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Role of human topoisomerase IB on ionizing radiation induced damage

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ABSTRACT

Ionizing radiation can induce DNA strand breaks' formation both through direct ionization and through induction of oxidative stress. The resistance to radiation is mostly associated with the efficacy of DNA repair system. The ionizing radiation damage response of human topoisomerase IB, that is the selective target of camptothecin and derivatives widely used for various cancers often in association of radiotherapy, has been investigated treating with 30 Gy of X-rays a *Saccharomyces cerevisiae* strain in which the endogenous topoisomerase IB, not essential in this organism, has been deleted and a similar strain which overexpresses the human enzyme. The results show that before irradiation the genetic damage is significantly lower in cells containing human topoisomerase, but soon after irradiation the amount of DNA breaks in these cells is larger than in cells not containing the enzyme. Kinetic analysis of DNA repair rate as well as colonies growth demonstrate that cells containing human topoisomerase display a more efficient rescue. Finally, ionizing radiation induces in the *Saccharomyces* cells an increase of enzymatic activity and of the amount of the enzyme bound to the DNA indicating a direct role of topoisomerase IB in the mechanism of nucleic acid repair.

1. Introduction

Ionizing radiation (IR) can induce various types of DNA damage both through direct ionization as well as through induction of oxidative stress due to the formation of reactive oxygen species (ROS), which ultimately cause damage to the DNA molecules such as DNA single (SSB) and double strand breaks (DSB) formation [1,2]. Remarkably, IR-induced DSB are generated with equal efficiency in all examined prokaryotic and eukaryotic cells (~0.005 DSB/Mbase/Gy irradiation) [3]. Thus the resistance and the rescue to IR-induced damage is mostly associated with the efficacy of DNA repair system, which involves different types of enzymes and signal molecules, many of which are not yet identified [4]. These enzymes represent target for molecules that, modulating their activity, may increase or decrease the efficiency of radiation-induced DNA repair and consequently cell viability. Topoisomerases (Tops) are ubiquitous and essential enzymes that solve the DNA topological problems deriving from replication, transcription, chromatin assembly, recombination, and chromosome segregation by introducing transient breaks into the helix [5]. Tops are grouped into two types: type I Tops (Tops I) usually monomeric, that cleave one strand of duplex DNA, type II Tops usually dimeric that cleave both DNA strands to resolve its topology. Tops I are further classified into two subfamilies: type IA (Top IA) that requires magnesium ions and relaxes only negatively supercoiled DNA and type IB (Top IB) not requiring metal ion and relaxing both positive and negative supercoils [6]. Human Top IB (hTop IB) is of significant medical interest being the selective target of antitumor drugs such as camptothecin and its water-soluble derivatives, widely used for treating cancers some times in association with radiotherapy [7]. There are several evidences that hTop IB is involved in DNA repair, although contradicting results have been reported [8–10]. In order to further clarify the role of hTop IB in DNA damage response, we have compared the effect of a 30 Gy of X-rays irradiation of a *Saccharomyces cerevisiae* strain, in which the endogenous Top IB, not essential in this organism [11,12], has been deleted (Topo−) and a similar strain over-expressing hTop IB (Topo+).

Data reported in this paper indicate that hTop IB does not confer to the cells radio-resistance, but the kinetic analysis of both DNA repair rate as well as colonies growth, demonstrates that the cells containing hTop IB show a more efficient rescue from IR injury.

2. Materials and methods

2.1. Yeast strains and plasmids

S. cerevisiae strain EKY3 (ura3–52, his3D200, leu2D1, TRP1D63, Top1:TRP1, MATa) that has been described previously [13,14] and plasmid YCpGAL1-hTop1 in which the hTop1 is expressed under the galactose-inducible promoter were used [15]. EKY3 cells were transformed with YCpGAL1-hTop1 and YCpGAL1 (without hTop1,

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control) and grown on synthetic complete SC uracil-medium with 2% dextrose and diluted 1:100 in SC uracil-medium with 2% raffinose. At an optical density of A595 = 1.0, the cells were induced with 2% galactose for 6 h [16].

2.2. Cells irradiation

Cells were exposed to 30 Gy of X-rays delivered at a dose of 1 Gy/min using CHF 320 G generator (Gilardoni, Mandello del Lario, LC, Italy) equipped with a Cu filter of 0.5 mm, operating at 250 keV, 15 mA.

2.3. Cell survival assay

After 10, 30 and 60 min from radiation exposure a total of 150 cells were plated on solid SC uracil-medium with 2% dextrose, respectively and the survival fractions were determined by counting the colony forming units (CFUs) grown at 30 °C.

2.4. Comet assay

The induction of DNA breaks and the repair kinetic was assessed with Comet assay, slightly modifying the method previously utilized for lymphocytes [17]. Aliquots of $1–2 \times 10^6$ cells were harvested immediately or at 1, 2 or 20 h after IR exposure, centrifuged (10 min at 8000 rpm, 4 °C) and 40 μl of pellet were mixed with 360 μl of 0.5% w/v low melting agarose (in PBS buffer) at 38 °C, containing approximately 2 mg/ml zymolyase 100T (Lyticase, US Biological, Z1004). This mixture was spread over four agarose coated slides (0.1% w/v normal melting agarose in PBS buffer), covered with a cover slip and incubated for 20 min at 30 °C to allow the enzymatic degradation of cell wall; then slides were let for 10 min on ice to solidify the agarose layer. All further procedures were performed at 4 °C with dim light. Cover slips were removed and slides were incubated in lysis solution (300 mM NaOH, 2.5 M NaCl, 0.05% w/v lauroylsarcosine, 100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10) for 20 min in order to remove cellular proteins, membranes and liberate DNA. The slides were rinsed three times for 20 min each in electrophoresis buffer (300 mM NaOH, 1 mM Na_2-EDTA, pH 13) to eliminate lysis solution, then placed on the horizontal gel box filled with freshly made buffer and submitted for 10 min to electrophoresis at 0.7 V/cm. After neutralization with Tris 0.4 M, pH 7.5, slides were dehydrated with ethanol series (70%, 85%, 100%) and dried at RT [18]. For microscopic analysis slides were stained with ethidium bromide (10 μg/ml) and immediately analyzed at 400× magnification by a fluorescent Axiolab Zeiss microscope (Carl Zeiss AG, Oberkochen, Germany). Due to the small dimension of the yeast's nuclei it was not possible to utilize image analysis softwares and DNA breaks were measured by the visual scoring technique [19] and expressed as arbitrary units (A.U.).

2.5. Analysis of hTop IB relaxation activity

The relaxation assay was carried out as described previously [20]. In brief, 30 or 60 min after IR exposure, cells were harvested by centrifugation (2500 rpm for 5 min) and resuspended in 0.5 ml of solution 1 (0.9 M Sorbitol, 0.1 M Tris–HCl pH: 8, 0.1 M EDTA). After the addition of 50 μl 1 M DTT the cell suspension was centrifuged and re-suspended in 500 μl of solution 2 (0.9 M Sorbitol, 0.1 M Tris–HCl pH 7.5, 0.1 M EDTA). 5 μl of zymolyase were added to cells that were incubated at 37 °C for 30 min with gentle shaking and then centrifuged at 2500 rpm for 10 min. Protein determination was achieved by Lowry method [21]. The crude protein extract was incubated in 30 μl reaction volume containing 0.5 μg of negatively supercoiled pBlue Script KS II (+) DNA and reaction buffer (20 mM Tris–HCl, 0.1 mM Na_2EDTA, 5 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl, pH 7.5). Reactions were stopped with a final concentration of 0.5% SDS after 1 h at 37 °C. The samples were electrophoresed in a horizontal 1% agarose gel box (in 50 mM Tris, 45 mM boric acid, 1 mM EDTA). The gel was stained with ethidium bromide (5 μg/ml), destained with water and photographed under UV illumination.

2.6. Analysis of DNA-hTop IB binding

Ten and sixty minutes from IR exposure, cells were centrifuged, suspended in lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2% SDS, 2% β-mercaptoethanol) containing glass beads and heated at 90 °C for 10 min. The lysates were brought to neutral pH adding 4 M acetic acid and, after vortexing for 30 s for approximately 30 times, cells were heated again at 90 °C for 10 min to increase solubilization. After centrifugation was added 4 volumes of methanol, 1 volume of chloroform and 3 volumes of water. The cell suspension was vortexed and centrifuged at 9000*g* for 10 s after each addition. After adding water, samples were vortexed vigorously and centrifuged for 1 min at 9000*g*, then supernatant was carefully removed and discarded. Three volumes of methanol were added to the lower chloroform phase and the samples were mixed and again centrifuged for 2 min at 9000*g* to pellet the proteins, supernatant was removed and the protein pellet was dried [22]. For determination of proteins in cellular extract, pellets were solubilized with 0.1 M Tris–HCl and 0.1 M NaCl buffer and, after a brief sonication, assayed according to the Lowry procedure [22]. Aliquots of crude extract of proteins (50 μg) were separated by 10% SDS–polyacrylamide gel, transferred to nitrocellulose membrane (BioRad, Hercules, CA) at 4 °C, blocked in 10% BSA for 1 h with TBS-0.1% Tween 20 buffer, and incubated with antibody against hTop IB and successively with the goat anti-mouse-GAM-HRP conjugated (BioRad, Hercules, Ca) and revealed by the ECL chemiluminescence detection system (Amersham, Bucks, UK) according to the manufacturer's specifications [23].

The total amount of Top IB in the cell extract (both DNA-bound and not bound) was detected by WB, using a crude cell protein extract obtained as described in Section 2.5.

2.7. Statistical analysis

For the statistical design of the Comet assay experiments we conformed to the indications of Wiklund and Agurell [24]: experiments were replicates 3 times and at least 100 cells/slide 4 slides/culture were analyzed.

3. Results

3.1. Effect of hTop IB on IR exposed cells viability

The *S. cerevisiae* EKY3 strain is very resistant to IR, a property common to many yeast strains [25]. Ten minutes after the end of irradiation the number of the colonies formed by Topo+ cells is significantly lower than by Topo− ones (Fig. 1A), however the presence of the enzyme triggers cells' recovery and 30 and 60 min after irradiation the number of the Topo+ cells is significantly increased. On the other hand the number of viable Topo− cells is almost constant in the 10–60 min range (Fig. 1A).

3.2. Effect of hTop IB on radiation-induced DNA breaks

The amount of SBs in yeast cells has been analyzed before and after irradiation using the alkaline Comet assay. Fig. 1B shows that the basal level of DNA damage in not irradiated Topo− cells is lar-

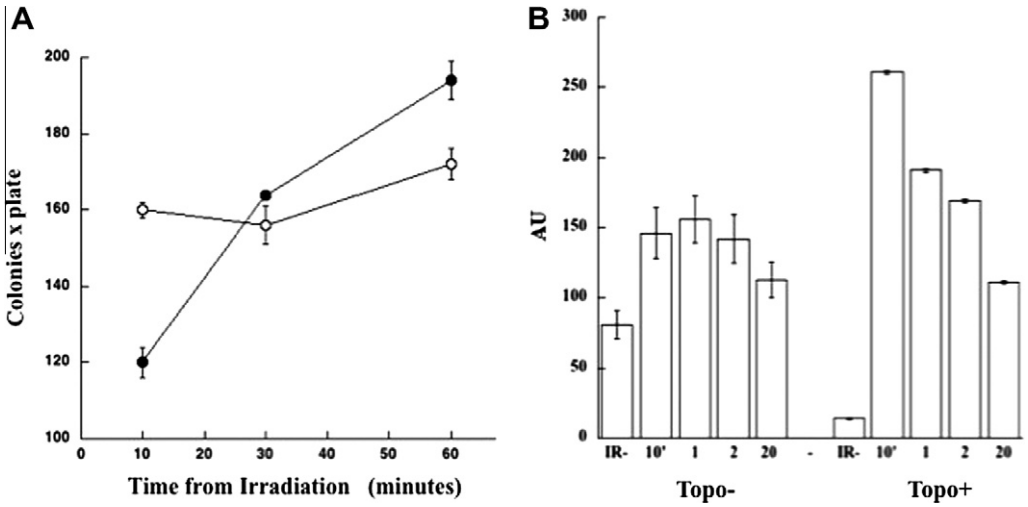

Fig. 1. (A) Number of colonies of Topo− (open circles) and Topo+ (closed circles) *Saccharomyces cerevisiae* cells at increasing time after a 30 Gy of X-rays; (2×10^2). Data represent the mean ± SEM of at least 3 independent experiments. (B) Amount of DNA breaks in the Topo− or Topo+ yeast cells at increasing time after a 30 Gy of X-rays in not irradiated (IR−) or irradiated (IR+) cells. Data represent the mean ± SEM of at least 3 independent experiments.

ger than in the Topo+ ones (80.81 ± 9.85 vs 14.27 ± 0.44), suggesting a role of hTop IB in preventing DNA damage induced by the basal oxidative metabolism, or in repairing it. Ten minutes after the end of the irradiation both Topo− and Topo+ cells show a significant increase of SBs compared to the not irradiated cells, that is more evident in Topo+ respect to Topo− (261.5 ± 1.3 vs 146.1 ± 17.80). One, two and twenty hours after the irradiation, the amount of SBs progressively decreases in the Topo+ cells (191.50 ± 1.50; 169 ± 1.40 and 111.50 ± 0.70, respectively), whereas in the Topo− cells the degree of damage remains in the same range up to 2 h, showing a slight decrease at 20 h (156.17 ± 19; 141.80 ± 17.2; 112.90 ± 13.78, respectively; Fig. 1B). These data indicate that the presence of hTop IB increases the IR-induced SBs immediately after the irradiation, however after 1 h the presence of the enzyme stimulates the rate of DNA repair, underlying the important role of hTop IB in the repair mechanisms of IR-induced DNA strand breaks.

3.3. Effect of ionizing radiation on hTop IB binding to DNA

The total amount of hTop IB into the yeast cells remains constant after irradiation as evaluated by WB (Fig. 2A). The percentage of hTop IB involved in DNA binding after irradiation has been assessed through immunoblotting assay that permits to evaluate the percentage of the free enzyme (Fig. 2B), taking advantage of the fact that the hTop IB bound to DNA does not enter into the acrylamide gel. Evaluation at different time after cell irradiation shows that, 10 min after irradiation, the free hTop IB is much lower in the irradiated cells compared to the not irradiated ones, indicating that a large amount of enzyme is bound to DNA (Fig. 2). On the other hand, 60 min after the irradiation the band corresponding to the free hTop IB increases in the irradiated cells whilst it remains constant in the not irradiated ones (Fig. 2).

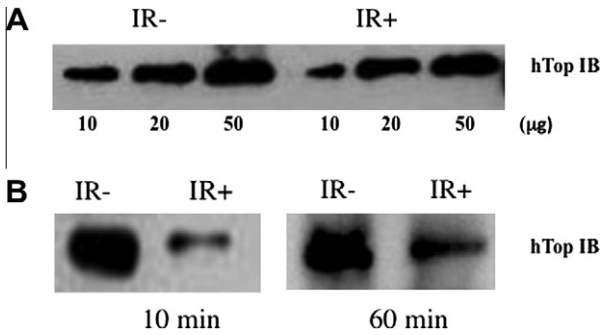

Fig. 2. (A) WB analysis of hTopo IB in extracts of not irradiated (IR−) or irradiated (IR+) yeast cell, observed 10 min after a 30 Gy. (B) Band depletion-immunoblotting experiments using whole lysates of irradiated (IR+) and not irradiated (IR−) Topo+ yeast cells. Experiments were performed twice in triplicate, giving comparable results.

3.4. Effect of radiation on hTop IB in vitro activity

To investigate if the irradiation is able to modify the hTop IB activity we performed a relaxation assay of supercoiled plasmid DNA using different amounts of protein extracts. The assay detects the different electrophoretic mobility of the supercoiled DNA plasmid relaxed by the enzyme. The data show that hTop IB is more active after the irradiation since 0.3 μg of irradiated cells extracts are sufficient to fully relax the supercoiled plasmid (Fig. 3 lane 15) whilst more than 1.2 μg of not irradiated cells is required to achieve the same degree of relaxation (Fig. 3 lane 7). The increase in relaxation activity in the irradiated Topo+ cells can be confidently attributed to an increased hTop IB activity since the same experiments carried out an the Topo− cells does not show any significative relaxation activity increase before and after the irradiation (Fig. 3 lanes 3 and 4).

4. Discussion

Aim of this research work is to analyze the role of hTop IB in the repair mechanisms of DNA breaks induced by IR. Despite the fact that hTop IB is the target for camptothecin-derived chemotherapeutic agents in the treatment of some tumours [7], little is known about the role of this enzyme in the repair of DNA damage induced by IR [9]. *S. cerevisiae* cells show a high resistance to the IR in comparison to animal cells [25] thus the yeast cells have been challenged with a single irradiation of 30 Gy, a dose strongly enough to induce an intermediates number of SBs, suitable to follow the repair process. Before the radiation exposure, Topo+ cells show a level of SBs significantly lower than Topo− cells, suggesting that the enzyme is involved in maintain the genome integrity, repairing the DNA damages induced by the basal oxidative metabolism

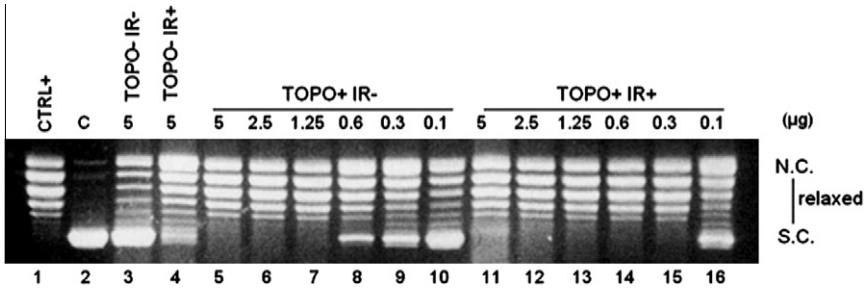

Fig. 3. *In vitro* relaxation of negative supercoiled plasmid DNA by cellular extract from not irradiated (IR−) and irradiated (IR+), Topo− and Topo+ yeast cells. The reaction products were resolved in an agarose gel and visualized with ethidium bromide. Lane 1, positive control reaction with DNA and purified Top IB. Lane 2, negative control reaction without enzyme. Lanes 3–4, activity of Topo− extract. Lanes 5–10, decreasing extract concentrations (5 μg–0.1 μg) of irradiated Topo− cells. Lanes 11–16, decreasing extract concentrations (5 μg–0.1 μg) of irradiated Topo+ cells. NC, nicked circular plasmid DNA. SC, supercoiled plasmid DNA. Experiments were performed at least 3 times in triplicate, giving comparable results.

(Fig. 1B). In contrast, 10 min after exposure to X-rays, the number of SBs is lower in the Topo− cells compared to the Topo+ ones. This apparently contradictory result can be explained considering that, when hTop IB cuts in proximity of a DNA base damaged by the IR, it cannot undergo the religation reaction remaining trapped to DNA and causing an irreversible damage [9]. At increasing times after the irradiation, the number of DNA breaks decreases greatly in Topo+ cells while it remains constant in Topo− cells, indicating a faster repair rate in cells bearing the enzyme (Fig. 1B). These results are in agreement with the cell viability assay showing, immediately after the irradiation, a number of Topo+ cells larger than Topo− cells (Fig. 1A), likely because the covalent non-repaired breaks bring to cell death. Once the repair machinery is restored, Topo+ cells show a faster reproductive fitness comparing to Topo− cells (Fig. 1A). This interpretation is also supported by the increase of concentration of hTop IB bound to DNA during the irradiation followed by a decrease 1 h after the irradiation, as demonstrated by the shift assay experiment (Fig. 2B). In line with this observation, the analysis of cellular extracts early after the irradiation shows a two–three times increase of hTop IB activity compared to the not irradiated ones (Fig. 3), although the total concentration of the enzyme remains unchanged (Fig. 2A), suggesting a IR-induced post-translational modification of the enzyme. Recently, it has been demonstrate that protein kinase CK2, enhances Top I-DNA activity upon phosphorylation of serine residues [26]. As a matter of fact, CK2 is related to the DNA damage induced by different agents such as UV and free radicals [27] suggesting that hTop IB could be one of its targets after cells exposure to IR.

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