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IN VIVO STIMULATION BY ANTITUMOR DRUGS OF THE TOPOISOMERASE II INDUCED CLEAVAGE SITES IN c-myc PROTOONCOGENE

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Summary. Several antitumor drugs including DNA intercalative and non intercalative agents induce in vitro and in vivo double-stranded DNA breaks by stabilization of a topoisomerase II-DNA complex [1][2]. In order to locate cleavage sites in an actively transcribed oncogene, N417 cells, originating from a human small cell lung carcinoma and containing 45-50 copies of c-myc oncogene, were treated with mAMSA, 9 hydroxyellipticine and VM 26. The presence of DNA lesions in c-myc was investigated by Southern blot hybridization with a human c-myc probe. In addition to normal bands, DNA patterns of drug treated-cells revealed the presence of new bands most likely corresponding to topoisomerase II-mediated cleavage as these bands were not found in untreated control DNA and in DNA treated with oAMSA, a biologically inactive stereoisomer of mAMSA. Major cleavage sites induced by drugs in the N417 cell c-myc locus were located in the 5' end of the c-myc exon 1 closely to some DNAse I hypersensitive sites which are assumed to reflect an activity of the gene. Therefore our data suggest that TopoII-mediated drug activity correlates with gene activity.

In vitro studies have shown that some anticancer drugs such as mAMSA (4'-(9-acridinylamino)-methanesulfon-m-anisidide), 9-OH-El (9-hydroxy ellipticine) and VM 26 (4' demethyl epipodophyllotoxin tenylidene-β-D-glucoside) blocks the rejoining reaction of DNA topoisomerase II (TopoII) by stabilizing a reversible enzyme-DNA complex, termed the cleavable complex [1][3][4]. Protein denaturant treatment of this cleavable complex leads to a double-stranded DNA break. Therefore it has

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been proposed that the cytotoxic action of these antitumor drugs is due to the formation of this cleavable complex.

The role of TopoII in cell proliferation has been recently emphasized : levels of TopoII activity are increased in regenerating hepatocytes and in tumor cells [5][6]. Several lines of evidence have also assessed the requirement of TopoII for transcriptional activity of genes [7]. The enzyme could affect the changes in chromatin structure accompanying gene activation or inactivation [8]. These results prompted us to investigate the effects of antitumor TopoII-mediated drugs on c-myc oncogene, a gene that is involved in cell proliferation processes and has been shown to be in a permanent state of transcriptional activity [9]. For this purpose, we used a human tumor cell line containing amplified and overexpressed c-myc gene as any TopoII-mediated drug effect was more likely to be appreciated than in cells containing a gene single copy. Our results show that some of the cleavage sites resulting from TopoII-drug stimulation map at DNAse I hypersensitive sites in the 5' end of c-myc.

METHODS

Small cell lung carcinoma cell line NCI-N417 was provided by Dr. D. Carney (Mater Misericordiae Hospital, Dublin). Cells were grown as tumor cells aggregates in previously described conditions [10].

mAMSA and oAMSA were provided by Dr. B. Baguley (Auckland Medical School, New Zealand), VM 26 by Sandoz Laboratories and 9-OH-El by Dr. N. Dat Xuong. mAMSA and VM 26 (20 mM) were dissolved in dimethyl sulfoxide and 9-OH-El (1 mM) in 0.1 N HCl. Cells in exponential growth (3-4 days of culture) were exposed to drugs for 3 hours at 37°C in fresh medium without foetal calf serum. Cells were washed with 50 mM Tris pH 7.9, 1 mM EDTA and immediately lysed with 2 % SDS, 50 mM Tris pH 7.9, 20 mM EDTA. Proteinase K was added to final concentration of 1 mg/ml for 4 hours at 50°C. Lysate was then treated twice with phenol and DNA extracted with ether, precipitated with ethanol, dried and then resuspended in 10 mM Tris pH 7.9, 0.1 mM EDTA as already described [11]. Procedure for cell nuclei isolation and treatment with pancreatic DNAse I have been already described in detail by Dyson et al [12].

Samples of cell DNA (2.5 µg) were digested to completion by EcoRI restriction endonuclease and electrophoresed in horizontal 1.2 % agarose slab gel as previously described [13]. The DNA fragments were denatured, transferred onto a GeneScreenPlus membrane (New England Nuclear) [13] and hybridized with the human c-myc probe (EcoRI-Cla I fragment, third exon) [14]. The probe was labeled with ^{32}P dCTP (2-3000 Ci/mmole) to a specific activity of 2-4 10^8 cpm/µg using the nick translation technique [11]. Hybridizations were performed in stringent condition [15] and

hybrids were revealed by autoradiography (Kodak XAR5 film). Phage λ -Hind III and ØX174-Hae III DNA fragments were used as gel calibration markers.

RESULTS

N417 cells, in exponential phase of growth were submitted in separated experiments to treatment with mAMSA, 9-OH-El and VM 26 at various concentrations (see Methods). These doses were previously shown to induce the formation of double-stranded DNA breaks in vitro [1][3][4] or in vivo by alkaline elution technique [2].

The DNA preparations were digested to completion with EcoRI restriction endonuclease and analyzed by Southern blot hybridization using human c-myc probe (third exon). In results presented in figure 1A, genomic DNA from untreated N417 cells revealed the presence on autoradiogramms of a germline 12.5 kb DNA (lane 1) usually observed for the human c-myc gene [16]. In addition to this band, genomic DNAs from drug

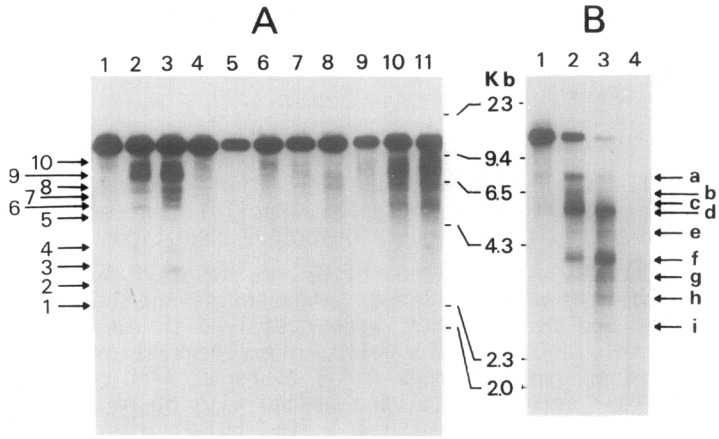

Figure 1. **A.** Cleavage sites induced in vivo by antitumor drugs in the amplified c-myc gene of N417 cells. DNA preparations (2.5 µg) from drug treated N417 cells (see Methods) were digested with EcoRI and analyzed by Southern blot hybridization using a ^{32}P-labeled c-myc probe (third exon). **1** : Untreated cells. **2 - 3** : mAMSA treated cells (5, 20 µM). **4** : oAMSA (5 µM). **5** : EthBr (50 µM). **6** : EthBr (50 µM) + mAMSA (5 µM) (see Results). **7 - 8** : 9-OH-El (1, 10 µM). **9** to **11** : VM 26 (0.25, 2.5, 5 µM).

B. DNAse I hypersensitive sites determined in the amplified c-myc gene of N417 isolated nuclei. DNA preparations (2.5 µg) were digested with EcoRI and analyzed by Southern blot hybridization using ^{32}P-labeled c-myc probe (third exon). **1** : Control nuclei. **2 - 4** : nuclei treated for 5 min. at 37°C with 0.2, 0.5. and 1 µg/ml of DNAse I.
The blots were exposed to Kodak XAR5 film to 3 days. DNA length fragments are given in kilobase pairs (Kb).

treated cells presented several hybridization bands of smaller size (lanes 2 to 11) that could be related to the in vivo c-myc gene cleavage products generated by drugs. mAMSA generated 6 to 7 bands of high intensity and some other bands of lower intensity (lanes 2 and 3). The intensity of bands increased with drug concentration indicating that cleavage sites were not generated at random (a new band arrow 7 lane 3 appeared for 20 μM of mAMSA). A similar cleavage pattern was observed with VM 26 (lanes 9, 10 and 11) and with 9-OH-El (lanes 7 and 8) (in that case the band intensity was fainter). In contrast, oAMSA (4'-(9-acridinylamino) methanesulfon-o-anisidide), a biologically inactive mAMSA stereoisomer induced only few cleavage bands of weak intensity (compare lanes 2 and 4 corresponding to 5 μM of mAMSA and oAMSA respectively).

In order to demonstrate whether an other intercalative drug with a high affinity for DNA was able to stimulate in vivo double-stranded DNA cleavage of the c-myc gene, ethidium bromide (EthBr) was used in the same conditions as mAMSA. The results showed that EthBr (50 μM) did not induce the production of cleavage bands (lane 5). Furthermore when N417 cells were pretreated for 30 min. with 50 μM EthBr and then incubated with 5 μM of mAMSA for 3 hours, a complete inhibition of c-myc gene cleavage was observed (compare results of lane 6 and of lane 2).

Cleavage sites generated by drugs were mapped in the c-myc locus (figure 2A). The cleavage sites do not seem to be arranged at random. Sites of preferential cleavage (generating the most intense bands), were located in the 5' part of the c-myc locus (arrows 7-8-9-10) and in the introns (arrows 1-2-4-5-6) ; however some sites were very close to exons. One cleavage site was located in the second exon (arrow 3).

DNAse I hypersensitive sites in c-myc gene were determined on N417 isolated nuclei [12] (figure 1B). Drug-stimulated TopoII cleavage sites and DNAse I hypersensitive sites clearly appear to map within the same regions of the c-myc 5' end (figure 2B). In contrast, the two DNAse I

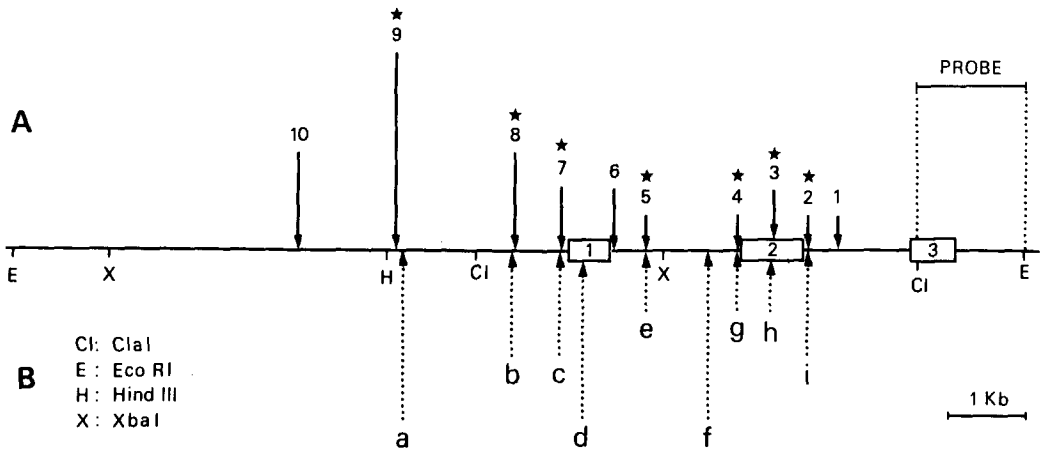

Figure 2. Mapping of mAMSA-induced cleavage sites and DNAse I hypersensitive sites in the c-myc locus of N417 cells. The restriction map was in agreement with that determined by Gazin et al [22]. The three c-myc exons are positioned in boxes **1**, **2** and **3**.
A. Full arrows indicate the position of the cleavage sites induced by mAMSA (20 µM). **B.** Dotted arrows indicate the position of the DNase I hypersensitive sites determined on N417 isolated nuclei.
Arrow size roughly reflects the importance of cleavage products.

hypersensitive sites in exon I (arrow d) and intron I (arrow f) have no TopoII cleavage site equivalents.

DISCUSSION

In this report, we have shown that antitumor drugs induce DNA breaks *in vivo* in the c-myc oncogene. These breaks are not randomly generated as only discrete bands were observed, their intensity increasing in function of drug concentrations. In case of a random cleavage, any increase in drug concentration would have generated a wide spectrum of heterogeneously sized fragments. Several arguments support the notion that these breaks are actually produced by drug stimulation of TopoII activity.

- In previous results obtained *in vitro* with calf thymus TopoII and a complete human c-myc gene inserted in a λ vector as DNA substrate, mAMSA highly stimulated the double-stranded cleavage of the c-myc gene [17]. The cleavage sites obtained *in vitro* and *in vivo* map at mostly similar places.

- oAMSA was not active on the cleavage process, consistent with experiments indicating that this drug does not stimulate TopoII activity in vitro [1], whereas mAMSA does it.

- When cells were exposed to EthBr prior to mAMSA treatment, DNA breakage was inhibited. EthBr has been already shown to inhibit the formation of the TopoII-DNA cleavable complex in vitro and in vivo [18].

Drug-induced preferential cleavage sites map within a region located 5' to the first c-myc exon, that is implicated in the control of c-myc expression. Other minor sites are located in introns and exons of the gene. Whatever the biological significance of these sites, their presence could be correlated with TopoII recognition consensus sequences [19].

Some TopoII cleavage sites (stars in figure 2A) map clearly within DNAse I hypersensitive sites or very closely to these sites. Similar observations have been already reported in vivo for SV40 chromatin [20] and in vitro system consisting of Drosophila melanogaster TopoII and 87A7 heat shock locus [21]. This situation argues in favor of common targets for DNAse I and TopoII or is likely to be related to an open structure of chromatin [8] in transcriptionally active genes (or parts of genes), since such a structure would be more accessible to both enzymes. To get insight on this point, it will be interesting to study TopoII drug-stimulated activity on genes which are alternatively active or inactive. In that respect, it is interesting that DNA from drug-treated N417 cells probed with a human β_1 globin pseudogene (supposed to be inactive in this situation) did not reveal the presence of cleavage bands (unpublished results).

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