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, (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-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.

Materials and Methods

Materials. The following chemicals were obtained from the indicated companies: RPMI 1640 medium, phenylmethylsulfonyl
The viability of HL-60 cells was determined by trypan blue exclusion. HL-60 cells were incubated at 5 x 10^5/0.1 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 10^4/mL.

**Assay for DNA fragmentation.** Cells were washed once with PBS (-) and lysed with 50 µL lysis 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 protease K for 1-2 hours at 50°C, and then mixed with 50 µL NaI solution [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-pNA (p-nitroanilide), caspase 8 (IETD-pNA) or caspase 9 (LEHD-pNA)) 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). After incubation, the membranes were washed and probed with 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 deoxyribonucleotide triphosphates, 10 units RNase inhibitor using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using the QuantiTect...
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 NaHCO₃/Na₂CO₃ (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 O₂⁻ 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 H₂O) 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 NO₂ 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 MnO.

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 O₂⁻ (Figure 6B) (IC₅₀>340 µM), NO (Figure 6C) (IC₅₀>340 µM) or DPPH (Figure 6D) (IC₅₀>340 µM) radicals. On the other hand, positive controls, such as gallic acid (IC₅₀=0.15 µM) and ferulic acid (IC₅₀= 5.2 µM), effectively scavenged the O₂⁻, 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 αβ-

Table I. Tumor specificity of doxorubicin.

<table>
<thead>
<tr>
<th>Normal cells</th>
<th>CC₅₀ (µM)</th>
<th>Tumor cell lines</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF</td>
<td>&gt;100</td>
<td>HSC-2</td>
<td>0.9</td>
</tr>
<tr>
<td>HPC</td>
<td>&gt;100</td>
<td>HSC-3</td>
<td>0.75</td>
</tr>
<tr>
<td>HPLF</td>
<td>&gt;100</td>
<td>HSG</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL-60</td>
<td>2.0</td>
</tr>
<tr>
<td>Doxorubicin (Exp. I)</td>
<td>&gt;100</td>
<td>&lt;1.0</td>
<td>0.47</td>
</tr>
<tr>
<td>Doxorubicin (Exp. II)</td>
<td>N.D</td>
<td>7.0</td>
<td>0.68</td>
</tr>
<tr>
<td>Doxorubicin (Exp. III)</td>
<td>233</td>
<td>2.9</td>
<td>500</td>
</tr>
</tbody>
</table>

SI=ΣCC₅₀ (normal)/ ΣCC₅₀ (tumor) x (Correction for number of cell type)
N.D., not determined
unsaturated ketones (TS=0.4-4) (22), 8 hydroxyketones (TS=1-18) (23), 23'-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 A405 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 O2- 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 H2O2 to form a strong

![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.](image1)

![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.](image2)
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
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.

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References


