Tumor-specific Cytotoxicity and Type of Cell Death Induced by β-Cyclodextrin Benzaldehyde Inclusion Compound

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Abstract. *The cytotoxicity of β-cyclodextrin benzaldehyde inclusion compound (CDBA) against human normal and cancer cell lines was investigated. CDBA showed slightly higher cytotoxicity against human tumor cell lines, as compared to normal cells, with a tumor-specificity index of 2.2. Human myelogenous leukemia cell lines (HL-60, ML-1, KG-1) were the most sensitive to CDBA, followed by human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4) and human glioblastoma (T98G, U87MG). Human normal cells (gingival fibroblasts, pulp cells, periodontal ligament fibroblasts) were the most resistant. CDBA induced internucleosomal DNA fragmentation in HL-60 cells and caspase-3, -8, -9 activation, but to a much lesser extent than that attained by UV irradiation or actinomycin D. On the other hand, CDBA did not induce DNA fragmentation, nor caspase activation in HSC-2, HSC-4 or T98G cells. Electron microscopy demonstrated that CDBA induced the destruction of mitochondrial structure and digestion of broken organelles by secondary lysosomes in all of these cells. CDBA also increased the number of acidic organelles as judged by acridine orange staining. The present study suggests that CDBA induces autophagic cell death in cancer cell lines.*

Benzaldehyde is an antitumor substance isolated from the volatile fraction of figs (1). Benzaldehyde and its derivatives have shown antitumor activity in mice against Ehrlich carcinoma, adenocarcinoma 755 and colon 38; however, they were not active against several other implanted tumors (2-4). Although benzaldehyde derivatives, sush as β-cyclodextrin benzaldehyde inclusion compound (CDBA), 4,6-benzaldehyde-α-D-glucose and sodium 5,6-benzylidene-L-ascorbate (SBA) have shown only weak antitumor activity against tumors implanted in mice, administration of CDBA to patients with advanced, inoperable carcinoma induced remarkable necrotic changes in the tumors (5-7). We have also reported that sodium 5,6-benzylidene-L-ascorbate (SBA) induced the degeneration of human spontaneous lung cancer and rat chemically induced hepatocellular carcinoma and the antitumor activity of SBA is not mediated *via* immumopotentiation activity of the hosts (8). It has been reported that the cytotoxicity and protein synthesis inhibition by deuterated benzaldehyde derivative zilascorb(^{2}H) (sodium 5,6-benzylidene-d_1-L-ascorbic acid sodium salt) was modified by aminotriazole, suggesting an important role of H_2O_2 in benzaldehyde-mediated cytotoxicity (9).

However, the mechanism of induction of antitumor activity by treatment with benzaldehyde derivatives is unclear. Here, whether CDBA induces tumor-specific cytotoxic activity was investigated using three human normal oral cells (gingival fibrobast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF), human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4), human glioblastoma (T98G, U87MG) and human myelogenous leukemia (HL-60, ML-1, KG-1) cell lines, and if so, which type of cell death is induced by CDBA in these tumor cell lines.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the companies indicated: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal

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Key Words: β-Cyclodextrin benzaldehyde inclusion compound, type of cell death, oral squamous cell carcinoma.

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). CDBA was provided by Ichijokai Hospital, Chiba, Japan.

Cell culture. HL-60 cells were provided by Prof. K. Nakaya, Showa University. ML-1 and KG-1 cells were provided by Prof. K. Takeda, Tokyo University of Science. HSC-2 and HSC-4 cells were obtained from Prof. M. Nagumo, Showa University; HSC-3 cells were provided by Prof. Y. Ohmori, Meikai University; T98G and U87MG cells were provided by Dr. M. Iida, Showa University, Japan. Normal oral cells (HGF, HPC, HPLF) were prepared from periodontal tissues, according to the guidelines of the Intramural Ethics Committee (No. 0206) after obtaining the informed consent from the patients (10).

Since normal oral cells have limited lifespan, all of them ceasing proliferation at the 20 population doubling level (PDL)(10), these cells were used at 5-9 PDL in the present study. HL-60, ML-1 and KG-1 cells were cultured at 37˚C in RPMI 1640 medium supplemented with 10% heat-inactivated FBS as suspension culture in the tissue culture dish (Falcon 100x20 mm style; Becton Dickinson Labware, NJ, USA), under a humidified 5% CO_2 atmosphere. The other eight adherent cells (three normal cells and five tumor cell lines) were cultured at 37˚C in DMEM supplemented with 10% heat-inactivated FBS as monolayer culture. 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. Five adherent tumor cell lines were similarly trypsinized and subcultured twice a week.

Assay for cytotoxic activity. Cells were initially inoculated at $3x10^3$/0.1 mL in 96-microwell plates (Becton Dickenson) and incubated for 48 hours before treatment. Near-confluent cells were treated for 48 h without (control: medium alone) or with different concentrations of CDBA (0.031-8 mM), with three replicate wells for each concentration. 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 cell number) was measured by a microplate reader (Multiskan Biochromatic, Labsystem, Osaka, Japan). The viable cell number of HL-60, ML-1 and KG-1 cells in suspension culture was determined by cell count with a hemocytometer after staining with 0.15% trypan blue. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve, and the mean value of CC_{50} against each cell line was calculated from 3-6 independent experiments. The tumor-specificity index (TS) was measured using the following equation: $TS=[CC_{50}(HGF)+CC_{50}(HPC)+CC_{50}(HPLF)]/[CC_{50}(HSC-2)+CC_{50}(HSC-3)+CC_{50}(HSC-4)+CC_{50}(T98G)+CC_{50}(U87MG)+CC_{50}(HL-60)+CC_{50}(ML-1)+CC_{50}(KG-1)]$ x (8/3).

Assay for DNA fragmentation. HSC-2, HSC-4 and T98G cells ($1x10^5$/3 mL) were inoculated in 6-well tissue culture plates (Becton Dickenson) and incubated for 48 hours before the treatment. These near-confluent cells, or HL-60 cells ($5x10^5$/mL) were then treated for 6 or 24 hours with CDBA (0, 1, 2 or 4 mM). They were then collected by scraping with a rubber policeman on ice, 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˚C 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, 1 mM EDTA, 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 weight marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic cells induced by UV irradiation ($6J/m^2$/min, 1 min) as described previously (11), followed by 6 h incubation in regular culture medium, was run in parallel as a positive control. After staining with ethidium bromide, DNA was visualized by UV irradiation and photographed by CCD camera (Bio Doc Inc, UVP, Inc., Upland, CA, USA).

Assay for caspase activation. HSC-2, HSC-4 and T98G cells ($5x10^5$/10 mL) were inoculated in a tissue culture plates (Falcon) and incubated for 48 hours before the treatment. These near-confluent cells, or HL-60 cells ($3x10^6$) were then treated for 6 or 24 hours with CDBA (0, 1, 2 or 4 mM), or 0.1 μg/mL actinomycin D (positive control). 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 xg, the supernatant was collected. Lysate (50 μL, equivalent to 100 μg

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

	CC_{50} of CDBA (mM)
Normal cells	
HGF	2.3±0.91 (n=5)
HPC	2.5±0.60 (n=5)
HPLF	1.6±0.52 (n=5)
	Mean=2.1
Human oral squamous cell carcinoma cell lines	
HSC-2	0.67±0.35 (n=5)
HSC-3	1.1±0.41 (n=4)
HSC-4	0.83±0.35 (n=5)
	Mean=0.87
Human glioblastoma cell lines	
T98G	1.1±0.38 (n=3)
U87MG	2.0±0.91 (n=3)
	Mean=1.6
Human leukemia cell lines	
HL-60	1.0±0.35 (n=5)
ML-1	0.52±0.17 (n=3)
KG-1	0.44±0.24 (n=3)
	Mean=0.65
Tumor-specificity index	2.2

Each value represents mean±S.D. from 3-5 independent experiments.

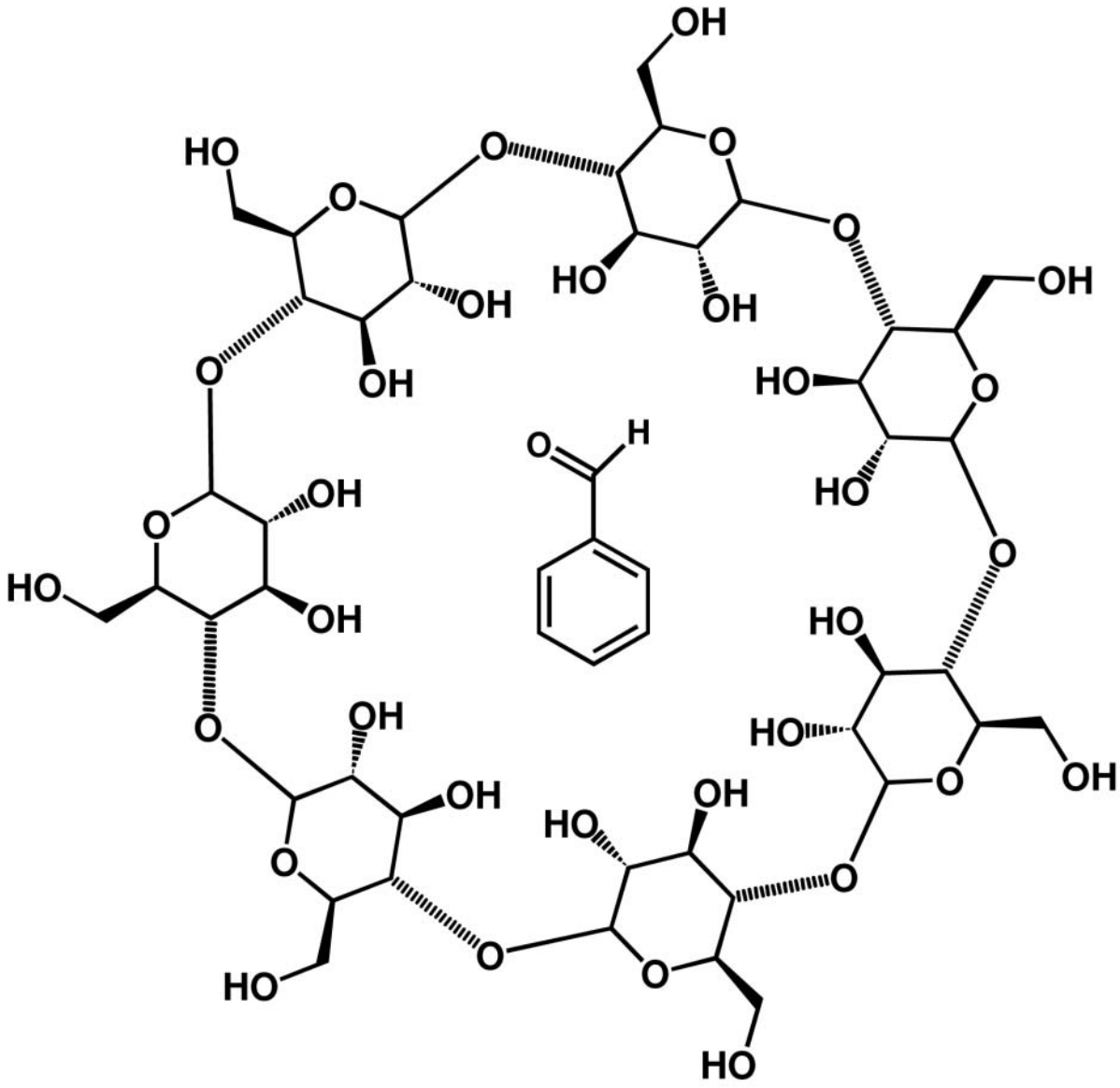

Figure 1. *Chemical structure of CDBA.*

protein) was mixed with 50 μL 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-pNA (*p*-nitroanilide), caspase-8 (IETD-pNA) or caspase-9 (LEHD-pNA). After incubation for 4 h at 37˚C, the absorbance at 405 nm of the liberated chromophore pNA was measured by a microplate reader (12).

Detection of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were stained with acridine orange (Sigma Chemical Co., St. Louis, MO, USA) as described elsewhere (13). HSC-4 cells were treated for 2 or 20 hours without (control), or with CDBA (2 mM) and then stained with 1 μg/mL acridine orange for 20 minutes. After washing once with PBS(–), samples were then examined under a Laser Scanning Microscope LSM510 (Clontech Laboratories, Inc., Mountain View, CA, USA), using the following filter: excitation filter 488 nm, emission filter 505-530 nm and >650 nm, to detect the dot-like granular distribution of autophagolysosome in the cytoplasm.

Electron microscopy. Cells were detached 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˚C, post-fixed for one hour with 1% osmium tetraoxide-0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated, then embedded in Araldite 502 (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 to detect any changes in the fine cell structure (13).

Results

Tumor-specific cytotoxic action of CDBA. CDBA showed slightly higher cytotoxicity against tumor cells as compared with normal cells with a tumor-specificity index of 2.2. There was a considerable variation of sensitivity to CDBA among cells used (Table I). The sensitivity to CDBA was roughly in the following order: myelogenous leukemia (mean CC_{50}=0.65 mM) (the most sensitive) >oral squamous cell carcinoma (mean CC_{50}=0.87 mM) >glioblastoma (mean CC_{50}=1.6 mM) > normal cells (mean CC_{50}=2.1 mM)(the most resistant).

Type of cell death. CDBA induced internucleosomal DNA fragmentation in HL-60 cells, but to an extent much lesser than that induced by UV irradiation. CDBA did not induce any trace of DNA fragmentation in HSC-2, HSC-4 and T98G cells at 6 and 24 hours after treatment (Figure 2). CDBA only marginally enhanced the caspase activity in HL-60 cells (Figure 3D), but the extent of activation was much lower than that attained by actinomycin D (positive control). CDBA did not enhance the caspase activity in HSC-2 (A), HSC-4 (B) and T98G (C), in contrast to actinomycin D (Figure 3).

In HSC-2 cells, the number of secondary lysosomes with a variety of forms was found substantially increased at 2 mM CDBA using electron microscopy (Figure 4C), and at 4 mM, they changed to a multivesicular body which is suggested as the last stage of phagocytosomal digestion (Figure 4D) (14). In HSC-4 cells, vacuolated mitochondria were evident at 1 and 2 mM CDBA (Figure 4F, G) and they were digested by secondary lysosomes (Figure 4H). In T98G cells, no fine structural changes were evident (Figure 4I-L). In HL-60 cells, very large secondary lysosomes accompanying membranous vesicles with broken organelles were apparent at 2 and 4 mM (Figure 4O, P). Fine structural changes in HSC-2, HSC-4 and HL-60 cells suggest the induction of phagocytosis and its digestion in the secondary lysosome by CDBA at the concentration of more than 2 mM. This suggests the occurrence of autophagic cell death in at least three out of four tumor cell lines. Acridine orange staining shows the increase in the number of granular acidic organelles in the cytoplasm of CDBA treated cells (Figure 5), supporting the induction of autophagy.

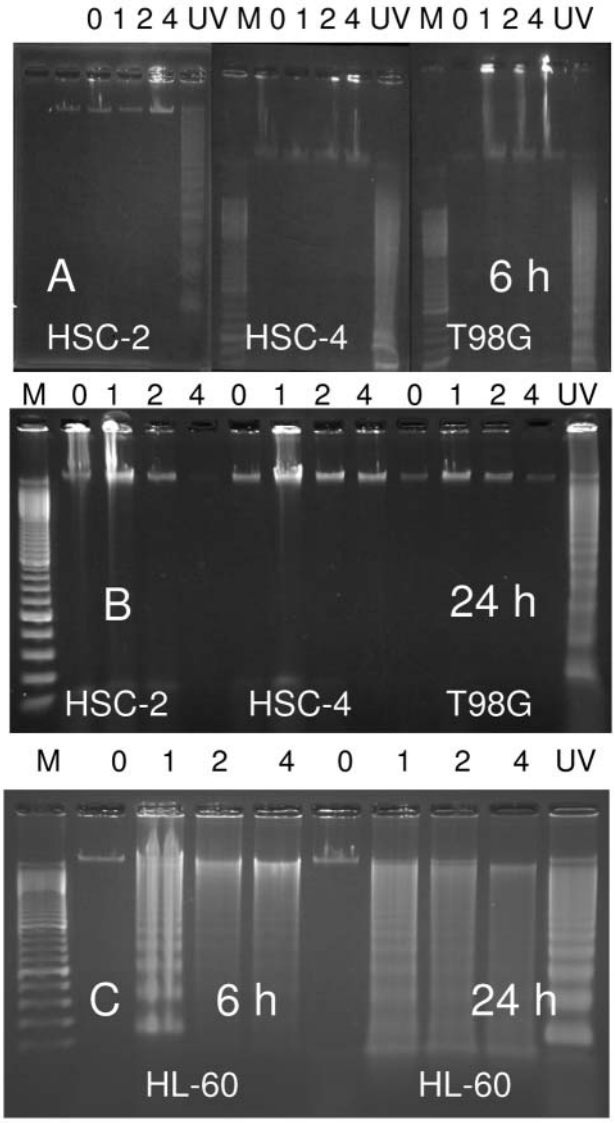

Figure 2. *Effect of CDBA on the induction of DNA fragmentation. HSC-2, HSC-4, T-98G and HL-60 cells were incubated for 6 or 24 hours without (control) or with 1, 2 or 4 mM of CDBA. DNA was then extracted and applied to agarose gel electrophoresis. M, DNA marker; UV, DNA from apoptotic HL-60 cells induced by UV-irradiation.*

Discussion

The present study demonstrated that CDBA showed slightly higher cytotoxicity against human oral squamous cell carcinoma, human glioblastoma and human myelogenous leukemia cell lines than against human normal oral cells. This provides evidence for the antitumor potential of CDBA against human carcinoma cell lines. HL-60 cells were the most sensitive to CDBA and underwent a low level of apoptosis as judged by only marginal induction of internucleosomal DNA fragmentation and caspase activation. However, human carcinoma cell lines showed considerable variation in their sensitivity to CDBA. Human glioblastoma cells (T98G, U87MG) were resistant to CDBA, as compared to oral squamous cell carcinoma (HSC-2, HSC-4). CDBA induced similar change in the fine structure of three out of four tumor cell lines, *i.e.*, the disruption of mitochondria structure and the digestion of

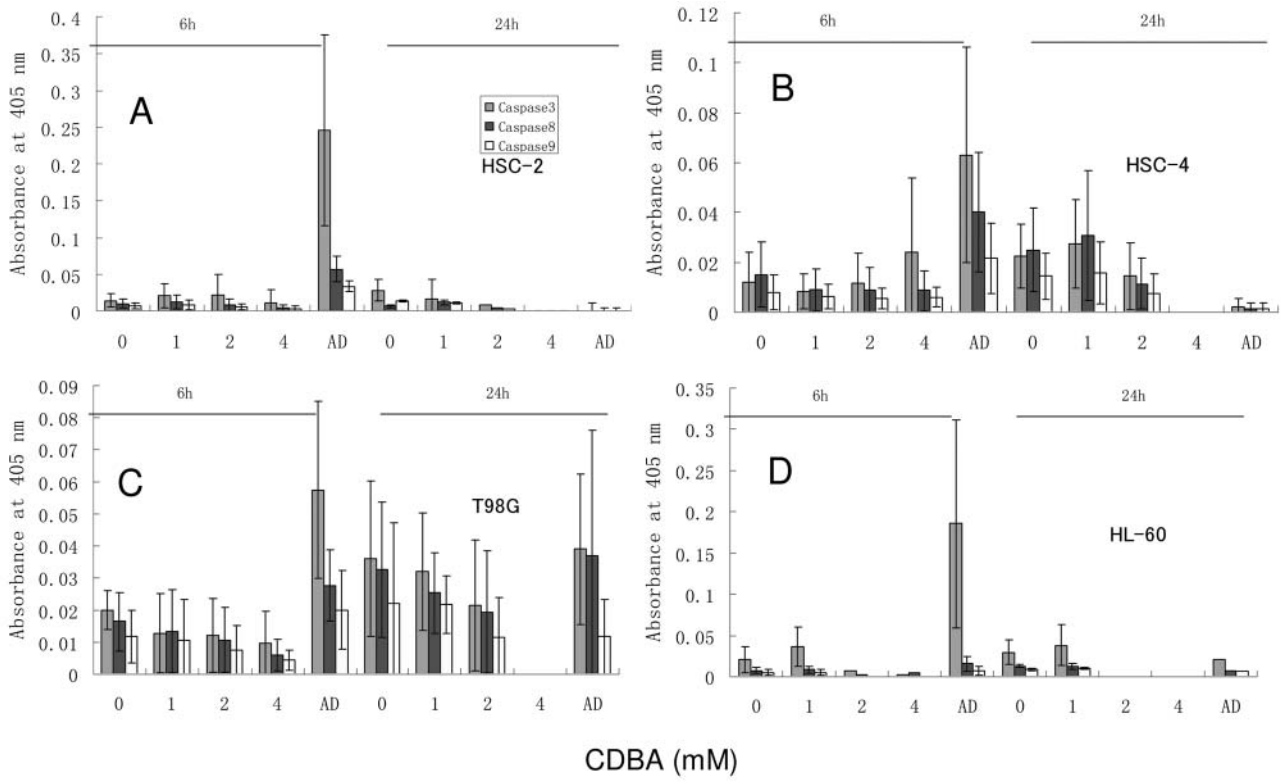

Figure 3. *Effect of CDBA on caspase activity. HSC-2, HSC-4, T98G and HL-60 cells were incubated for 6 or 24 hours without (control) or with 1, 2, or 4 mM CDBA, and caspase activity (expressed as 405 nm of cleaved product for each substrate) was determined. Each value represents mean±S.D. from 3 independent experiments. AD: Actinomycin D.*

broken organelles by the secondary lysosome. These observations suggest the occurrence of autophagic cell death. It has recently been reported that glioblastoma cell lines are prone to autophagic cell death (type II programmed cell death) characterized by autophagosome formation upon treatment with chemotherapeutic agents, radiations and toxicants (15-17). These results further confirm our proposal that the type of cell death is determined not only by the type of cells, but also by the unique structure of the inducers (18).

Cyclodextrins are produced from starch by means of enzymatic conversion. Over the last few years they have found a wide range of applications in food, pharmaceutical and chemical industries, as well as in agriculture and environmental engineering. Cyclodextrins are able to form host–guest complexes with hydrophobic molecules given the unique nature imparted by their structure. However, due to the lower solubility of CDBA at higher concentrations in the cell culture system used here, it was inevitable for us to use CDBA in suspension. This might have caused the lower tumor-specific index, compared to benzaldehyde (TS=20) (Sakagami *et al.*, unpublished data). The optimal condition for the release of benzaldehyde from the cyclodextrin–benzaldehyde complex at or near the target cancerous tissues or cells remains to be established.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (Sakagami, No. 19592156).

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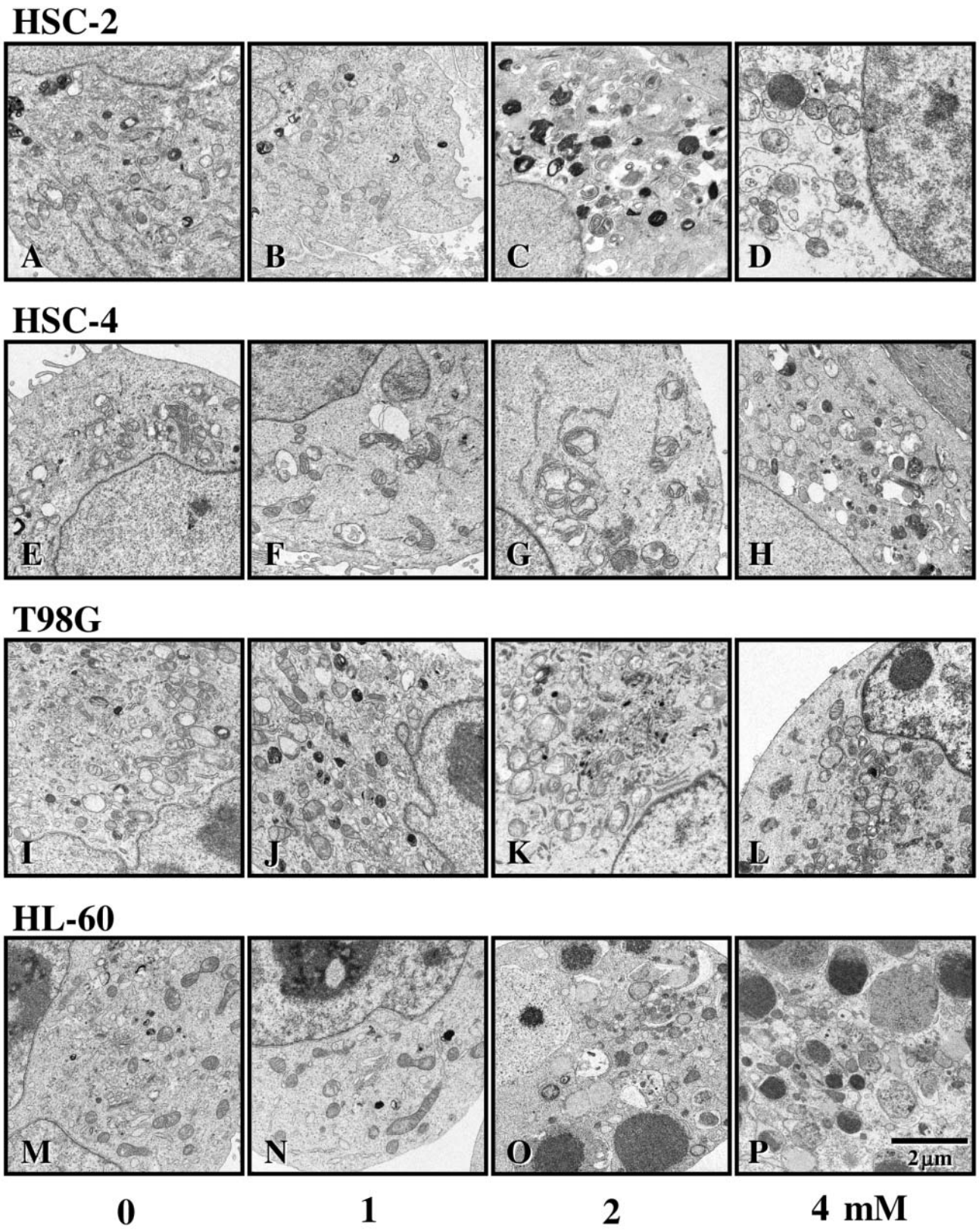

Figure 4. *Change in the fine structure of cells induced by CDBA. HSC-2 (A-D), HSC-4 (E-H), T98G (I-L) and HL-60 (M-P) cells were treated for 6 hours without 0 (A, E, I, M) (control) or with 1 (B, F, J, N), 2 (C, G, K, O) or 4 mM (D, H, L, P) CDBA and then processed for electron microscopy.*

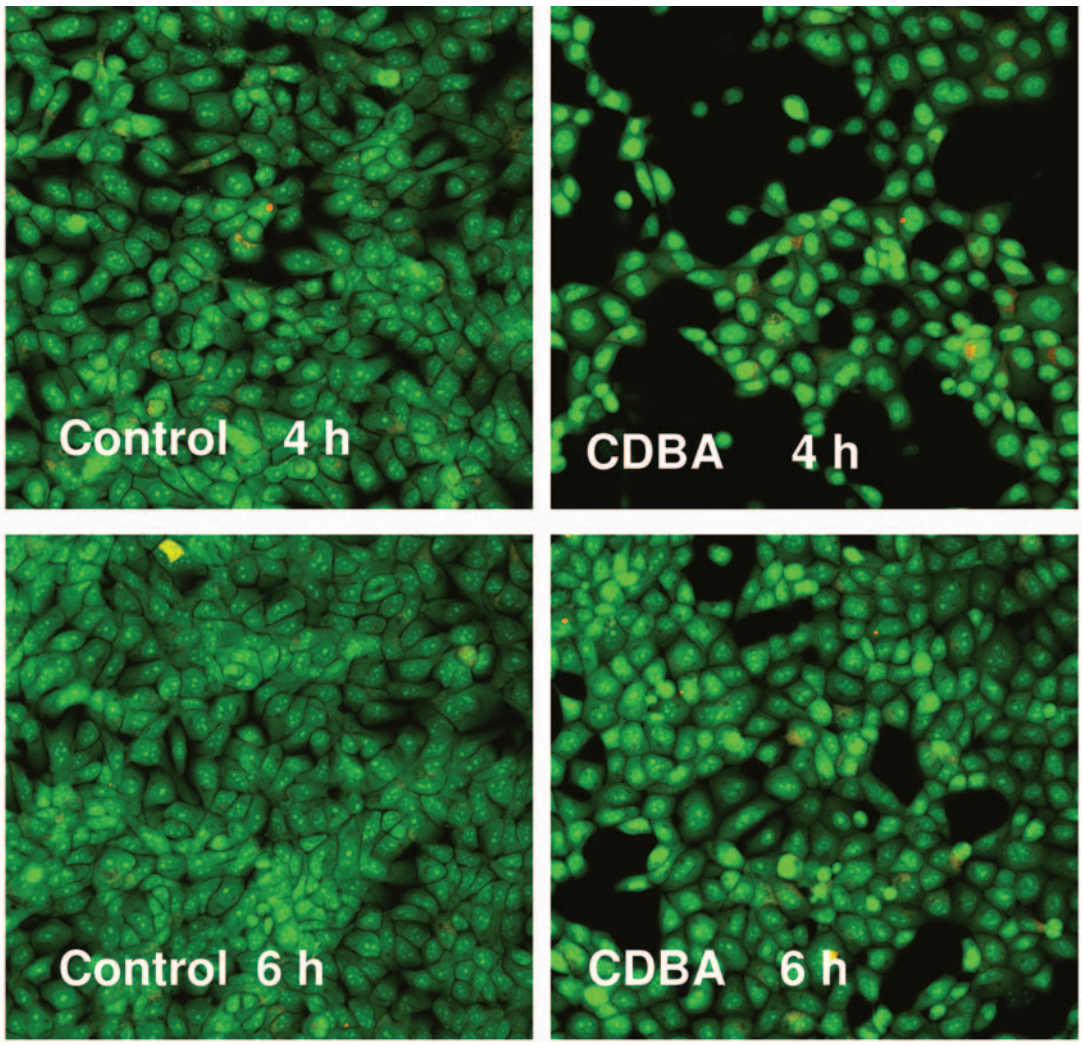

Figure 5. *Production of acidic vesicular organelles by CDBA. HSC-4 cells were treated for 4 or 6 hours without (control) or with 2 mM CDBA, and then stained with acridine orange. Magnification x200.*

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Received August 9, 2007
Revised November 15, 2007
Accepted December 17, 2007