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Doxorubicin-induced DNA breaks, topoisomerase II activity and gene expression in human melanoma cells

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Abstract—We have analyzed five human melanoma cell lines, displaying variable doxorubicin resistance (1- to 6-fold), for drug-induced DNA breaks, topoisomerase II activity and mRNA expression. Enhanced drug efflux was not the reason for doxorubicin resistance of these tumor cells although they overexpressed the transmembrane 170 kDa P-glycoprotein. Doxorubicin-induced DNA lesions (2-fold) and topoisomerase II activity (7-fold) were higher in HM-1 and G361 cells than in the less doxorubicin-sensitive NH and FCCM-9 cells. Topoisomerase II mRNA expression was also 2-fold higher in HM-1 and G361 cells. Doxorubicin-induced DNA breaks and topoisomerase II activity inversely correlated with the degree of doxorubicin sensitivity. Southern blot analysis showed variation in the hybridization pattern of topoisomerase II gene in doxorubicin-resistant cells when compared to sensitive cells. This study portrays the low doxorubicin sensitivity of NH and FCCM-9 cells as "atypical" and emphasizes the importance of DNA damage and topoisomerase II activity in cellular low doxorubicin resistance.

Several studies have focussed on the mechanisms of cellular resistance [1], and drug efflux mediated by transmembrane 170 kDa P-glycoprotein [2, 3] and alteration in cellular detoxification have received considerable attention [4–6]. The importance of protein-associated DNA breaks and topoisomerase II activity in cellular drug resistance is also emphasized in many studies [7–12]. Our earlier studies with doxorubicin-resistant murine leukemic P388/R84 cells show that rapid drug efflux and glutathione detoxification partially account for doxorubicin resistance of this cell line [13–16]. We also observed reduced doxorubicin-induced DNA damage in P388/R84 cells as compared to doxorubicin-sensitive P388 cells [17] even if doxorubicin retention was enhanced in P388/R84 cells by trifluoperazine or verapamil. These observations suggested that besides rapid drug efflux and detoxification, DNA damage and repair may also contribute to doxorubicin resistance of P388/R84 cells [17]. Protein-associated DNA strand breaks and cross-links occur in cells treated with doxorubicin and a variety of DNA intercalators, and association of topoisomerase II with protein-associated DNA breaks has been well-documented [7–12, 18–21]. The relative importance of drug interference in topoisomerase II function has been emphasized in several studies on tumor cell resistance to 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (VP-16), and doxorubicin [17, 19, 20, 22, 23]. In the present study, we have characterized five human melanoma cell lines, with varying levels of low doxorubicin resistance, for drug-induced DNA breaks, and for topoisomerase II activity and gene expression.

Materials and Methods

Drug and reagents. Doxorubicin hydrochloride (Adriamycin®, NSC-123127) was obtained from Adria Laboratories, Inc. (Columbus, OH). Stock solutions were prepared and diluted before each experiment with Ca^{2+}- and Mg^{2+}-free 0.05 M phosphate-buffered saline (PBS).* The recombinant plasmid pBShTOP2 carrying the human topoisomerase II gene was a gift from Dr. J. C. Wang, Harvard University, Cambridge, MA.

Cell lines. FCCM-2 and FCCM-9 cell lines were established in this laboratory from tumors of two separate melanoma patients. Human melanoma HM-1, G361 and NH cell lines were obtained from Dr. Michael Wick, Dana Farber Cancer Center, Boston, MA. The characteristics of these cell lines have been reported elsewhere [24]. Cells, as monolayer cultures, were maintained in RPMI-1640 medium containing 25 mM HEPES, 10% fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL) and growth factors: bombesin (10 nM) (Sigma, St. Louis, MO) and 1% SGF-9 (Scott Lab., Fiskeville, RI). Cells were trypsinized with 0.025% trypsin–0.02% EDTA for 5 min at room temperature; then they were resuspended in RPMI-1640 medium containing 10% fetal bovine serum and monodispersed cells were taken for all experiments.

Soft agar assay. Doxorubicin cytotoxicity was determined by a soft agar colony forming assay. The details of the assay procedure have been published previously [13]. Colonies containing more than 50 cells were counted from three different wells for determining IC_{50} and plating efficiency. The plating efficiency varied from 15 to 35%.

Alkaline elution of DNA fragments. The alkaline elution procedure of Kohn *et al.* [25] was followed for estimation of DNA strand breaks in cells after doxorubicin treatment. Drug-treated ([*methyl*-^{14}C]thymidine labeled) and untreated (internal standard, [^{3}H]thymidine labeled and γ-irradiated) cells (1×10^6) were placed on 2 μM polyvinyl chloride filters (25 mm diameter, Millipore Corp., Bedford, MA) and washed with 5 mL of ice-cold PBS. The cells were lysed at room temperature in 5 mL of 0.2% Sarkosyl solution (pH 10) containing 2 M NaCl, 0.04 M disodium EDTA and 0.5 mg/mL proteinase K (Bethesda Research Laboratories, Gaithersburg, MD), and DNA on the filter was eluted with 0.02 M tetrahydroammonium hydroxide–EDTA buffer (pH 12.2). The details of the alkaline elution assay and the quantitation of DNA breaks as rad equivalents have been reported previously [17, 26].

Topoisomerase II activity. The nuclear extracts of cells from log-phase cultures were prepared according to the procedure of Sullivan *et al.* [27]. The protein content of the nuclear extract was determined by the method of Bradford [28]. The reaction mixture (50 μL) for the topoisomerase II assay consisted of: 50 mM Tris–HCl (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM EDTA, 30 μg/mL bovine serum albumin, 1 mM ATP, 0.4 μg of supercoiled pBR322 DNA and aliquots of nuclear extract. The reaction was carried out at 37° for 30 min and stopped by the addition of 10 μL of 2% sodium dodecyl sulfate (SDS) containing 0.05% bromophenol blue

* Abbreviations: PBS, phosphate-buffered saline; MDR, multidrug resistance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; and VCR, vincristine.

Table 1. Doxorubicin toxicity, DNA damage, topoisomerase II activity and gene expression of human melanoma cell lines

Cell line	IC_{50} (μM)	Relative resistance* (fold)	DNA breaks† (rad equivalent) Doxorubicin (μM) 0.1	0.5	1.0	Topoisomerase II Activity‡ (units/μg protein)	mRNA expression§ (arbitrary units)
HM-1	0.09 ± 0.02	1.0	44 ± 15	76 ± 14	121 ± 38	89 ± 19	39.4 ± 3.4
G361	0.15 ± 0.02	1.7	36 ± 4	64 ± 22	111 ± 41	72 ± 25	32.0 ± 3.0
FCCM-2	0.24 ± 0.10	2.7	62 ± 2	87 ± 14	120 ± 2	72 ± 25	18.0 ± 2.0
NH	0.35 ± 0.08	3.9	15 ± 7‖	26 ± 14‖	70 ± 31	17 ± 6	20.4 ± 1.9
FCCM-9	0.55 ± 0.04	6.1	19 ± 6‖	27 ± 9‖	67 ± 14	12 ± 7	16.3 ± 2.2

Values are means ± SD (N = 3).
* On the basis of HM-1 cytotoxicity.
† By alkaline assay procedure after doxorubicin treatment of cells for 1 hr at 37°.
‡ Amount of protein required for complete relaxation of 0.4 μg supercoiled pBR322 DNA.
§ By slot blot hybridization with human topoisomerase II cDNA probe.
‖ DNA breakage was statistically different ($P \leq 0.05$ by Student's t-test) from that in HM-1 cells.

and 30% Ficoll. The DNA in the assay mixture was analyzed by electrophoresis on 1.2% agarose gel in 89 mM Tris-borate buffer (pH 8), stained with 2 μg/mL ethidium bromide and photographed under UV light. The relaxation assay was carried out with serially diluted nuclear extracts, and the amount of protein required for complete relaxation of 0.4 μg supercoiled DNA (1 unit of enzyme activity) was determined by interpolation.

Preparation of topoisomerase II gene probe, RNA extraction and slot blot hybridization. The topoisomerase II cDNA is a 5.6 kb insert cloned at the *Sma*I site of Bluescript plasmid vector. The recombinant plasmid was digested with *Eco*RI (Boehringer Mannheim Biochemicals, Indianapolis, IN) and electrophoresed on 1% agarose gel. The 5.4 kb fragment of pBShTOP2 was electroeluted, purified by passage through an Elutip-D column (Schleicher & Schuell, Keene, NH) and concentrated by ethanol precipitation [29]. The DNA fragments were labeled with [^{32}P]dCTP (3000 Ci/mmol, New England Nuclear, Boston, MA) by the random primer method [30] using hexadeoxyribonucleotides (Pharmacia, Piscataway, NJ) and Klenow fragment of DNA polymerase (Boehringer Mannheim Biochemicals).

RNA was extracted from cells according to the procedure of Chirgwin *et al.* [31] and its integrity was examined by monitoring 18S and 28S bands in the ethidium bromide stained gels. For slot blot analysis, RNA was diluted serially and spotted on nitrocellulose filters in a Bio-Dot slot format apparatus (Bio-Rad, Richmond, CA). The filters were dried and baked at 80° for 2 hr and prehybridized and hybridized with ^{32}P-labeled topoisomerase II cDNA probe [24]. RNA blots hybridized with β-actin probe served as the control. The blots were washed, autoradiographed, and scanned in a Zeineh soft laser densitometer under tungsten light; values are expressed in arbitrary units.

Southern blot hybridization. Genomic DNA extracted from the cell lines was digested with *Eco*RI [29]. The digested DNA (20 μg) was fractionated on 1% agarose gel and stained with ethidium bromide. DNA in the gel was denatured, neutralized and transferred to nitrocellulose filters [29, 32]. The filters, after baking at 80° for 2 hr, were prehybridized and hybridized with the topoisomerase II cDNA probe [24].

Results and Discussion

Doxorubicin toxicity of melanoma cells. The five human melanoma cell lines differed 1- to 6-fold in their sensitivity to doxorubicin (Table 1). The FCCM-9 cells were 6-fold less sensitive when compared to HM-1 cells. The other cell lines, G361, FCCM-2 and NH, were 2-, 3-, and 4-fold less sensitive to doxorubicin than the HM-1 cell line. Flow cytometry analysis showed that cellular doxorubicin

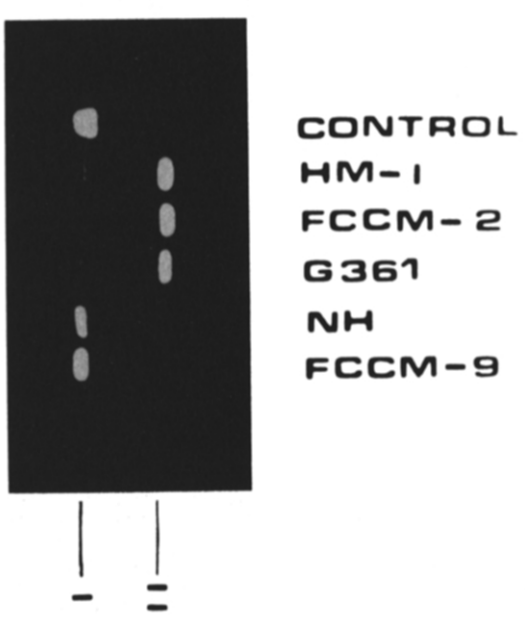

Fig. 1. Topoisomerase II activity of human melanoma cells. The relaxation assay mixture (50 μL) contained: supercoiled pBR322 DNA (0.4 μg), Tris–HCl (50 mM, pH 7.5), KCl (85 mM), $MgCl_2$ (10 mM), EDTA (0.5 mM), ATP (1 mM), dithiothreitol (10 mM), BSA (30 μg/mL) and nuclear extract (2.5 ng protein). The reaction was carried out at 37° for 30 min. Key: supercoiled DNA (I) relaxed DNA (II). Control: no enzyme.

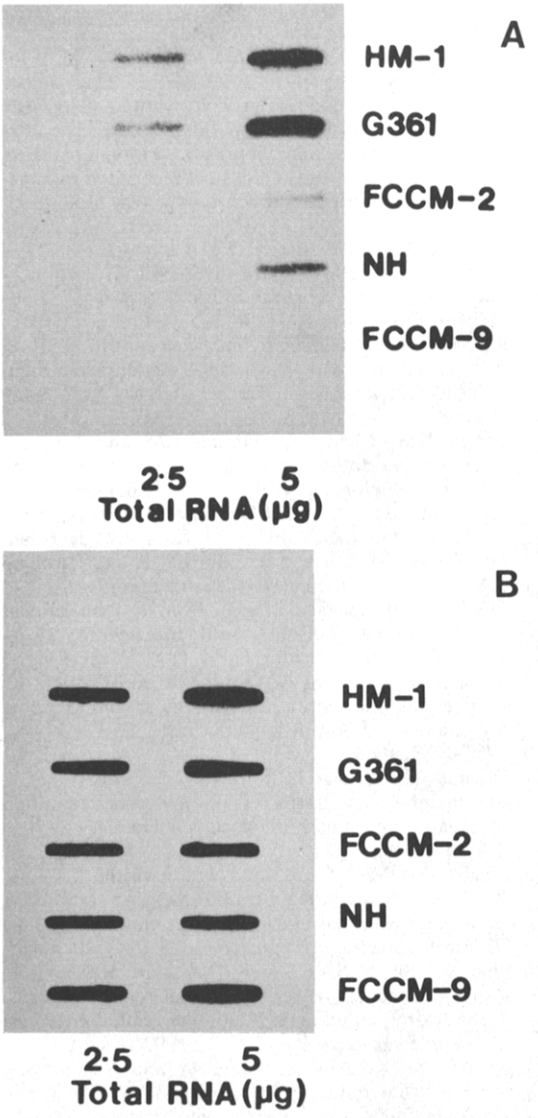

Fig. 2. Slot blot analysis of total RNA of melanoma cells hybridized with topoisomerase II (A) and β-actin (B) cDNA probes. Serial dilutions (2.5 and 5 μg) of RNA were spotted on nitrocellulose filters and hybridized with random primer labeled probes. A slot blot of β-actin was used as the internal control. The relative values derived from densitometric scans of slot blots are given in Table 1.

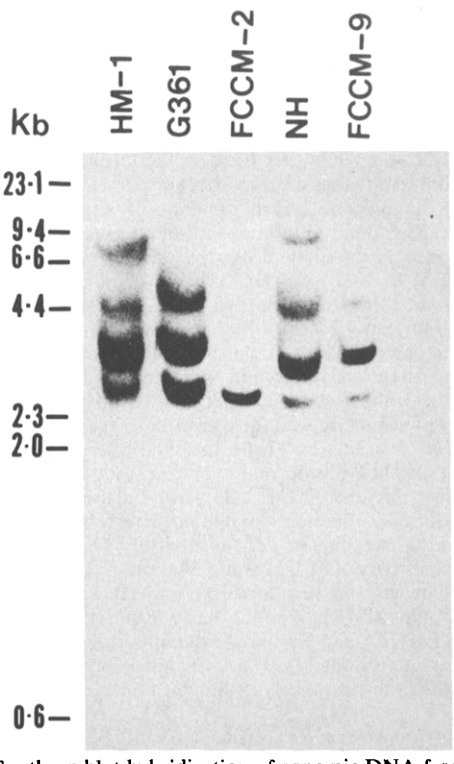

Fig. 3. Southern blot hybridization of genomic DNA from melanoma cells with human topoisomerase II cDNA probe. *Eco*RI digested DNA (20 μg) was fractionated on 1% agarose gel, blotted onto the nitrocellulose filter, and probed with a ^{32}P-labeled random primer probe of topoisomerase II cDNA (5.4 kb).

retention was relatively high in all cell lines compared to retention in doxorubicin-sensitive murine leukemic P388 cells. The peak fluorescence channel values of cellular doxorubicin in melanoma cell lines were 111 (FCCM-9), 110 (NH), 106 (FCCM-2), 108 (G361), and 113 (HM-1), compared to 98 and 31 for P388 and P388/R84 cells, respectively [24]. Co-incubation with trifluoperazine or verapamil did not enhance the cellular doxorubicin retention or cytotoxicity. However, the less doxorubicin-sensitive cell lines such as FCCM-9, FCCM-2, and NH have high expression of P-glycoprotein as revealed by their reaction to C219 monoclonal antibody both in the percentage of C219 positive cells (27–63%) and peak fluorescence channel values (PFCV = 24–54) compared to positive control CH^{R}C5 cells (68%; and PFCV = 69) [24]. It appeared that the differential doxorubicin sensitivity of these melanoma cell lines was not due to low cellular doxorubicin retention but to some other mechanism.

Doxorubicin-induced DNA breaks and topoisomerase II activity. The amount of DNA breaks in different melanoma cells generated by doxorubicin treatment is given in Table 1. DNA breaks were approximately 2-fold higher in HM-1, G361 and FCCM-2 cells compared to NH and FCCM-9 cells at all three doxorubicin levels tested. The topoisomerase II activity of the five melanoma cell lines is given in Fig. 1 and Table 1. The HM-1, G361 and FCCM-2 cells had 5- to 7-fold higher activity than NH and FCCM-9 cells. The doxorubicin sensitivity of these cells appeared to be inversely correlated with DNA break frequency and topoisomerase II activity. The NH and FCCM-9 cells, which were relatively less sensitive to doxorubicin, had less topoisomerase II activity and lower DNA breaks (upon doxorubicin treatment) than HM-1 and G361 cells. No such correlation was noted, however, for FCCM-2 cells.

Expression of topoisomerase II gene. The expression of topoisomerase II and β-actin genes in human melanoma cells is shown in Fig. 2. The mean densitometric readings of the autoradiographs (Table 1) showed that the topoisomerase II mRNA expression was about 2-fold higher in HM-1 and G361 cells than in the relatively less sensitive FCCM-2, NH, and FCCM-9 cells. The variation in the number of DNA lesions and in the activity and expression of topoisomerase II gene caused by doxorubicin treatment, and also the appearance of an inverse correlation of these effects with the doxorubicin sensitivity of cells, suggested that lower DNA damage in association with a

lowered function of topoisomerase II contributed to the lower sensitivity of NH and FCCM-9 cells to doxorubicin.

Southern blot analysis of genomic DNAs of melanoma cell lines with the topoisomerase II cDNA probe (Fig. 3) showed hybridization to four *Eco*RI fragments in the range of 9.4, 4.4, 3.1, and 2.5 kb in all cell lines except FCCM-2. In FCCM-2, only one band at 2.5 kb was visible. We also observed alteration in the hybridization intensity of the 9.4, 4.4, and 2.5 kb fragments in FCCM-9. Single base change point mutations in the topoisomerase II gene have been reported in drug-resistant HL-60/AMSA [33] and VM-26/VCR sublines [34].

In conclusion, the five melanoma cell lines established from tumor biopsies displayed variability in their sensitivity to doxorubicin. Cellular doxorubicin retention was similar in these cell lines and was more than the retention in doxorubicin-sensitive P388 cells [24]. Enhanced drug efflux was not the reason for doxorubicin resistance of FCCM-9, NH, and FCCM-2 cells, in spite of their overexpression of 170 kDa P-glycoprotein and thus they cannot be classified as "classical" MDR cells [24]. Furthermore, glutathione content, although variable in these cells, did not correlate with the degree of sensitivity.* The low doxorubicin sensitivity of FCCM-9 and NH cells is due to reduced DNA damage and low topoisomerase II activity, and is thus "atypical" [35, 36]. Our data concur with the finding of Fry *et al.* [37] and emphasize the importance of DNA breaks, and topoisomerase II activity and expression in differential doxorubicin toxicity of human tumor cells.

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