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This paper is dedicated to Dr. Gerald WOGAN, Massachusetts Institute of Technology, in appreciation for his outstanding introduction into the world of DNA lesions

DNA topoisomerases as repair enzymes: Mechanism(s) of action and regulation by p53[o*]

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DNA topoisomerases regulate the organization of DNA and are important targets for many clinically used antineoplastic agents. In addition, DNA topoisomerases modulate the cellular sensitivity toward a number of DNA damaging agents. Increased topoisomerase II activities were shown to contribute to the resistance of both nitrogen mustard- and cisplatin-resistant cells. Similarly, cells with decreased topoisomerase II levels show increased sensitivity to cisplatin, carmustine, mitomycin C and nitrogen mustard. Recent studies propose that topoisomerases may be involved in damage recognition and DNA repair at several different levels including: 1) the initial recognition of DNA lesions; 2) DNA recombination; and 3) regulation of DNA structure. The stress-activated oncogene suppressor protein p53 can modulate the activity of at least three different human topoisomerases, either directly by molecular

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Abbreviations: ATP, adenosine 5′-triphosphate; BCNU, carmustine, *N,N′*-bis(2-chloroethyl)-*N*-nitrosourea; EDTA, ethylenediaminetetraacetic acid; 9-OHE, 9-hydroxyellipticine; PMSF, phenylmethylsulphonyl fluoride.

associations or by transcriptional regulation. Since DNA topoisomerases have considerable recombinase activities, inappropriately activated topoisomerases in tumor cells lacking functional p53 may contribute to the genetic instability of these cells.

DNA topoisomerases are predominantly nuclear enzymes that regulate the topology of DNA and are essential for the viability of all proliferating eukaryotic cells. There are two major groups of topoisomerases, the type I and type II enzymes. Both types are targets for many commonly used antineoplastic agents which act by stabilizing covalent DNA-topoisomerase complexes. In addition, DNA topoisomerases modulate the sensitivity toward alkylating agents due to their involvement in DNA repair. In this paper, the repair functions of DNA topoisomerases as well as their regulation by the oncogene suppressor protein p53 are discussed.

MATERIALS AND METHODS

Drugs and chemicals. Cisplatin, mitomycin C and *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) were obtained from Lilly (St. Cloud, France), Laboratoire Choay (Gentilly, France), and Bristol (Paris-La Défense, France), respectively. [^{3}H]Thymidine and [^{14}C]leucine were obtained from Amersham (Buckinghamshire, U.K.). All other chemicals were of reagent grade.

DNA substrates, enzymes and antibodies. Supercoiled plasmid pBR322 DNA and DNase I were purchased from Boehringer Mannheim (Mannheim, Germany). Highly catenated kinetoplast DNA was isolated from *Crithidia fasciculata* kindly provided by Dr. Guy Riou (Institut Gustave Roussy, Villejuif, France) and purified by cesium chloride/ethidium bromide density centrifugation. The Ab-1 (clone 421) antibody which recognizes both wild type and mutant p53 was purchased from Oncogene Science (Cambridge, MA, U.S.A.).

Cell culture and colony formation assays. The Chinese hamster fibrosarcoma cell line DC-3F and the 9-hydroxyellipticine resistant subline DC-3F/9-OHE were grown as described previously [1]. The M1 murine myeloid leukemia cell line (clone S6) and its transfectant LTR-13 have been characterized earlier [2]. The parental M1 cells, which express neither p53 protein nor mRNA were stably transfected with a temperature-sensitive p53 mutant (LTR-13: Ala→Val at position 135), which shows wild type conformation at 32°C and mutant conformation at 37°C [3]. Cytotoxicity was determined by colony formation after 3 h drug exposure followed by incubation in a drug-free medium as described previously [4].

Alkaline elution and KCl/SDS precipitation. Interstrand DNA cross-links were measured by alkaline elution as described by Kohn *et al.* [5]. Briefly, cells were labeled with [^{14}C] or [^{3}H]thymidine, treated with various doses of cisplatin for 3 h, incubated for 21 h in a drug-free medium and irradiated with 300 rads in ice-cold Hanks' balanced salt solution containing 0.02% EDTA. Cells were then collected on membrane filters, lysed with sodium dodecyl sulfate (SDS) and treated with proteinase K. DNA was eluted in the presence of 0.1% SDS. Under these conditions, only DNA interstrand cross-links were assayed.

The formation of camptothecin-stimulated DNA-protein complexes in living cells was quantitated by the KCl/SDS coprecipitation assay as previously described in detail [6].

Nuclear extracts and decatenation. Nuclear extracts were prepared from about 5 $\times 10^7$ exponentially growing cells as earlier described [7]. Decatenation was carried out in a mixture containing 50 mM Tris/HCl, pH 8, 10 mM $MgCl_2$, 125 mM KCl, 5 mM EDTA, 5 mM dithiothreitol, 1 mM ATP and 200 ng of kinetoplast DNA. Reactions were initiated by the addition of nuclear extracts and allowed to proceed at 30°C for the indicated times. Reac-

tions were stopped by addition of 1% SDS, 0.5% bromophenol blue and 30% glycerol. The samples were electrophoresed in 1.2% agarose gels at 5 V/cm for 4 h in Tris/borate EDTA buffer, pH 8.3. Liberated minicircles were quantified by densitometric scanning of photographic negatives of the ethidium bromide-stained agarose gels.

Immunoprecipitation and DNA relaxation assay. About 5×10^7 S6 or LTR-13 cells were incubated at 32°C for 45 min and cellular extracts were prepared as described by Gobert *et al.* [8]. One milliliter of nuclear extract (about 2.5 mg protein) was incubated for 1 h on ice in the presence of 10 μl of Ab-1 antibody. Protein A-Sepharose beads (100 μl) were then added and the immunoprecipitates incubated for an additional 1 h under gentle agitation. The immunoprecipitates were washed twice as described previously [8] and the pellets were resuspended in 40 μl of 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 200 mM KCl, 1 mM PMSF, 1 mM β-mercaptoethanol and 50% (v/v) glycerol. The catalytic activity of the immunoprecipitates was determined by relaxation of supercoiled DNA in the presence of 10 mM $MgCl_2$ as described previously [8]. Relaxed DNA was quantified by densitometric scanning of photographic negatives of the ethidium bromide-stained agarose gels.

DNase I digestion. Cells labeled with [^{3}H]thymidine as described above were washed with reaction buffer (RB: 10 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.5) and allowed to swell in RB for 5 min on ice. Nonidet NP40 was added to 0.5% and nuclei were collected by centrifugation, washed with RB and resuspended in RB at 8 absorbance units (260 nm) per ml. DNase I was added at 240 U/ml. After digestion, samples were mixed with equal volumes of cold 10% perchloric acid, chilled on ice for 30 min and centrifuged at 2000 × ***g*** for 20 min. Radioactivity in the supernatant fraction was then determined by liquid scintillation counting.

RESULTS AND DISCUSSION

DNA topoisomerases: functions and activities

DNA topoisomerases catalyze many types of interconversions between topological isoforms of DNA through transient DNA cleavage, strand passing and religation (for a recent review, see [9]). Higher eukaryotes have four different DNA topoisomerases. There are topoisomerases I and III, which are both type I enzymes, and topoisomerases IIα and IIβ, two isoforms which belong to the type II family. Both type I and type II enzymes can relax DNA. In addition, topoisomerase II can decatenate intertwined DNA molecules. Whereas little is known about the biological functions of topoisomerases III and IIβ, topoisomerases I and IIα have been extensively studied.

Topoisomerase I is predominantly located in the nucleolar region in proliferating cells [10]. The enzyme is associated with genes and regions undergoing active transcription by RNA polymerase I or II [11, 12] and is involved in the elongation step. In addition, topoisomerase I is a component of the transcription factor II D complex (TFIID) and serves both to repress basal transcription and to stimulate activated transcription. The repressor activity is specific for promoters that contain a TATA motif and seems to rely on protein-protein interactions rather than on catalytic activity [13]. Recent results suggest that topoisomerase I may also possess a protein kinase activity, which is specific for serine residues in splicing factors containing an Arg-Ser motif [14].

While topoisomerase I is involved in transcription at several different levels, the main functions of topoisomerase II seem to be in mitosis where the enzyme is needed for chromosome condensation and separation of intertwined DNA molecules [15–18]. In addi-

tion to its catalytic activity, topoisomerase II may play a structural role in maintaining the chromatin structure of both interphase cells [19] and mitotic chromosomes, where the enzyme is associated with the DNA at the matrix attachment regions (MARs), which maintain the DNA loops [20–22]. Recent results show that the α isoform is specifically associated with the nuclear matrix during DNA synthesis [23] and with the centromeres during metaphase [24].

The amount and stability of topoisomerase I and IIβ show no significant fluctuations across the cell cycle [25, 26]. In contrast, topoisomerase IIα is regulated in a highly proliferation- and cell cycle-dependent manner with the highest protein levels present during late G2/early M, and the lowest during early G1 phase (for a recent review, see [27]). The activity of topoisomerase I and II is regulated by phosphorylation as well as by formation of molecular complexes with other proteins such as e.g. casein kinase II (for review, see [28]). Finally, both types of enzymes are substrates for poly(ADP-ribose)polymerase and poly-(ADP)-ribosylation is associated with a reduction of catalytic activity.

Variations in topoisomerase activities influence the cellular sensitivity toward DNA damaging agents

Cells resistant to DNA damaging agents often show altered DNA topoisomerase II activities. The resistance of human Burkitt lymphoma cells to nitrogen mustard was associated with decreased nitrogen mustard induced DNA interstrand crosslinking [29, 30]. These cells also showed an about 4-fold increased topoisomerase II activity whereas the topoisomerase I activity was not changed. Interestingly, after one month in the absence of selection pressure the nitrogen mustard resistance was reduced from 10- to 3-fold. This was accompanied by increased formation of nitrogen mustard-induced DNA crosslinking and a decrease of the topoisomerase II activity to a level comparable to that of the parental cell line [29].

The topoisomerase activities were also studied in L1210 murine leukemia cells that are about 15-fold resistant to cisplatin [31]. These cells showed increased sensitivity toward topoisomerase II inhibitors such as amsacrine, which was associated with a 3-fold increase in topoisomerase II activity. In contrast, no changes were observed for topoisomerase I. Sequential treatment with amsacrine and cisplatin led to an additive effect of the two drugs in the sensitive, parental cells but a supra-additive effect in the resistant cells. These results strongly suggest a role for topoisomerase II in the cisplatin-resistance of the resistant cells [31].

To further study the role of topoisomerase II, the cross-resistance toward alkylating agents was determined for cells resistant to the topoisomerase II inhibitor, 9-hydr-

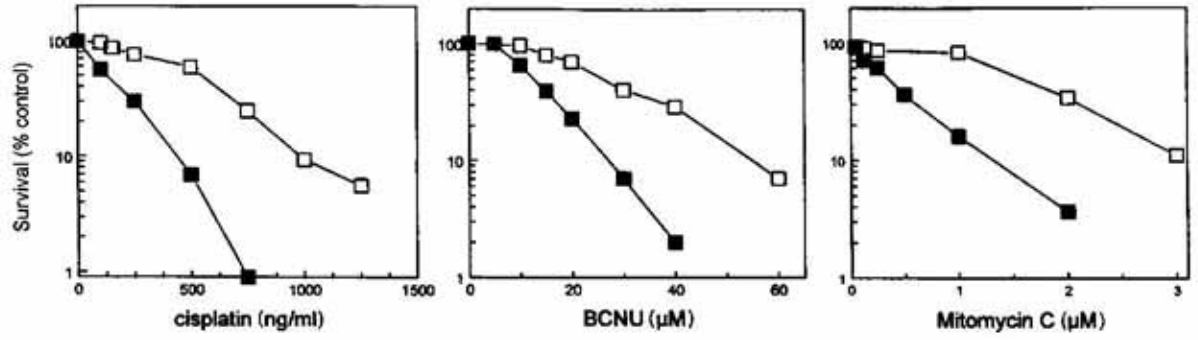

Figure 1. The cytotoxicity of cisplatin, mitomycin C and BCNU toward parental DC-3F cells (□) and 9-hydroxyellipticine-resistant DC-3F/9-OHE cells (■) was determined after 3 h of drug exposure followed by colony formation in the absence of drug.

oxyellipticine. In the 9-OHE-resistant cells the expression of the topoisomerase IIα protein is decreased to about $^1/_4$, whereas the topoisomerase IIβ isoform is lost [32]. The results show that the decrease in topoisomerase II activities is associated with hypersensitivity (collateral sensitivity) toward cisplatin, mitomycin C and BCNU (Fig. 1). Cisplatin treatment results in the formation of poorly toxic, monofunctional adducts which subsequently are converted into highly toxic, bifunctional

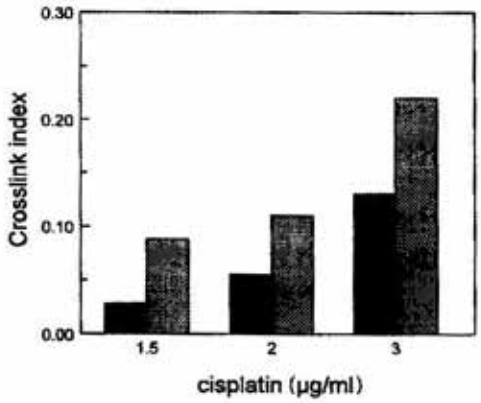

Figure 2. DNA interstrand cross-links.

Cells were treated for 3 h with cisplatin followed by 21 h incubation in the absence of drug and then analyzed by alkaline elution. The cross-link index was calculated as described by Kohn *et al.* [5]. DC-3F (■) and DC-3F/9-OHE (▣) cells.

adducts. These DNA interstrand crosslinks can be quantitated by alkaline elution under deproteinizing conditions [5]. Figure 2 shows that the hypersensitivity of DC-3F/9-OHE cells toward cisplatin is associated with increased formation of DNA interstrand crosslinks in comparison to the parental DC-3F cells.

To further investigate the relationship between topoisomerase II expression and the resistance toward alkylating agents, Chinese hamster topoisomerase IIα was expressed in mouse mammary tumor cells [33]. The topoisomerase II-transfectants were 5- to 10-fold more resistant to cisplatin and nitrogen mustard. Together, these results strongly suggest a causal relationship between topoisomerase II expression and resistance toward alkylating agents.

Topoisomerase activities involved in DNA repair

To better understand the molecular basis for the involvement of topoisomerases in DNA repair, numerous studies have been carried out in which the DNA repair process was studied in the presence of topoisomerase inhibitors. In general, difficulties in interpretation of such studies can be explained by the fact that most clinically used topoisomerase inhibitors, both directly and indirectly, lead to formation of DNA strand breaks. However, recent studies propose that topoisomerases may be involved in DNA repair at several different levels including i) the initial recognition of DNA lesions, ii) recombination and iii) regulation of DNA structure.

DNA damage recognition

Recent results suggest that the ability of topoisomerase II to recognize specific DNA structures also includes the ability to recognize certain types of DNA lesions. *In vitro* studies show that topoisomerase II binds to cisplatin-treated DNA with higher affinity than to untreated DNA. Interestingly, increased binding was only observed in the presence of Mg^{2+}, which indicates that covalent rather than non-covalent DNA-topoisomerase interactions are involved [33]. Topoisomerase II can also recognize endogenous DNA lesions such as apurinic sites, apyrimidinic sites and deaminated cytosines (i.e. uracil-guanine mismatches) [34, 35]. Apurinic sites stimulate topoisomerase II mediated DNA cleavage by 10- to 18-fold when located within the 4-base overhang generated by enzyme-mediated cleavage [34]. Apyrimidinic sites and deaminated cytosines also stimulate topoisomerase II-mediated DNA cleavage but to a lesser degree than do apurinic sites. In addition to topoisomerase II, topoisomerase I can recognize

DNA damage. Uracils immediately 3′ to the topoisomerase I cleavage site stimulate topoisomerase I-mediated DNA cleavage [35] whereas the presence of mismatches or apurinic sites immediately 3′ to the cleavage site results in the formation of irreversible topoisomerase I-DNA complexes [36].

Recombination

Topoisomerase I and II are recombinases with both intra- and intermolecular DNA strand transfer activities [37, 38]. Studies in yeast expressing different levels of topoisomerase I show a direct association between topoisomerase I levels and the illegitimate integration of nonhomologous, foreign DNA. The hot spot sequences for integration correspond to sequences previously identified as topoisomerase I preferred cleavage sites [39]. Topoisomerase II has been implicated in some forms of illegitimate recombination in humans. Treatment of patients with the topoisomerase II inhibitor etoposide may result in secondary leukemias that are characterized by translocations at chromosome band 11q23 close to topoisomerase II cleavage sites [40]. Recent studies suggest that spontaneous DNA damage such as apurinic sites, apyrimidinic sites and deaminated cytosine residues may stimulate topoisomerase II-mediated cleavage and recombination [41].

Regulation of DNA structure

DNA topoisomerases might also influence the formation of interstrand cross-links through regulation of DNA structure. It has been shown that bifunctional cisplatin lesions are formed at a higher rate on supercoiled plasmids than on relaxed ones [42]. This may, at least in part, explain the association between topoisomerase II activity and DNA cross-link formation *in vivo*, since cells with lower topoisomerase activities are likely to have a higher degree of superhelical density. Alternatively, a decrease in topoisomerase II activity may lead to changes in chromatin structure and organisation as described for differentiating erythroleukemia cells, where a decrease in topoisomerase II activity is associated with a more compact chromatin structure [43]. As discussed earlier in this paper, 9-hydroxyellipticine-resistant cells have decreased topoisomerase II levels and show increased sensitivity to alkylating agents. In addition, in these cells the catalytic activity of topoisomerase II is decreased about 3-fold (Fig. 3A) and their modified chromatin structure is less accessible to DNase I digestion

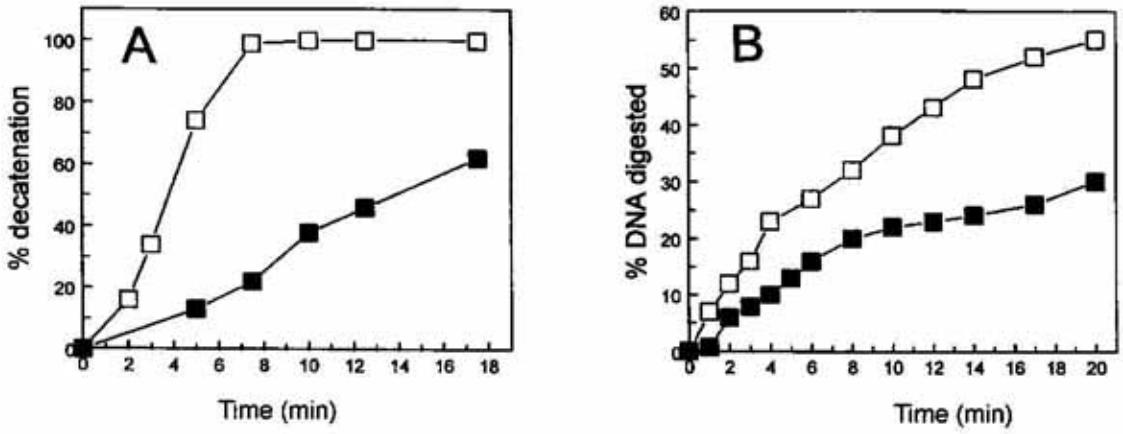

Figure 3. Topoisomerase II activity and nuclear structure in DC-3F (□) and DC-3F/9-OHE (■) cells.

A. Topoisomerase II activities in nuclear extracts from parental and 9-hydroxyellipticine-resistant DC-3F cells was determined by decatenation. B. The accessibility of chromatin in isolated nuclei from parental and 9-hydroxyellipticine-resistant cells was determined by DNase I digestion.

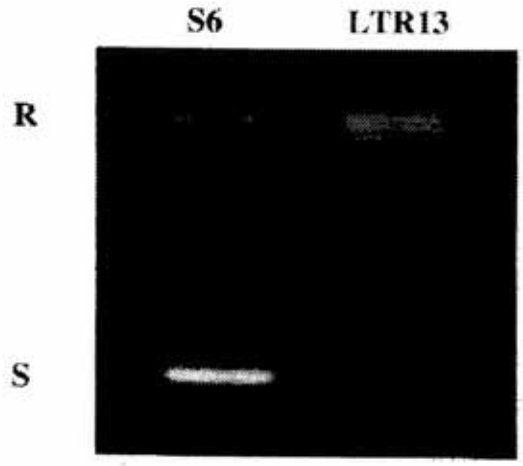

Figure 4. Association of p53 and topoisomerase I in cells expressing no (S6) or wild-type p53 (LTR-13 at 32°C).

The catalytic activity of p53 immunoprecipitates of nuclear extracts from S6 or LTR-13 cells was determined by relaxation of supercoiled DNA. S, supercoiled DNA; R, relaxed DNA.

(Fig. 3B). Interestingly, previous studies showed that increased topoisomerase II activities in nitrogen mustard-resistant cells were accompanied by a more open chromatin structure that showed enhanced sensitivity to DNase I digestion [30]. Together, these findings strongly support a causal relationship between topoisomerase II activity and chromatin structure which may influence the repair of monoadducts, and thereby the subsequent formation of interstrand DNA cross-links.

Regulation of DNA topoisomerases during DNA repair

Usually, DNA damage triggers multiple cascades which affect DNA repair, cell cycle progression and apoptosis. One of the components implicated in the regulation of these stress-induced pathways is the oncogene suppressor protein p53. Recent results suggest that p53 also is able to modulate topoisomerase activities either directly by molecular associations or by transcriptional regulation. Experiments with reporter genes suggest that p53 expression leads to transcriptional down-regulation of topoisomerase IIα [44, 45]. Another report describes the formation of molecular complexes between topoisomerase IIβ and p53 [46]. Finally, p53 and topoisomerase I form molecular complexes *in vitro* which stimulate the catalytic activity of topoisomerase I [8]. To determine if p53 and topoisomerase I also associate in living cells, p53 antibodies were added to myeloid leukemia cells expressing either no (S6) or wild type p53 (LTR-13 at 32°C). The results (Fig. 4) show that topoisomerase I activity copurified with p53 from LTR-13 cells as shown by relaxation of supercoiled DNA. The topoisomerase activity was not due to non-specific coprecipitation of topoisomerase since no similar activity was present in immunoprecipitates from the parental S6 cells, which lack p53. Similar results were observed for MCF7 cells which have wild-type p53. Mitomycin C treatment of MCF7 cells results in a time-dependent translocation of p53 from the cytoplasm towards the nucleus. This is accompanied by an increase in topoisomerase I activity which, at least in part, is due to the formation of molecular complexes between topoisomerase I and p53 [8]. These results suggest that the p53-mediated response to DNA damage may involve regulation of topoisomerase activities. It follows, that topoisomerase activities may be inappropriately regulated in about 50% of human tumors which lack functional p53 which could contribute to the overall genetic instability of these tumors.

In conclusion, there is convincing evidence that DNA topoisomerases play an important role in the repair of both endogenous and therapeutically-induced DNA damage. The repair-associated topoisomerase activity is, at least in part, regulated by the tumor suppressor protein p53. Since DNA topoisomerases have considerable recombinase activity, inappropriately modulated topoisomerases in tumor cells lacking functional p53 may contribute to the genetic instability of such cells.

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