Tumor-specific Cytotoxicity and Type of Cell Death Induced by Peplomycin in Oral Squamous Cell Carcinoma Cell Lines

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Abstract. *The antitumor antibiotic peplomycin showed higher cytostatic antiproliferative effect on five cultured human oral squamous cell carcinoma (OSCC) cell lines (HSC-2, HSC-3, HSC-4, Ca9-22 and NA), as compared with three human oral normal cells (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF). Although the antiproliferative activity of peplomycin declined with increasing cell density, peplomycin showed tumor-specific cytotoxicity at any cell density. The five OSCC cell lines showed considerable differences in sensitivity against peplomycin; the HSC-2 cells were the most sensitive, followed by the NA, HSC-3, Ca9-22 and HSC-4 cells. Peplomycin did not induce internucleosomal DNA fragmentation in any of the five OSCC cell lines, and only slightly modified caspase-3, -8 and -9 activities in the HSC-2, Ca9-22 and NA cell lines. Electron microscopy revealed that peplomycin induced the vacuolation of mitochondria accompanying electron lucent matrices lacking cristae and the enlargement of the endoplasmic reticulum in the HSC-2 cells. These data suggest that the anti-proliferative effect of peplomycin is time-dependent, and therefore prolonged treatment with peplomycin in combination with cytotoxic chemotherapeutic agents may induce greater cytotoxic action.*

Chemotherapy for malignant tumors has been advanced by the development of a variety of effective anticancer agents. Understanding the cellular processes involved and the molecular mechanisms responsible for the cell death induced by anticancer drugs may aid in the development of more effective and tumor-selective chemotherapy. Peplomycin (Figure 1), an antitumor antibiotic which has less pulmonary toxicity than the natural bleomycin preparation, has been used for the treatment of breast, lung, stomach, intestinal, testicular, cholioepithelial, seminal and oral carcinoma. A single administration of peplomycin to oral cancer patients is rare, possibly due to its weak antitumor activity, the immunosuppressive activity and the effects on lung function (1). Peplomycin has rather been administered in combination with cisplatin together with irradiation (2, 3) or hyperthermia (4), producing significant therapeutic effect accompanied by apoptotic cell death in the surgical specimens of oral squamous cell carcinoma (OSCC) (3). Similarly, combination treatment of peplomycin and cisplatin has shown an antitumor effect on nude mice transplated with OSCC cells, inhibiting metastasis and tumor stage progression (5). There are relatively few publications on the *in vitro* antitumor potency of peplomycin targeted against human oral cancer cells. Peplomycin has shown higher cytotoxicity than bleomycin against neoplastic cells derived from the salivary gland (HSG) and melanoma cells (6), whereas peplomycin and bleomycin showed comparable cytotoxicity against OSCC cell lines (7). Peplomycin induced apoptosis markers such as nuclear condensation and fragmentation of chromatin in OSCC SSCKN cells, but not in SCCTF cells, indicating the heterogenous response of OSCC cell lines to peplomycin (8). However, most previous studies have not investigated the cytotoxicity of peplomycin against human normal cells, and therefore the tumor-specificity of peplomycin has not been known until recently (7, 9). We have established an *in vitro* screening system to search for highly tumor-specific cytotoxic compounds, using human normal and cancer cells (10). We have found that doxorubicin (adriamycin), a popular anthracycline antibiotic, nocobactines and cyclic α,β-unsaturated ketones showed

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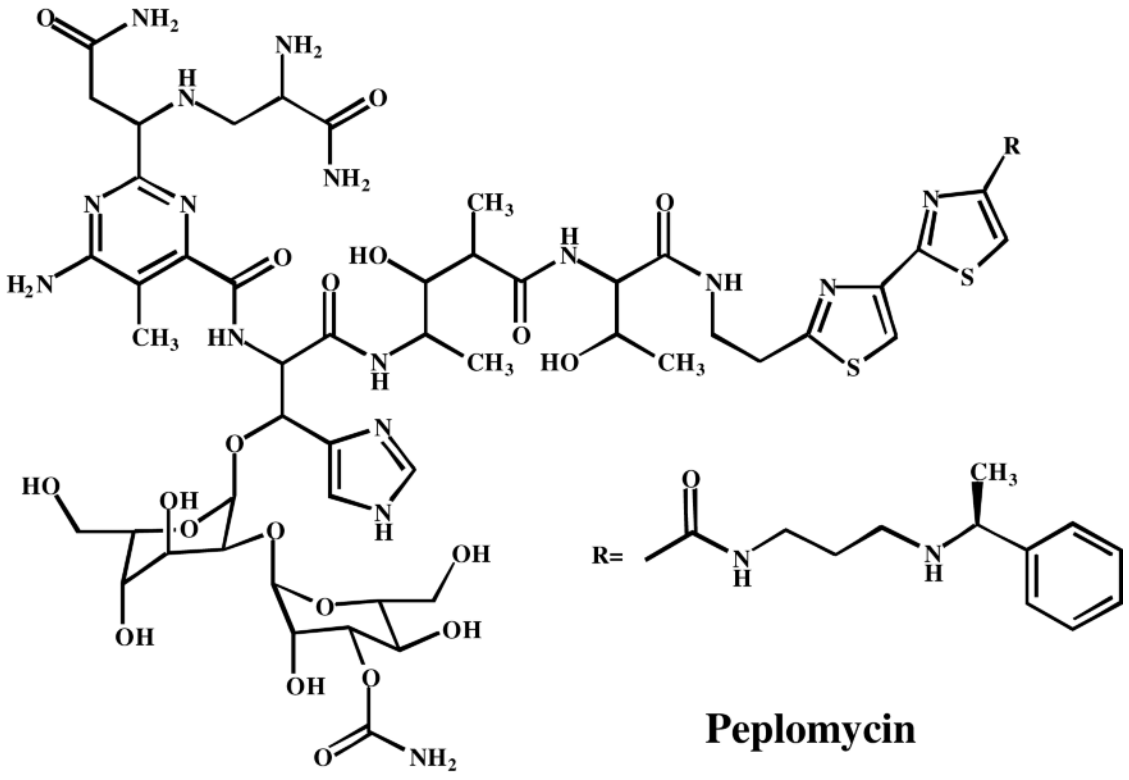

Figure 1. *Chemical structure of peplomycin.*

highly selective cytotoxicity against OSCC cell lines compared with human normal oral cells, yielding a tumor-specific index (TS) of 80-200 (11). Other antitumor antibiotics such as mitomycin (TS>22.7), bleomycin (TS>3.7) and peplomycin (TS>4.0) showed one order lower tumor-specific cytotoxicity (7) and therefore the cell death induced by peplomycin has not been analysed further. However, this comparative study used only 24 hours treatment time (7), and therefore the 50% cytotoxic concentration (CC_{50}) could not be determined accurately, due to the cytostatic activity of bleomycin and peplomycin revealed by the present study.

In the present study, the tumor-specific cytotoxic activity of peplomycin was re-evaluated using three human normal oral cells (gingival fibrobast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF) and five human OSCC cell lines (HSC-2, HSC-3, HSC-4, Ca9-22 and NA). The treatment time with peplomycin was prolonged to 48 hours (2 cell cycles) to make it possible to calculate the CC_{50} value for both normal and tumor cells. Since the cytotoxic activity of peplomycin may be influenced by the cell density (crowdedness), the cytotoxic activity of peplomycin against cells that had been seeded at three different cell densities, low (sparse), intermediate, and high (dense) was investigated. Since there are at least three types of cell death, apoptosis, autophagy and necrosis (12-14), the type of cell death induced by peplomycin was investigated, using several apoptosis markers and electron microscopy.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); RPMI-1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem Co., St. Louis, MO, USA) and peplomycin (Wako Pure Chem. Ind., Ltd., Osaka, Japan).

Cell culture. The HSC-2, HSC-4, Ca9-22 and NA cells were obtained from Professor M. Nagumo, Showa University, Japan, and the HSC-3 cells were provided by Professor Y. Ohmori, Meikai University, Japan. The normal oral cells (HGF, HPC, HPLF) were prepared from the periodontal tissues, according to the guideline of the Intramural Ethic Committee (No. 0206), after obtaining the informed consent from the patients. Since normal oral cells have a limited lifespan, all of them ceasing proliferation at the 20 population doubling level (PDL) (15), these cells were used at the 5-9 PDL in the present study. The eight adherent cells (three normal cells and five tumor cells) were cultured in DMEM supplemented with 10% heat-inactivated FBS. The normal cells were detached by 0.25% trypsin-0.025% EDTA-2Na in phosphate-buffered saline without Mg and Ca (PBS(–)) and subcultured at a 1:4 split ratio

once a week, with one medium change in between. The five adherent tumor cell lines were similarly trypsinized and subcultured twice a week.

Assay for viable cell number. Near-confluent cells were treated for 48 hours with various concentrations of peplomycin. 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 hours 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 cell number) was measured by a microplate reader (Multiskan Biochromatic, Labsystem, Osaka, Japan) with a Star/DOT Matrix printer JL-10. The CC_{50} was determined from the dose-response curve, and the mean value of CC_{50} against each cell lines was calculated from 3-6 independent experiments. The TS was measured by the following equation:
$TS=(CC_{50}[HGF]+CC_{50}[HPC]+CC_{50}[HPLF])/(CC_{50}[HSC\text{-}2]+CC_{50}[HSC\text{-}3]+ CC_{50}[HPS\text{-}4] + CC_{50}[Ca9\text{-}22]+ CC_{50}[NA])\times (5/3)$.

Assay for DNA fragmentation. The HSC-2, HSC-3, HSC-4, Ca9-22 and NA cells were collected by scraping with a rubber policeman on ice, 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 hours at 50˚C with 0.4 mg/mL RNase A and 0.8 mg/ml proteinase K. The DNA was extracted with 50 μl NaI solution (40 mM Tris-HCl [pH 8.0], 7.6 M NaI, 2 mM EDTA-2Na), and precipitated with 1 mL of 70% ethanol. The DNA was dissolved in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl [pH 8.0], 89 mM boric acid, 2 mM EDTA). A DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic human promyelocytic leukemia HL-60 cells induced by UV irradiation (6 J/m^2/min, 1 min), followed by 6 hours incubation in regular culture medium, were run in parallel as positive controls (16). After staining with ethidium bromide, the DNA was visualized by UV irradiation, and photographed by CCD camera (Bio Doc-It, UVP, Inc., Upland, CA, USA).

Assay for caspase activation. The cells were washed twice 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×g, the supernatant was collected. The lysate (50 μL, equivalent to 100 μg protein) was mixed with 50 μL 2× reaction buffer (MBL) containing substrates for caspase-3 (DEVD-*p*-nitroanilide (pNA)), caspase-8 (IETD-pNA) or caspase-9 (LEHD-pNA). After incubation for 4 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore pNA was measured by microplate reader (17).

Electron microscopy. The cells were detached by trypsin-EDTA, and pelleted by centrifugation at 1,000 rpm for 5 min. The cells were fixed for 1 hour with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C, postfixed for one hour with 1% osmium tetraoxide-0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated, then embedded in Araldite 502 (CIBA-GEIGY, Basel, Switzerland). 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 (18).

Table I. *Cytotoxic activity of peplomycin against cultured human normal and tumor cells.*

	CC_{50} (μM)		
	High	Intermediate	Low
HGF	232.0±4.3	221.5±8.5	175.5±10.5
HPC	182.3±12.9	177.0±28.7	122.3±21.1
HPLF	185.0±24.1	142.5±29.5	105.6±33.5
(mean)	200	180	134
HSC-2	6.3±1.5	5.9±1.9	1.9±0.28
HSC-3	20.6±1.5	21.3±13.5	8.8±0.9
HSC-4	38.3±4.4	24.8±28.3	15.5±6.5
Ca9-22	45.2±21.8	12.8±8.9	9.3±6.4
NA	12.4±5.5	4.5±3.0	4.3±4.8
(mean)	25	14	8
TS	8.1	13	16.9

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

Results

Tumor-specific cytostatic activity of peplomycin. Peplomycin displayed cytostatic, but not cytotoxic activity against all eight cells after 48 hours incubation (Figure 2). The antiproliferative effect of peplomycin was enhanced when the cell density was decreased from high to intermediate, and intermediate to low in all eight cells (Table I). However, the antiproliferative effect of peplomycin on the five human OSCC cell lines (HSC-2, HSC-3, HSC-4, Ca9-22, NA) was always higher than that on the three human normal oral cells (HGF, HPC, HPLF), yielding TS values of 8.1, 13.0 and 16.9 for the high, intermediate and low cell densities, respectively.

Type of cell death. Peplomycin treatment (6 or 24 hours) did not induce clear-cut internucleosomal DNA fragmentation in any of the five tumors cell lines, in contrast to the UV-induced apoptotic HL-60 cells where the typical laddering pattern of DNA fragmentation was observed (Figure 3).

The highest concentration of peplomycin (4 μM) slightly activated caspase-3, but not caspase-8 and caspase-9 in the HSC-2, Ca9-22 and NA cells after 24 hours (Figure 4A, B, C). In contrast, a lower concentration of peplomycin (2 μM) slightly inactivated the caspase-3, -8 and -9, especially in the NA cells (Figure 4C).

Changes in the ultrastructure. The electron microscopic findings are shown in Figure 5. When the HSC-2 cells were cultured with peplomycin for 6 hours, slight vacuolation of mitochondria accompanying electron lucent matrices lacking cristae were observed at the higher concentration

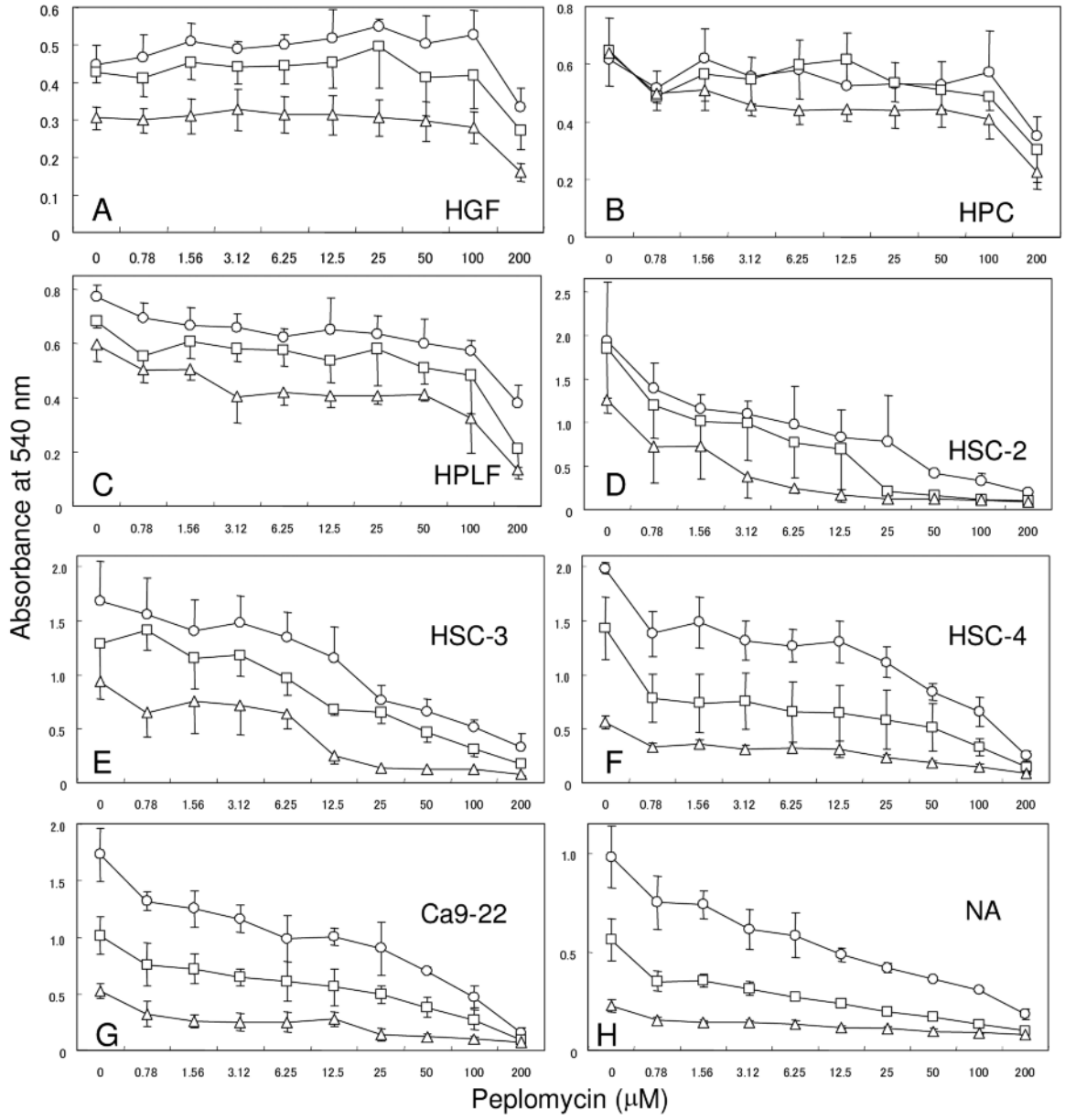

Figure 2. *Cytostatic activity of peplomycin against human normal and tumor cells. Normal cells (A-C) were inoculated at 1:1.5 (○), 1:3 (□) or 1:6 (△) split ratio. OSCC cell lines (D-H) were inoculated at 6×10^3 (○), 3×10^3 (□) and 1.5×10^3 (△) in 0.1 mL medium (96-microwell plate). After incubation for 48 hours to allow the complete adherence to plastic plate and recovery from trypsin damage, media were replaced with fresh medium without (control) or with the indicated concentrations of peplomycin. After incubation for 48 hours, the viable cell number was then determined by MTT method, and expressed as % of control. Each value represents mean±S.D. from 3 independent experiments.*

(Figure 5C). No ultrastructural changes were found in the control cells (Figure 5A) or at the lower concentration (Figure 5B). When they were cultured with peplomycin for 24 hours, almost all the mitochondria exhibited abnormal structure including vacuolation at both concentrations (Figure 5E, F). In addition, enlargement of the endoplasmic reticulum was recognized. The cells cultured for 24 hours without peplomycin showed normal structure (Figure 5D).

Discussion

The present study demonstrated that peplomycin displayed a higher anti-proliferative effect on human oral tumor cell lines, than on human normal oral cells. This tumor-specificity provided further evidence for the antitumor potential of peplomycin against OSCC cell lines. However, the five OSCC cell lines showed considerable variation in

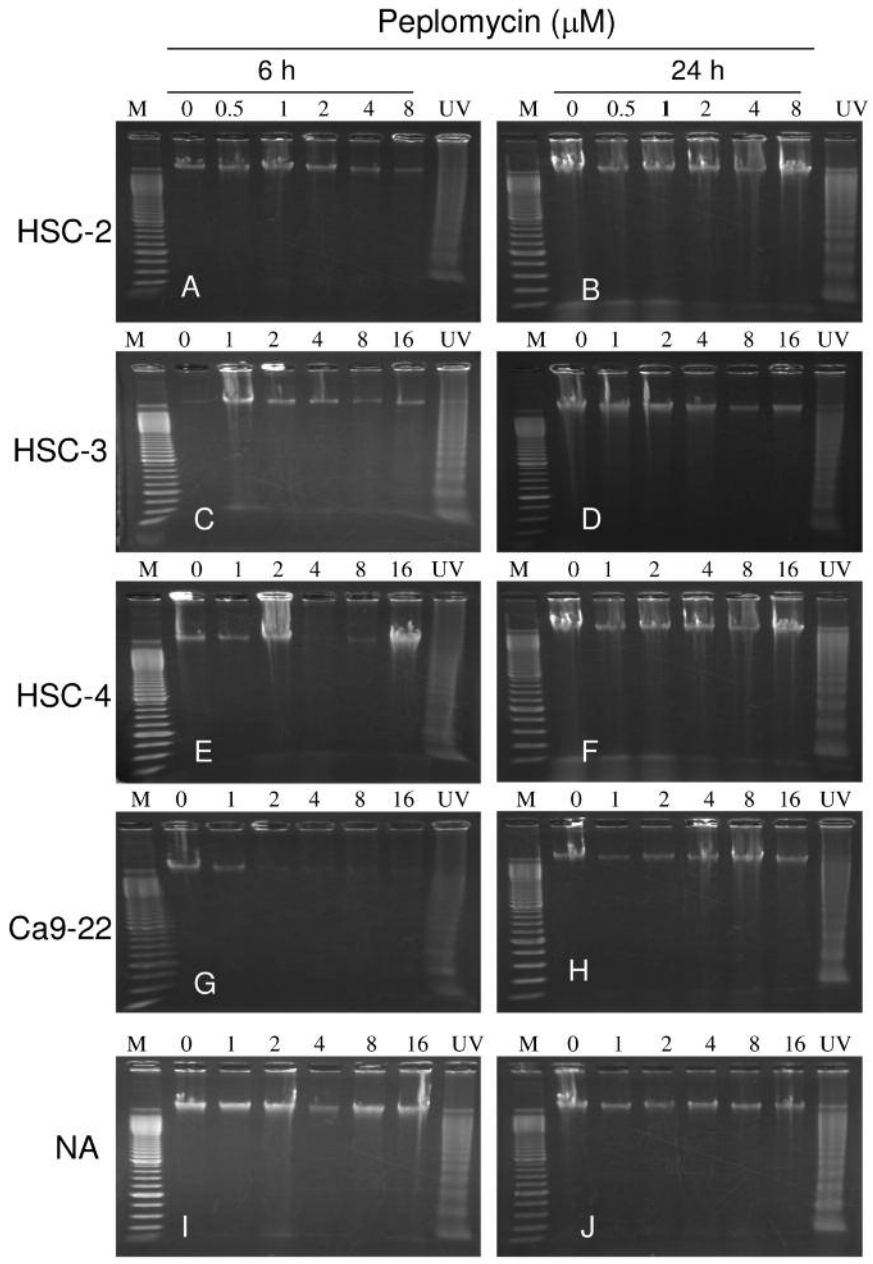

Figure 3. *Effect of peplomycin on the induction of DNA fragmentation. HSC-2 cells were incubated for 6 or 24 hours without (control) or with 0.5, 1, 2, 4 or 8 μM of peplomycin. HSC-3, HSC-4, Ca9-22 and NA cells were incubated for 6 or 24 hours without (control) or with 1, 2, 4, 8 or 16 μM of peplomycin. DNA was then extracted and subjected to agarose gel electrophoresis. M, DNA marker; UV, DNA from apoptotic HL-60 cells induced by UV-irradiation.*

sensitivity to peplomycin. The HSC-2 cells were the most sensitive (CC_{50}=6.3, 5.9, 1.9 mM, at high, intermediate and low cell density, respectively), followed by the NA cells then the HSC-3, HSC-4 and Ca9-22 cells (Table I). Peplomycin displayed a cytostatic, but not a cytotoxic effect, still leaving 10-40% of viable cells in the total cell population even after 2 cell cycles (48 hours) (Figure 2). This suggested that the anti-proliferative effect of peplomycin was time-dependent, and therefore prolonged treatment with peplomycin in combination with cytotoxic chemotherapeutic agents may induce a greater cytotoxic action.

Peplomycin did not induce internucleosomal DNA fragmentation (Figure 3) nor caspase activation (Figure 4) in any of the five OSCC cell lines, but did induce vacuolation of the mitochondria and enlargement of the endoplasmic reticulum in the most sensitive HSC-2 cells

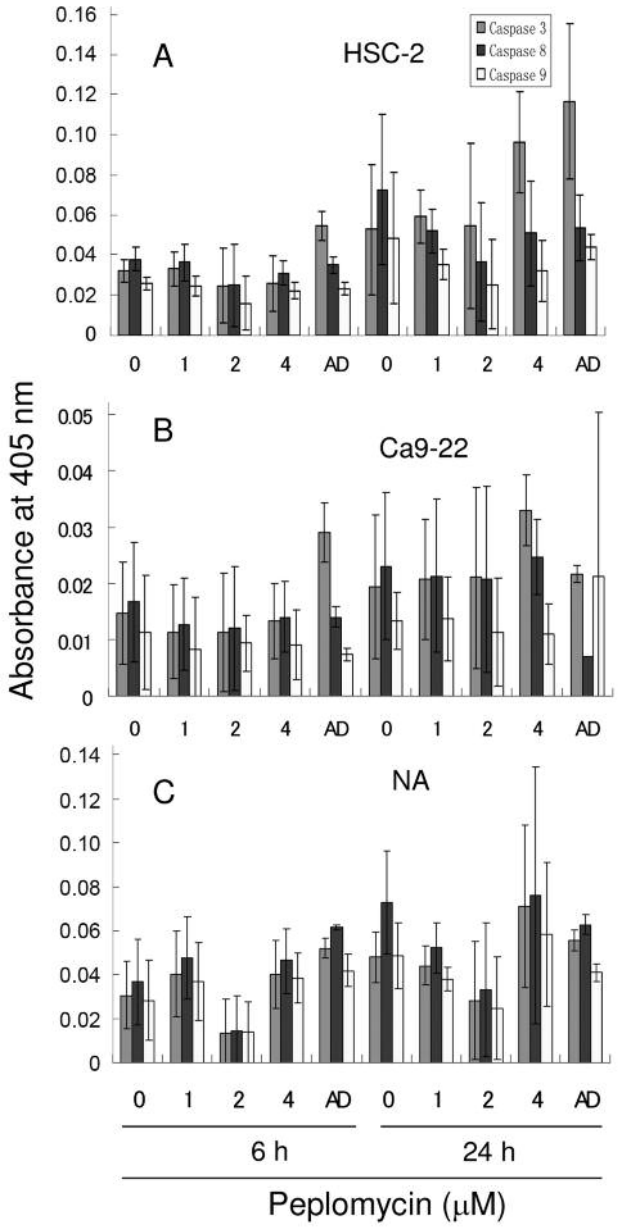

Figure 4. *Effect of peplomycin on the caspase activity. HSC-2 (A), Ca9-22 (B) and NA cells (C) were incubated for 6 or 24 hours without (control) or with 1, 2, or 4 μM peplomycin, and caspase activity (expressed as 405 nm of cleaved product for each substrate) was determined. AD: actinomycin D (1 μg/mL).*

(Figure 5). These results suggest that peplomycin may induce mitochondrial dysfunction. There was no clear-cut indication of autophagy, since the secondary lysosomes could not be detected. Further investigation is required to identify the type of cell death induced by peplomycin in OSCC cell lines. However, the present study demonstrated that peplomycin does not always induce apoptosis in OSCC cell lines (19). The type of cell death may be determined by both the chemical structure of the inducer (14) and the drug-sensitivity or malignancy of the target cells (8).

The caspase activity declined at lower peplomycin concentrations, and returned to or exceeded the control level at the highest concentration in the HSC-2, Ca9-22 and NA cells. This dose-dependent bimodal action of peplomycin may be the so-called "hormesis" (20). The presence of an anti-apoptotic mechanism (decline of caspase activity) may produce diverse heterogenic responses in the human OSCC cell lines to peplomycin.

The mechanism by which peplomycin induces cell death is unclear. It has been reported that peplomycin induced the elevation of the intracellular level of reactive oxygen species, resulted in the increase of pro-apoptotic protein BAD and the decrease of anti-apoptotic protein Bcl-2 *via* the enhancement of their phosphorylation and the decrease or increase of their ubiquitination, respectively (21). However, this mechanism may not apply in the cells used here, since they were not be destined for apoptosis. The cytotoxic activity of peplomycin against OSCC cells has been reported to be correlated with the level of the epidermal growth factor (EGF) expressed on the cells, in contrast to the absence of such correlation between adriamycin and the EGF receptor (22), suggesting a different point of action between peplomycin and adriamycin. The sensitivity of peplomycin has been augmented by over expression of caveolin-1 (23) and in transforming growth factor (TGF)-β stimulated clone-22 (24). It remains to be investigated whether changes in these protein expressions are related to the tumor-specificity and the type of cell death.

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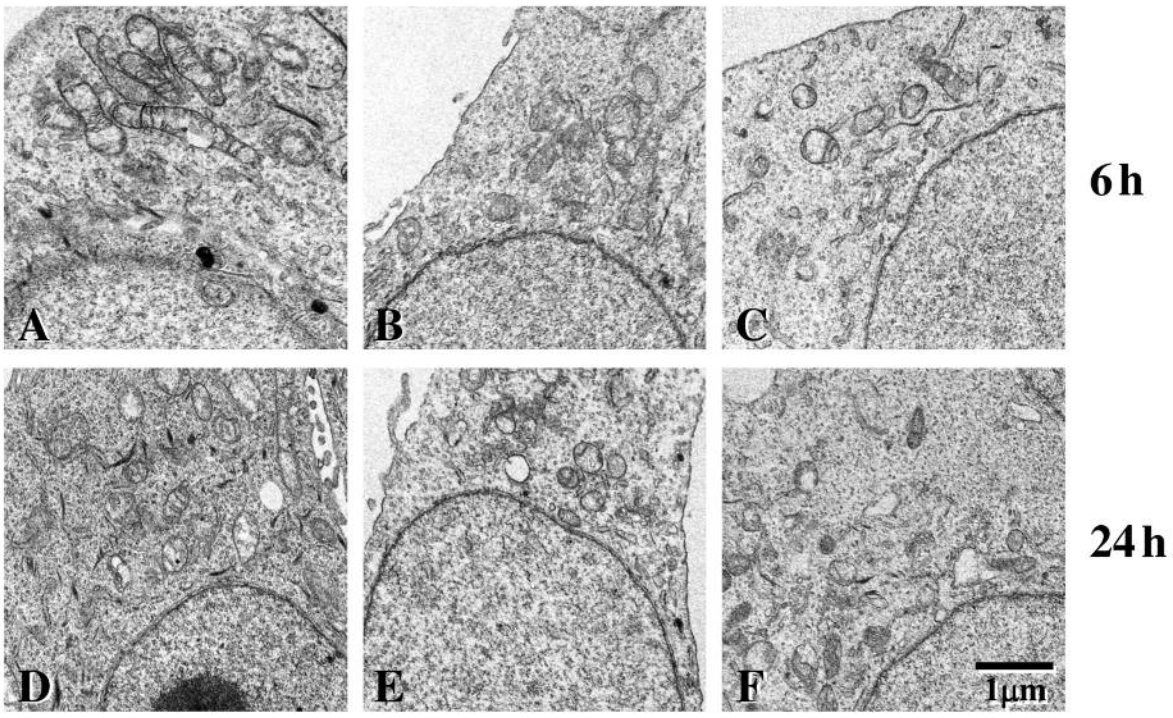

Figure 5. *Change in the fine structure of HSC-2 cells induced by peplomycin. HSC-2 cells were treated for 6 or 24 hours with 0 (A, D), 3 (B, E) or 6 (C, F) mM peplomycin, and then processed for electron microscopy.*

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