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Induction of Tumor-specific Cytotoxicity and Apoptosis by Doxorubicin

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Abstract. *Doxorubicin (adriamycin), an anthracycline antibiotic, showed higher cytotoxic activity against human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG, promyelocytic leukemia HL-60) than against normal human cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF). Doxorubicin activated caspases 3, 8 and 9 in both HSC-2 and HL-60 cells, but induced internucleosomal DNA fragmentation only in HL-60 cells. Western blot analysis showed that doxorubicin did not significantly change the intracellular concentration of Bcl-2, Bax and Bad in HL-60 cells. Real-time PCR analysis showed that HPC cells expressed the highest amount of mdr1 mRNA, followed by HSC-2 > HGF > HSC-3 > HPLF > HSG > HL-60. ESR spectroscopy showed that doxorubicin produced no discernible radical under alkaline conditions (pH 7.4 to 10.5) except at pH 12.5, and it did not scavenge O_2^-, NO and DPPH radicals. The present study demonstrates that doxorubicin induces the tumor-specific cytotoxicity and some, but not all, apoptosis markers possibly by a radical-independent mechanism, and that mdr1 expression in the tumor cells is not related to the tumor specificity of doxorubicin.*

Doxorubicin, (8*S-cis*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)-oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione) (Figure 1), is an anthracycline antibiotic isolated from *Streptomyces peucetius* var. *caesius* and has the same anthraquinone chromophore and glycoside structure as daunomycin (1). Pharmacokinetics (2) and chemotherapeutic (3) and macrophage-mediated immunomodulation (4) activities of doxorubicin have been reported. The cytotoxicity of doxorubicin appears to be due to its ability to intercalate with DNA (especially with guanine residue) (5), interact with plasma membranes (6) and take part in oxidation-reduction reactions (7). Doxorubicin has been used for the treatment of cancer of the bladder (8), breast (in combination with other anticancer agents) (9) and prostate (10). Doxorubicin is suspected to be a human carcinogen.

We first investigated whether doxorubicin shows tumor-specific cytotoxicity against human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG, promyelocytic leukemia HL-60), in comparison with that against normal oral human cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF). Since there is some relationship between cytotoxic activity and radical generation/scavenging activity in some antioxidants (11-13), we also investigated whether doxorubicin produces radicals under alkaline conditions, and scavenges superoxide anion (O_2^-) [generated by hypoxanthine-xanthine oxidase (HX-XOD) reaction in the presence of spin-trapping agent 5,5-dimethyl-1-pyrroline 1-oxide (DMPO)], NO [generated by 1-hydroxyl-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) in the presence of spin-trapping agent 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO)] and diphenyl-2-picrylhydrazyl (DPPH) radical, using ESR spectroscopy.

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Key Words: Tumor specificity, apoptosis, doxorubicin, DNA fragmentation, mdr1.

Materials and Methods

Materials. The following chemicals were obtained from the indicated companies: RPMI 1640 medium, phenylmethylsulfonyl

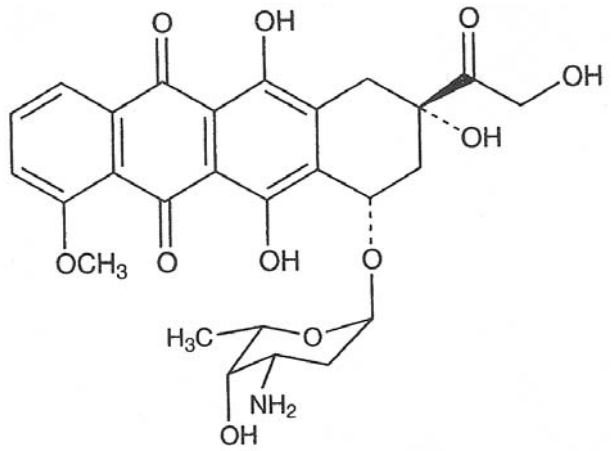

Figure 1. *Structure of doxorubicin.*

fluoride (PMSF), HX, XOD, diethylenetriaminepentaacetic acid (DETAPAC), DPPH (Sigma Chem. Ind., St. Louis, MO, USA); fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA); dimethyl sulfoxide (DMSO), doxorubicin hydrochloride (MW 580) (Wako Pure Chem. Ind., Osaka, Japan); gallic acid, ferulic acid (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan); DMPO, carboxy-PTIO, NOC-7, TEMP (Dojin, Kumamoto, Japan).

Cell culture. Three human oral tumor cell lines (HSC-2, HSC-3, HSG) and three human normal cells (HGF, HPC, HPLF) were cultured in DMEM supplemented with 10% heat-inactivated FBS. The HL-60 cells were cultured in RPMI 1640 supplemented with 10% FBS. Normal cells were prepared from the periodontal tissues, according to the guidelines of Meikai University Ethics Committee, Japan (No. 206), after obtaining informed consent from the patients. Since normal cells have a limited *in vitro* life-span (14), they were used at 5-8 population doubling level (PDL).

Assay for cytotoxic activity. Cells (other than HL-60 cells) were incubated at 5-6 x 10^3 cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 48 hours, the medium was removed by suction with an aspirator, and replaced with 0.1 mL of fresh medium containing various concentrations of test compounds. Near confluent cells were incubated for another 24 hours, and the relative viable cell number was then determined by MTT method. In brief, cells were replaced with fresh culture medium containing 0.2 mg/mL MTT and incubated for another 4 hours. The cells were lysed with 0.1 mL of DMSO, and the absorbance at 540 nm (A_{540}) of the cell lysate was determined, using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). The A_{540} of control cells were usually in the range of 0.40 to 0.90. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. Tumor specificity (TS) was determined by the following equation:

$$TS = \frac{[CC_{50}\,(HGF) + CC_{50}\,(HPC) + CC_{50}\,(HPLF)] \times (4/3)}{CC_{50}\,(HSC\text{-}2) + CC_{50}\,(HSC\text{-}3) + CC_{50}\,(HSG) + CC_{50}\,(HL\text{-}60)}$$

The viability of HL-60 cells was determined by trypan blue exclusion. HL-60 cells were incubated at 5 x 10^4/0.1 mL in 96-microwell, and various concentrations of test compounds were added. After incubation for 24 hours, the viable cell number was determined, as described previously. The density of control cells at harvest was in the range of 8-9 x 10^5/mL.

Assay for DNA fragmentation. Cells were washed once with PBS (-) and lysed with 50 μL lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate solution]. The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1-2 hours at 50˚C, and then mixed with 50 μL NaI sodium [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0] and then 250 μL of ethanol. After centrifugation for 20 minutes at 20,000 xg, the precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0]. The sample (10-20 μL) was applied to 2% agarose gel electrophoresis in TBE buffer [89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0]. A DNA molecular marker (Takara, Bio, Tokyo, Japan) and DNA from apoptotic HL-60 cells induced by UV irradiation were used for calibration (15). The DNA fragmentation pattern was examined in a photograph taken under UV illumination.

Assay for caspase activation. Cells were washed twice with ice-cold PBS (-) and lysed in solution (MBL, Nagoya, Japan). After standing for 10 minutes on ice and centrifugation for 5 minutes at 10,000 xg, the supernatant was collected. The lysate (50 μL, equivalent to 200 μg protein) was mixed with 50 μL of 2 x reaction buffer (MBL) containing substrates for caspase 3 (DEVD-*p*NA (*p*-nitroanilide)), caspase 8 (IETD-*p*NA) or caspase 9 (LEHD-*p*NA). After incubation for 2 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by microplate reader.

Western blotting. The cell pellets were lysed with 100 μL of lysis buffer [10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF]. The protein in the cell lysate was determined by Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS-0.005% bromphenol blue, 0.6% 2-β mercaptoethanol) and aliquots equivalent to 20 μg protein were applied to 15% SDS polyacrylamide gel electrophoresis, then transferred to PVDF membrane (Immobilon P, Millipore Corp, Bedford, MA, USA). The membranes were then blocked with 5% skim milk in PBS(-) plus 0.05% Tween 20 and incubated with anti-Bcl-2 antibody (1:1000), anti-Bax antibody (1:1000), anti-Actin antibody (1:1000) or anti-Bad antibody (1:1000) (Santa Cruz, Delaware, Cam, USA) for 90 minutes at room temperature or overnight at 4˚C, before incubation with horseradish peroxidase-conjugated anti-rabbit IgG for 1 hour at room temperature. Immunoblots were developed by Western Lightning™ Chemiluminescence reagent *plus* (Perkin Elmer Life Sciences, Boston, MA, USA).

Assay for quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR). The total RNA of the cells was isolated using the ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan). Synthesis of cDNA was done with 5 μg total RNA, 1 μg oligo dT primer and 0.2 μmol deoxynucleotide triphosphates, 10 units RNase inhibitor using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using the QuantiTect

Table I. *Tumor specificity of doxorubicin.*

	CC_{50} (μM)							
	Normal cells			Tumor cell lines				
	HGF	HPC	HPLF	HSC-2	HSC-3	HSG	HL-60	SI
Doxorubicin (Exp. I)	>100	>100	>100	0.9	0.75	3.7	2.0	>54.1
Doxorubicin (Exp. II)	N.D	211	160	<1.0	7.0	<1.0	1.1	>73.0
Doxorubicin (Exp. III)	233	414	500	0.68	2.9	1.9	0.47	255.0

$SI=\Sigma CC_{50}$ (normal)/ ΣCC_{50} (tumor) x (Correction for number of cell type)
N.D., not determined

SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and analyzed on an iCycler iQ Real-Time PCR Detection System (Bio-Rad). The primer sequences of mdr1 were previously described (16). Quantitative values were obtained from the threshold PCR cycle number, where the increase in signal associated with an exponential growth for PCR product becomes detectable.

Assay for radical intensity. The radical intensity of test samples was determined at 25˚C in 0.1 M Tris-HCl (pH 7.4, 8.5), 0.1 M $NaHCO_3/Na_2CO_3$ (pH 10.0, 10.5) or 0.1 M KOH (pH 12.5), using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency). Instrument settings; center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT: gain, 500, time constant, 0.1 second; scanning time, 2 minutes.

The O_2^- scavenging activity was determined, using the HX and XOD reaction system (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) 50 μL, 0.5 mM DETAPAC 20 μL, 8% DMPO 30 μL, 0.1 M PB 20 μL sample (in H_2O) 50 μL, XOD (0.5 U/ml in PB) 30 μL]. The radical intensity was determined 1 minute after mixing.

The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 80 μM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4, in the presence of 30% DMSO (microwave power, 5 mW; modulation amplitude, 5 mT; gain, 250; time constant, 0.1 second). When NOC-7 (NO generator) and carboxy-PTIO (spin trapping agent) were mixed, NO was oxidized to NO_2 and carboxy-PTIO was reduced to carboxy-PTI, which produced seven-line signals. Nine signals of carboxy-PTIO and carboxy-PTI were not overlapped with each other, and so it was easy to distinguish the signals of carboxy-PTIO from that of carboxy-PTI (17, 18). NO radical intensity was defined as the ratio of signal intensity of the first peak of carboxy-PTI (indicated by asterisk in Figure 6C) to that of MnO.

The DPPH radical scavenging activity contained the reaction mixture (total volume: 200 μL) [100 μM DPPH 140 μL, H_2O 20 μL, sample in DMSO 40 μL]. After 1 minute, the mixture was subjected to ESR spectroscopy (19).

Results

Tumor specificity. Doxorubicin (Figure 1) showed higher cytotoxicity against human tumor cell lines (HSC-2, HSC-3, HSG, HL-60) than against human normal cell lines (HGF, HPC, HPLF), yielding a tumor specificity index (TS) of 54-255 (Table I).

Induction of apoptosis. Doxorubicin induced activation of caspases 3, 8 and 9 in both HL-60 (A) and HSC-2 cells (B) (Figure 2). The extent of maximum caspase activation by doxorubicin was comparable with that achieved by actinomycin D (positive control). However, doxorubicin induced internucleosomal DNA fragmentation only in HL-60 cells (A) and not in HSC-2 cells (B) (Figure 3).

Western blot analysis showed that doxorubicin did not apparently change the intracellular concentration of Bcl-2, Bax and Bad in HL-60 cells (Figure 4).

Real-time PCR analysis showed that HPC cells expressed the highest amount of mdr1 mRNA, followed by HSC-2 > HGF > HSC-3 > HPLF > HSG > HL-60 (Figure 5, Table II). On the other hand, changes in GAPDH mRNA between the cells were much smaller (Figure 5, Table II).

Radical scavenging activity. ESR spectroscopy showed that doxorubicin produced no radical under alkaline conditions (pH 7.4 to 10.5) (Figure 6A). However, elevation of the pH up to 12.5 caused the generation of detectable radical (Figure 6A), accompanied by bluish coloration of the reaction buffer (data not shown).

Doxorubicin did not scavenge O_2^- (Figure 6B) (IC_{50}>340 μM), NO (Figure 6C) (IC_{50}>340 μM) or DPPH (Figure 6D) (IC_{50}>340 μM) radicals. On the other hand, positive controls, such as gallic acid (IC_{50}=0.15 μM) and ferulic acid (IC_{50}= 5.2 μM), effectively scavenged the O_2^-, NO and DPPH radicals, respectively (data not shown).

Discussion

The present study demonstrated that doxorubicin induced considerably higher tumor-specific cytotoxicity (TS=54-255), as compared to other chemically defined compounds including 65 steroidal saponins (TS=0.8-17) (Sakagami *et al.*, in preparation), 21 coumarins (TS=1-6) (20), 11 flavonoids and stilbenes (TS=2-5) (Chowdhury *et al.*, in preparation), 10 opiates (TS=1-4) (21), 5 redox compounds (hydroquinone, vitamin C, gallic acid, catechin, dopamine) (TS=2-5), 26, α,β-

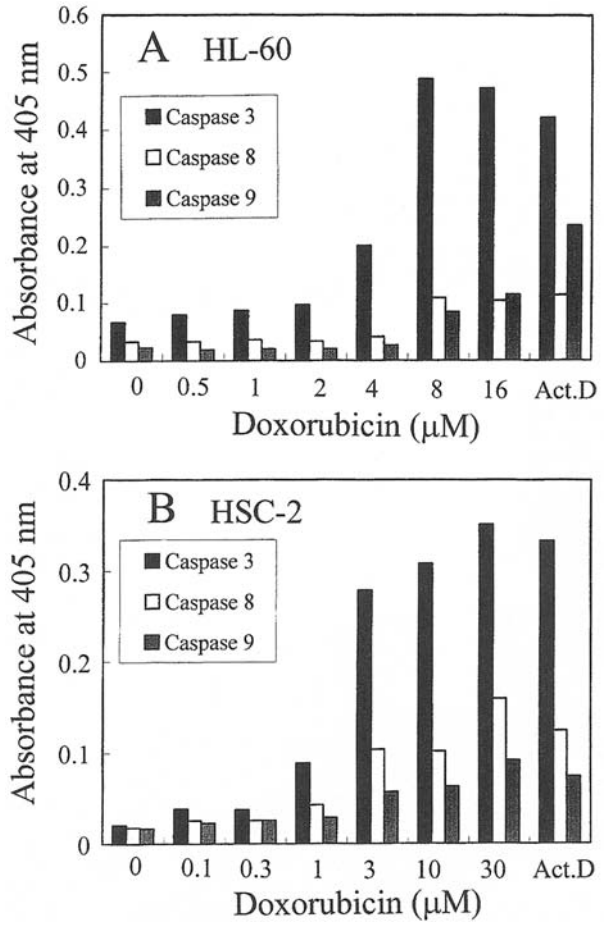

Figure 2. *Activation of caspases 3, 8 and 9 by doxorubicin. HL-60 (A) and HSC-2 (B) cells were incubated for 4 hours with the indicated concentrations of doxorubicin or 1 μg/mL actinomycin D (positive control), and the activities of caspases 3, 8 and 9 were assayed by substrate cleavage activity.*

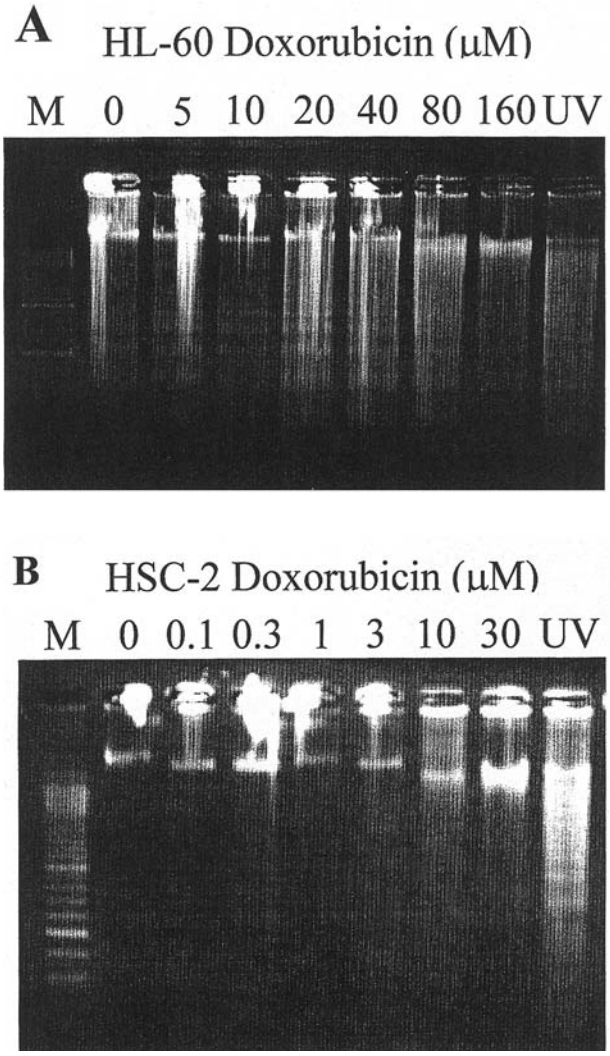

Figure 3. *Induction of DNA fragmentation by doxorubicin in HL-60 cells, but not in HSC-2 cells. HL-60 (A) and HSC-2 (B) cells were incubated for 6 hours with the indicated concentrations of doxorubicin, and DNA was extracted and applied to agarose gel electrophoresis. M, DNA marker; UV, UV irradiation.*

unsaturated ketones (TS=0.4-4) (22), 8 hydroxyketones (TS=1-18) (23), 23 β-diketones (TS=0.3-6) (24) and 6 styrylchromones (TS=0.8-23) (25). There was no close relationship between the tumor-specific cytotoxicity and apoptosis-inducing activity in these compounds (22-24). In this sense, it was apparent that doxorubicin had both the highest tumor-specificity and some apoptosis-inducing activity.

We found that doxorubicin did not induce DNA fragmentation in HSC-2 cells, although it activated caspases 3, 8 and 9. This suggests that the induction of internucleosomal DNA fragmentation requires the activation of caspases over a certain threshold level (compare the A_{405} between HL-60 and HSC-2 cells in Figures 2, 3). We also found that doxorubicin did not induce any apparent changes in the intracellular concentration of Bcl-2, Bax and Bad proteins, suggesting that the induction of apoptosis by doxorubicin may be incomplete. It remains to be investigated whether doxorubicin induces non-apoptotic cell death such as necrosis (characterized by cell swelling) or autophagy (characterized by vacuolization and expression of ATG 7 and beclin 1) (26-28).

ESR spectroscopy showed that doxorubicin did not generate the radical nor scavenged three popular radicals (both water soluble O_2^- and NO, and fat-soluble DPPH) near the physiological pH range, suggesting that a radical-mediated reaction is not involved in doxorubicin-induced cell death. However, the quinone moiety of the doxorubicin-DNA complex can react with H_2O_2 to form a strong

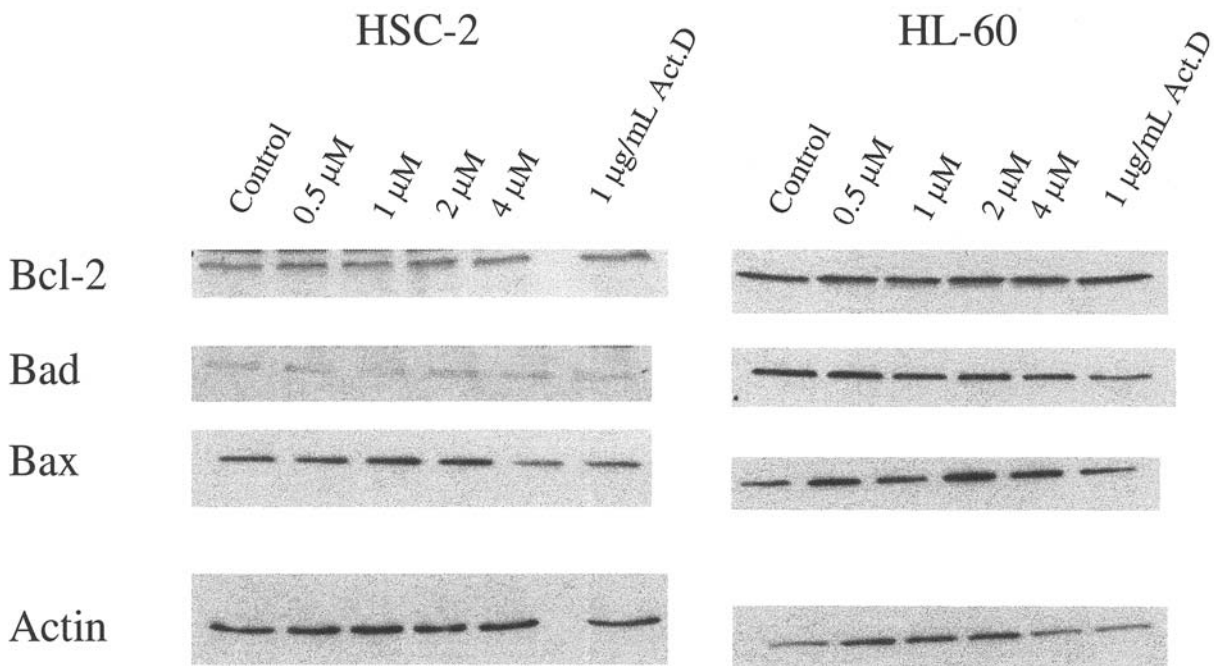

Figure 4. *Effect of doxorubicin on the intracellular concentration of Bcl-2, Bax, Bad and Actin in HL-60 cells. HL-60 cells were incubated for 6 hours with the indicated concentrations of doxorubicin, and proteins were extracted and applied to SDS-PAGE for the detection of Bcl-2, Bax, Bad and Actin by Western blot analysis.*

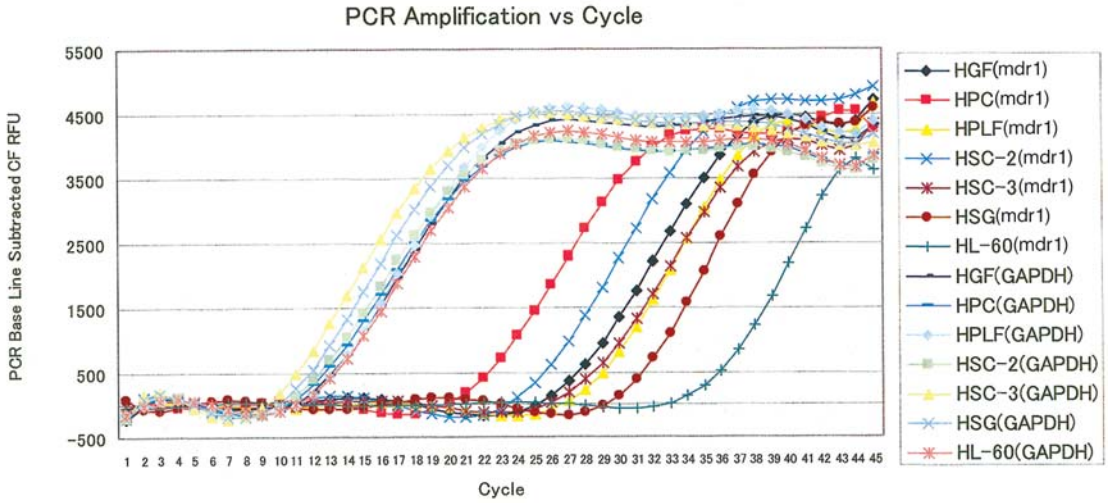

Figure 5. *Expression of mdr1 mRNA in normal and tumor cells. Total RNA was extracted from near confluent normal human cells (HGF, HPC, HPLF) or human tumor cell lines (HSC-2, HSC-3, HSG, HL-60) and subjected to quantitative real-time RT-PCR.*

oxidation in *in vitro* system (7). Therefore, the possibility of local generation of radical by doxorubicin cannot be eliminated at present.

Tumor cell lines may express lower amounts of P-glycoprotein than normal cells (29). To test this possibility, we made preliminary measurements of the expression of mdr1 mRNA. As expected, HL-60 cells, that are the most sensitive to many apoptosis-inducing drugs, expressed four orders less mdr1 mRNA as compared with normal human cells (HPC). However, HSC-2 cells, that also showed similar sensitivity to doxorubicin, expressed a comparable amount of mdr1 with normal cells. These data suggest that mdr1 expression in the tumor cells is not related to the tumor specificity of doxorubicin. It remains to compare the

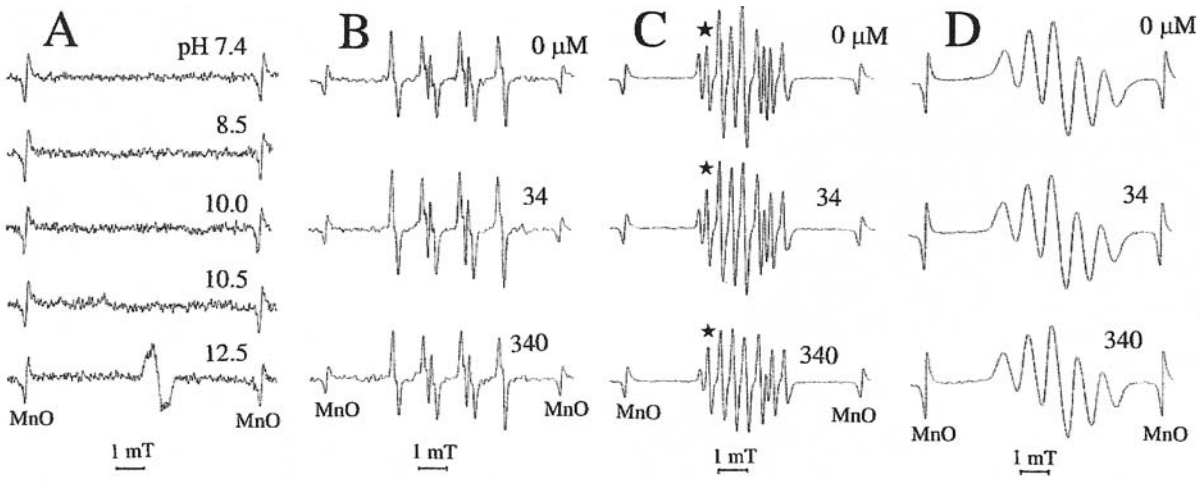

Figure 6. *ESR spectra of doxorubicin (final: 850 μM) in 0.1 M Tris-HCl (pH 7.4, 8.5), 0.1 M $NaHCO_3$/Na_2CO_3 (pH 10.0, 10.5) or 0.1 M KOH (pH 12.5) (A), of O_2^- (DMPO-OOH adduct produced in HX-XOD reaction) (B), NO (produced by NOC-7 with carboxy-PTIO) (C) or DPPH radical in the presence of the indicated concentrations of doxorubicin.*

Table II. *Quantification of mdr1 mRNA expression in normal human cells (HGF, HPC, HPLF) and human tumor cell lines (HSC-2, HSC-3, HSG, HL-60).*

Primer	Cell	Ratio of Expression
mdr1	HGF	1/39.4
	HPC	1
	HPLF	1/104
	HSC-2	1/10.6
	HSC-3	1/73.5
	HSG	1/588
	HL-60	1/12300
GAPDH	HGF	1/4.29
	HPC	1/3.25
	HPLF	1/4.59
	HSC-2	1/2.64
	HSC-3	1
	HSG	1/1.74
	HL-60	1/4.92

expression of not only mdr, but also mrp mRNA between normal and tumor cells during the cell death induction by doxorubicin. It has been recently shown that doxorubicin induced apoptosis characterized by activation of caspase 3 by both mitochondrial-dependent and -independent pathways in TGR-1 and HO15.19 rat fibroblasts, and that loss of MYC conferred them resistance to doxorubicin-induced apoptosis (30). This suggests that MYC may also participate in the resistance to doxorubicin.

Clinically, doxorubicin has been used almost exclusively for the treatment of malignant lymphoma, but not for that of oral carcinoma. Furthermore, the usefulness of doxorubicin is severely limited by cardiotoxicity arising from cumulative treatment (31). A more detailed study of the drug delivery system for doxorubicin is necessary for the efficient treatment of oral carcinoma.

Acknowledgements

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