Abstract. Three antitumor antibiotics, mitomycin C, bleomycin sulfate and peplomycin sulfate, were compared for their tumor-specific cytotoxicity, using human oral squamous cell lines (HSC-2, HSC-3, HSC-4, Ca9-22 and NA), human promyelocytic leukemic cell line HL-60 and human normal oral cell types (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF). Among these three compounds, mitomycin C showed the highest tumor-specificity, due to its higher cytotoxic activity against human oral tumor cell lines than bleomycin and peplomycin. However, there was considerable variation of drug sensitivity among the six tumor cell lines. Mitomycin C induced internucleosomal DNA fragmentation and caspase-3, -8 and -9 activation in HL-60 cells only after 24 h. On the other hand, mitomycin C induced no clear-cut DNA fragmentation in HCS-2 cells, although it activated caspase-3, -8 and -9 to a slightly higher extent. Western blot analysis demonstrated that mitomycin C did not induce any apparent change in the intracellular concentration of anti-apoptotic protein (Bcl-2) and pro-apoptotic proteins (Bax, Bad). Electron microscopy of mitomycin C-treated HL-60 cells showed intact mitochondria (as regards to integrity and size) and cell surface microvilli, without production of an apoptotic body or autophagosome, at an early stage after treatment. The present study suggests the incomplete induction of apoptosis or the induction of another type of cell death by mitomycin C treatment.

The ideal anticancer agents are those having higher cytotoxicity against tumor cell lines than against normal cells, regardless of the types of cell death. There has been no systematic study of the screening of a variety of natural and synthetic compounds for their tumor-specific cytotoxicity. A total of 500 compounds have been randomly screened for their tumor-specific cytotoxic activity, using several human normal and tumor cell lines as targets (1). Among them, doxorubicin, an anthracylin antibiotic, displayed the highest level of tumor specific cytotoxicity (evaluated by the tumor-specificity index (TS)=255) (2), followed by cyclic α,β-unsaturated ketones (TS>229) (3) and nocobactines (TS=44-80) (4). On the other hand, flavones, flavonols (3-hydroxyflavones) and isoprenoid-substituted flavonoids, benzophenones, xanthones, anthraquinones, phenylbutanone glucoside, stilbene glucoside, isoflavones, isoflavanones and stilbenes (TS=0.3-31.7), coumarin derivatives (TS=1.0-11.0), procyanidines and flavonoids (TS=1.0-7.4), hydrolysable tannins (TS=1.0-8.2), triterpene aglycones and glycosides (TS=0.65->2.8), cycloartane glycosides and chromones (TS=0.7-1.4), furuosta glucosides (TS=0.4-17.0), α,β-unsaturated ketones (TS=0.6-1.9), hydroxysterones (TS=1.0-17.6), β-diketones (TS=0.3-6.3), styrlichromones (TS=1.4-27.3), dihydroisoxazole and isoxazole derivatives (TS=0.9-1.6) showed much lower tumor-specificity (1). These findings urged us to investigate other classes of antitumor antibiotics for their antitumor potency against oral carcinoma.

Mitomycin C, bleomycin and peplomycin (Figure 1) are three frequently used antitumor antibiotics for the treatment of breast, lung, stomach, intestinal, testicular, choleiopethelial, seminal and oral carcinoma (5-7). In this study, the tumor-specific cytotoxic activity of these three compounds was compared, using three human normal oral cell types (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF), human oral
squamous cell carcinoma (HSC-2, HSC-3, HSC-4, Ca9-22 and NA) and human promyelocytic leukemia HL-60 cells.

Recently, various chemotherapeutic agents have been reported to induce at least three types of cell death: apoptosis (type I programmed cell death), autophagy (type II programmed cell death) and necrosis (8). Therefore, whether or not mitomycin C, which was found to be the most cytotoxic among three compounds tested, can induce various apoptosis-associated characteristics in highly sensitive HL-60 and HSC-2 cells was also investigated.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle medium (DMEM) (GIBCO BRL, Gland Island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA); RPMI1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bleomycin sulfate and phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem Co., St Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem Ind, Osaka, Japan); mitomycin C (Merck KGaA, Darmstadt, Germany); peplomycin sulfate (Nippon Kayaku Co., Ltd., Tokyo, Japan); RNase A and proteinase K (Boehringer Ingelheim GmbH, Ingelheim, Germany).

Cell culture. HL-60 cells were obtained from Prof. K. Nakaya, Showa University. HSC-2, HSC-4 and NA cells were obtained from Prof M Nagumo, Showa University, Japan, and HSC-3 and Ca9-22 cells were obtained from Prof. Y. Ohmori, Meikai University, Japan. Normal oral cells (HGF, HPC, HPLF) were prepared from the periodontal tissues, according to the guideline of Meikai University Ethic Committee (No. 0206), after obtaining the informed consent from the patients. Since normal oral cells have limited lifespan, all of them ceasing proliferation at the 20 population doubling level (PDL) (9), these cells were used at 5-9 PDL in the present study. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO₂ atmosphere. The other eight adherent cells
(three normal cells and five tumor cells) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS. Normal cells were prepared by detaching with 0.25% trypsin-0.025% EDTA-2Na in phosphate-buffered saline without Mg and Ca (PBS(-)) and subcultured at the 1:4 split ratio once a week, with one medium change in between. Five adherent tumor cell lines were similarly trypsinized and subcultured twice a week.

**Assay for cytotoxic activity.** Near-confluent cells were treated for 24 h with various concentrations of test samples. The relative viable cell number of adherent cells was then determined by the MTT method. In brief, the cells were washed once with PBS(-), and incubated for 4 h with 0.2 mg/mL of MTT in the culture medium. After removing the medium, the cells were lysed with 100 μL DMSO and the absorbance at 540 nm of the cell lysate (the relative viable number) was measured by a microplate reader (Multiskan Biochromatic, Labsystem, Osaka, Japan) with Star/DOT Matrix printer JL-10. The viable cell number of HL-60 cells in suspension culture was determined by cell count with a hemocytometer after staining with 0.15% trypan blue. The 50% cytotoxic concentration (CC₅₀) was determined from the dose-response curve, and the mean value of CC₅₀ against each cell was

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Figure 2. Tumor-specific cytotoxic activity of antitumor antibiotics. Cells were incubated for 24 hours without (control) or with the indicated concentrations of mitomycin C (■), bleomycin (◇) or peplomycin (▲). The viable cell number was then determined by MTT method, and expressed as % of control. Each value represents mean±S.D. was from 3 independent experiments.
calculated from 3–6 independent experiments. Tumor-specificity index (TS) was measured by the following equation: TS = [CC50 (HGF) + CC50 (HPC) + CC50 (HPLF)] / [CC50 (HSC-2) + CC50 (HSC-3) + CC50 (HSC-4) + CC50 (NA) + CC50 (Ca9-22) + CC50 (HL-60)] x (6/3).

Assay for DNA fragmentation. HSC-2 cells, collected by scraping with a rubber policeman, and HL-60 cells in suspension, were pelleted and washed once with PBS(–). They were lysed with 50 µL lysis buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium N-lauroylsarcosinate), and incubated for 2 h at 50 ºC with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K. DNA was extracted with 50 µL NaI solution [7.6 M NaI, 2 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], and precipitated with 1 mL of 70% ethanol. DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.5) and 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 (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic cells induced by UV irradiation (6 J/m²/min, 1 min), followed by 6 h incubation in regular culture medium, was run in parallel as a positive control (10). After staining with ethidium bromide, DNA was visualized by UV irradiation, and photographed by CCD camera (Bio Doc Inc, UVP).

Assay for caspase activation. Cells were washed with PBS– and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 10 min on ice and centrifugation for 20 min at 15,000 xg, the supernatant was collected. Lysate (50 µL, equivalent to 100 µg protein) was mixed with 50 µL 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-pNA (p-nitroaniline)), caspase-8 (IETD-pNA) or caspase-9 (LEHD-pNA). After incubation for 4 h at 37 ºC, the absorbance at 405 nm of the liberated chromophore pNA was measured by microplate reader (11).

Western blot analysis. 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) for 10 min in ice water, and then incubated for 55 min at 4 ºC with RT-5 ROTATOR (Titec, Saitama, Japan). Cell lysates were centrifuged at 16,000 xg for 20 min at 4 ºC to remove insoluble materials and the supernatant was collected. The protein concentration of the supernatant was determined by Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Cell lysates (containing 15 µg protein) were mixed with 2x sodium dodecyl sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-mercaptoethanol), boiled at 99 ºC for 5 min, and applied to SDS-12% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (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 overnight at 4 ºC and incubated with anti-Bcl-2 antibody (1:1,000), anti-Bax antibody (1:1,000), anti-Bad antibody (1:1,000) (Santa Cruz Biotechnology, Delaware, CA, USA) or anti-actin antibody (1:1,000) (Novus Biologicals, Inc. Littleton, CO, USA) for 90 min at room temperature, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Immunoblots were developed by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA) and analyzed on a Power Macintosh 7600/120, using the public domains NIH Image program.

| Table I. Tumor-specific cytotoxic activity of three antitumor antibiotics. |
|---------------------------------|------------------|------------------|
|                                | CC50 (µM)        |
|                                | Mitomycin C      | Bleomycin         | Peplomycin        |
| Normal cells                   |                  |                  |
| HGF                            | >308.0           | >185.6           | >400.0           |
| HPC                            | >323.3           | >200.0           | >400.0           |
| HPLF                           | >342.6           | >200.0           | >400.0           |
| Tumor cells                    |                  |                  |
| HSC-2                          | 3.5±0.9          | 4.6±4.6          | 9.9±7.0          |
| HSC-3                          | 9.7±9.7          | 6.3±1.7          | 25.2±25.5        |
| HSC-4                          | 18.0±6.2         | 77.4±43.3        | 175.8±134.7      |
| NA                             | 37.8±5.4         | 91.6±50.4        | 143.2±111.0      |
| Ca9-22                         | 16.4±7.6         | 111.6±72.2       | 216.9±178.4      |
| HL-60                          | 1.2±0.3          | 23.5±6.9         | 33.3±20.5        |
| TS                             | >22.7            | >3.7             | >4.0             |

Each value represents mean±S.D. from 3–6 independent experiments.

Electron microscopy. Cells were harvested by trypsin-EDTA, and pelleted by centrifugation at 1,000 rpm for 5 min. The cells were fixed for 1 h with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 ºC, postfixed for 1 h with 1% osmium tetraoxide-0.1 M cacodylate buffer (pH 7.4) at 4 ºC, dehydrated, then embedded in Araldite 502 (CIBA-GEIGY, Base, Swiss; NISSHIN EN Co., Ltd., Tokyo, Japan). Fine sections were stained with uranyl acetate and lead citrate, and then observed under a JEM-1210 transmission electron microscope (JEOL) at an accelerating voltage of 100 KV (12). The mitochondrial area (S) was calculated by the following equation: S = πab, where a and b lengths are the shorter and longer radiiuses of the mitochondria, respectively.

Calculation. The most stable structure of test compounds was calculated by CONFLEX (Conflux Co, Ltd, Tokyo). The optimization of the structure was carried out by the semiempirical molecular-orbital method (PM3), using CAChe Worksytem 4.9 (Fijitsu Co, Ltd, Tokyo). Octanol-water distribution coefficient (log P) was calculated by ACD-Log P (Fijitsu) (13).

Statistical treatment. Student’s t-test was used to assess the statistical significance between the two groups.

Results

Tumor-specificity. Mitomycin C, bleomycin sulfate and peplomycin sulfate dose-dependently reduced the viable cell number of 6 tumor lines (HSC-2, HSC-3, HSC-4, Ca9-22, NA and HL-60) (Figure 2). On the other hand, three normal cells (HGF, HPC and HPLF) were much more resistant. Mitomycin C reproducibly showed much higher cytotoxicity against tumor cell lines except NA cells, as compared with bleomycin sulfate and peplomycin sulfate. The TS of mitomycin C (TS>22.7) exceeded that of bleomycin (TS>3.7) and peplomycin (TS>4.0) (Table I).

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Variation of sensitivity among tumor cell lines. There was a considerable variation of drug-sensitivity among tumor cell lines (Table I). The sensitivity to mitomycin C was in the following order (from sensitive to resistant): HL-60 (CC50=1.2 μM) (the most sensitive) > HSC-2 > HSC-3 > Ca9-22 > HSC-4 > NA (CC50=37.8 μM) (the most resistant) (31.5-fold difference between HL-60 and NA). The sensitivity to bleomycin was in the following order: HSC-2 (CC50=4.6 μM) (the most sensitive) > HSC-3 > HL-60 > HSC-4 > NA > Ca9-22 (CC50=111.6 μM) (the most resistant) (24.3-fold difference between HSC-2 and Ca9-22). The sensitivity to peplomycin was in the following order: HSC-2 (CC50=9.9 μM) (the most sensitive) > HSC-3 > HL-60 > NA > HSC-4 > Ca9-22 (CC50=216.9 μM) (the most resistant) (21.9-fold difference between HSC-2 and Ca9-22).

Induction of apoptosis markers. The apoptosis-inducing activity of mitomycin C against highly sensitive HL-60 and HSC-2 cells was investigated. Mitomycin C (1~4 μM), as well as UV irradiation induced internucleosomal DNA fragmentation only after 24 h in HL-60 cells (Figure 3A). In contrast, mitomycin C (2~8 μM), and even UV irradiation, produced large DNA fragments, without induction of internucleosomal DNA fragmentation after 24 h in HSC-2 cells (Figure 3B).

Figure 4 shows that mitomycin C induced the activation of caspase-3, -8 and -9 in both HL-60 (A) and HSC-2 cells (B). The effect of mitomycin C was both time- and concentration-dependent. The most dramatic activation of caspases was observed after 24 h. Treatment of HL-60 cells for 24 h with mitomycin C (1~4 μM) activated caspase-3, -8 and -9, 16~33-, 3.0~3.7- and 5.0~7.6-fold, respectively (Figure 4A). Treatment of HSC-2 cells for 24 h with mitomycin C (2~8 μM) activated caspase-3, -8 and -9, 14~23-, 4 ~6- and 3- fold, respectively (Figure 4B).

Western blot analysis demonstrated that mitomycin C did not significantly change the intracellular concentration of anti-apoptotic protein (Bcl-2) and pro-apoptotic proteins (Bax, Bad) (Figure 5).

Electron microscopy of mitomycin C-treated HL-60 cells showed intact mitochondria (as regards to the integrity and size) (Table II) and cell surface microvilli, without production of an apoptotic body or autophagosome (Figure 6).

Discussion

The present study demonstrated that mitomycin C induced tumor-specific cytotoxicity against human oral tumor cell lines, in comparison with human normal oral cells. This provides evidence for the antitumor potential of mitomycin.
C against oral carcinoma cell lines. However, oral carcinoma cell lines showed considerable variation in sensitivity to mitomycin C. It remains to be investigated whether this variation related to variable expression of P-glycoprotein or/and MDR1 polymorphisms (14). It was found that mitomycin C did not induce any apoptosis markers (i.e., internucleosomal DNA fragmentation, caspase activation, production of an apoptotic body, loss of cell surface microvilli, increased expression of pro-apoptotic proteins (Bax, Bad), decreased expression of anti-apoptotic protein (Bcl-2)) at early stage (6 h after treatment with mitomycin C). This suggests that apoptosis may not be involved in the early onset of cell death induced by mitomycin C. It has recently been shown that malignant glioma cells are susceptible to autophagy (15), and that the

Table II. Effect of mitomycin C on the mitochondrial volume of HL-60 cells.

<table>
<thead>
<tr>
<th>Mitomycin C (μM)</th>
<th>Mitochondrial area (μm²)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.34±0.15 (n=20)</td>
</tr>
<tr>
<td>2</td>
<td>0.36±0.16 (n=20)</td>
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HL-60 cells were treated for 6 hours without (control) or with 2 μM mitomycin C, and then subjected to electron microscopy.

Figure 4. Activation of caspase-3, -8 and -9 by mitomycin C. HL-60 (A) and HSC-2 (B) cells were incubated for 4, 8 or 24 hours with the indicated concentrations of mitomycin C (1, 2, or 4 μM for HL-60 cells, 2, 4, or 8 μM for HSC-2 cells), and the activities of caspase-3, -8 and -9 were assayed by substrate cleavage activity. The absorbance at 405 nm of cleaved product pNA from the substrate of caspase-3, -8 and -9 in control cells was 0.0063, 0.004, 0.001 (HL-60) and 0.014, 0.011, 0.0080 (HSC-2), respectively. Control=HL-60 cells harvested before mitomycin C treatment. UV=HL-60 cells that were exposed to UV irradiation and cultured for 6 hours. ^p<0.10, ^p<0.05, ^p<0.02, ^p<0.01, ^p<0.001

Figure 5. Effect of mitomycin C on the intracellular concentration of Bcl-2, Bax and Bad proteins in HL-60 and HSC-2 cells. Near confluent cells were incubated for 4, 8, or 24 h with the indicated concentrations (μM) of mitomycin C. Cell lysate was applied to SDS-polyacrylamide gel electrophoresis and processed for western blot analysis for the expression of Bcl-2, Bax, Bad and Actin proteins. An example representative of three independent experiments is shown.
forced expression of Bcl-2 or BcL-X\textsubscript{L}, that produces unbalanced expression of Bcl-2/Bax-X\textsubscript{L} and Bax/Bak, induces autophagy in mouse fibroblasts (16). Therefore, the possibility of incomplete induction of apoptosis or the induction of another type of cell death by mitomycin C should be investigated. In the present study the density of nucleoli in the mitomycin C-treated HL-60 cells found to be slightly lower than that of control untreated cells (Figure 6). The biological significance of this phenomenon is unclear at present, and therefore a subject for future research.

Mitomycin C was not found to induce internucleosomal DNA fragmentation, although it activated the caspase activity in HSC-2 cells at a late stage of cell death induction (24 h after treatment). However, mitomycin C-treated HSC-2 cells were found to cleave the substrate for caspases more efficiently (absorbance at 405 nm of pNA (cleavage product)=0.31) than mitomycin C-treated HL-60 cells (absorbance at 405 nm of pNA=0.23) (Figure 4). This indicates that the extent of caspase activation itself is not a determinant of DNA fragmentation. The possibility should be considered that the lack of DNA fragmentation in mitomycin C-treated HSC-2 cells may be due to the lower expression of caspase-activated DNase (17), or the lower susceptibility of the chromatin structure to attack by DNase(s) (18), as compared with mitomycin C-treated HL-60 cells.

The cytotoxic activity of mitomycin was found to exceed that of bleomycin and peplomycin, but was one order less than that of anthracyclin antibiotics such as doxorubicin (2) and daunorubicin, idarubicin, mitoxantrone in a previous study (19). This may be explained by the difference in their log P values. We have previously reported that the optimal log P value for the expression of the maximum cytotoxicity was around 2~4 in eugenol (20), isoflavone (21), vitamin K\textsubscript{1}, K\textsubscript{2} and K\textsubscript{3} (22)-related compounds. As expected, the log P value of the most cytotoxic anthracyclines (doxorubicin, daunorubicin, idarubicin, mitoxantrone) was very close to 3.0 (3.1, 3.2, 3.0 and 3.4, respectively) (19). The log P value of mitomycin C, bleomycin and peplomycin was much less (-0.49, -3.62 and -1.32 respectively). This further confirms the usefulness of the log P value for the estimation of the cytotoxic activity of structurally-related compounds.

The growth potential of normal cells cultured in the presence of any antitumor antibiotics used here did not decrease to the 50% level of control (Figure 2), making the calculation of their tumor-specificity index difficult. Bleomycin and peplomycin were found to show one order lower cytotoxicity than mitomycin C. This may be overcome, if a synergistic cytotoxic effect by simultaneous treatment with other classes of antitumor agents could be produced. However, it should be considered that the antitumor potential of antitumor antibiotics may be modified by various physiological factors such as serum components or metals (19), when administered in vivo. It should be noted

Figure 6. Electron microscopy of control and dying HL-60 cells. HL-60 cells were incubated for 6 hours with 0 (A) or 2 (B) \textmu M mitomycin C, or were treated with UV irradiation (C), and then processed for the electron microscopy. Bar: 2 \textmu m.
that the serum we and many other investigators have used is FBS, heterologous to human origin. It remains to be investigated whether human serum, either from normal volunteers or cancer patients, may modify the cytotoxic action of mitomycin C.

We have provided evidence that three antitumor antibiotics (mitomycin C, bleomycin, peplomycin) showed some tumor-specific cytotoxicity, using three human normal cells and six tumor cell lines derived from different origins. It is essential to confirm the reproducibility of the present study in the normal and tumor cells derived from the same origin.

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References


