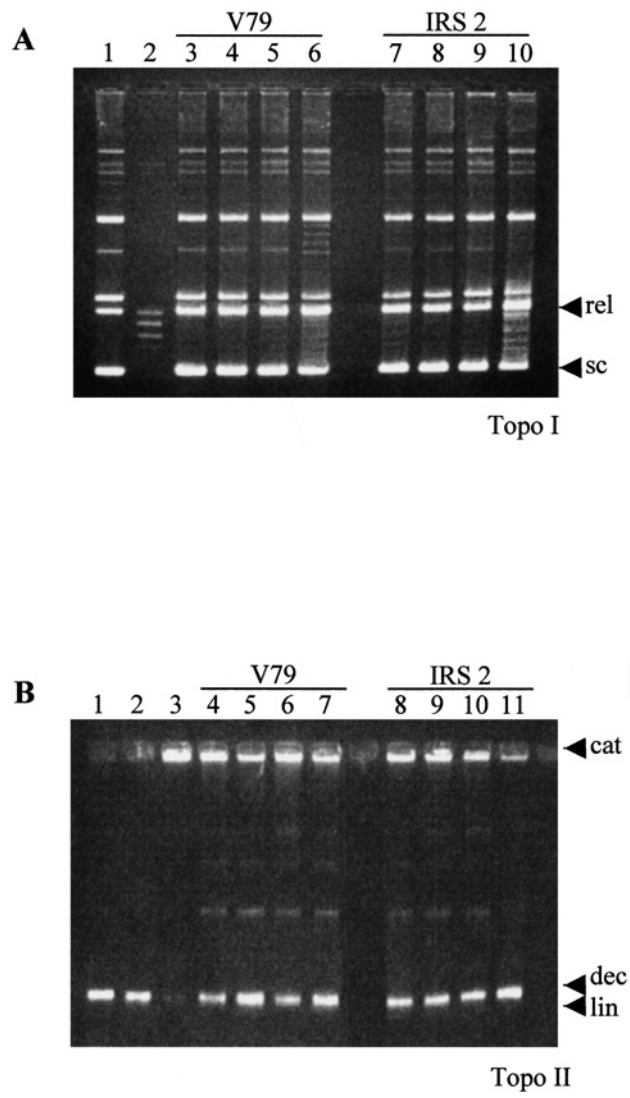

Fig. 3. Catalytic activities of topo I and topo II in nuclear extracts from G1 parental V79 and mutant cell line *irs* 2 unirradiated or after irradiation with 5 Gy of X-rays. (A) (topo I assay): *lanes 1* and *2*, marker supercoiled (sc) and relaxed (rel) pRYG plasmid DNA, respectively; *lanes 3, 5, 7* and *9*, unirradiated samples; *lanes 4* and *8*, samples harvested 15 min after irradiation; *lanes 6* and *10*, the same 1 h after irradiation. (B) (topo II assay): *lane 1* and *2*, linear (lin) and decatenated (dec) DNA markers, respectively; *lane 3*, marker catenated kinetoplast DNA (cat); *lanes 4, 6, 8* and *10* unirradiated cells; *lanes 5* and *9,* samples harvested 15 min after X-rays exposure; *lanes 7* and *11*, the same 1 h after irradiation.

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,

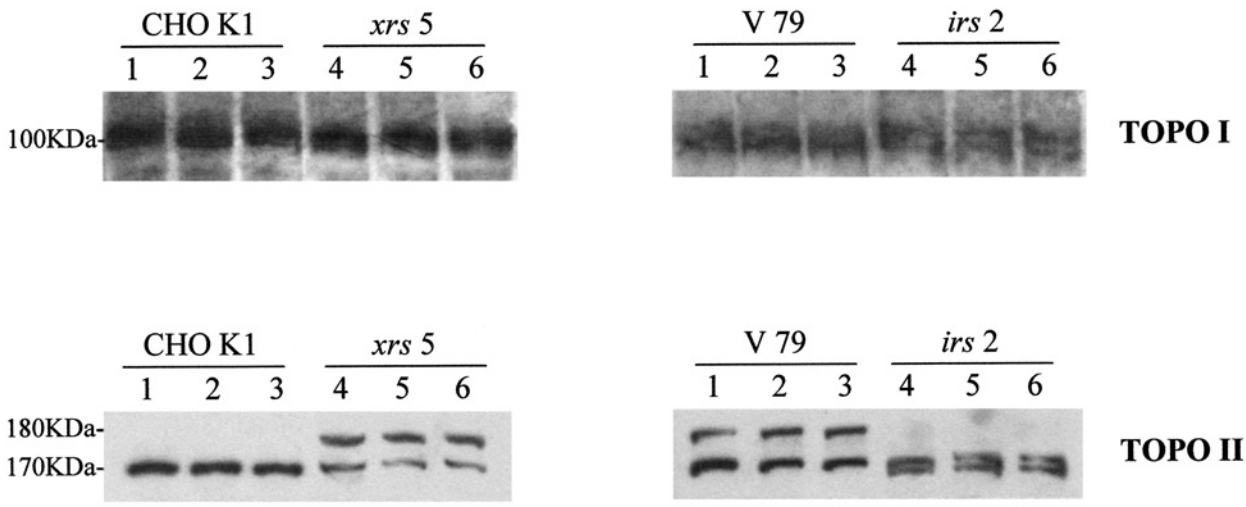

Fig. 4. Assessment of topoisomerase expression in unirradiated controls for comparison with irradiated cells (Western blotting). For each cell line analyzed, *lanes 1* and *4* show the control topoisomerase expression for the parental and the radiosensitive mutant, respectively. *Lanes 2* and *5* correspond 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.

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.

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