Tumor-specific Cytotoxicity and Type of Cell Death Induced by Sodium 5,6-Benzylidene-L-ascorbate

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Abstract. *The cytotoxic activity of sodium 5,6-benzylidene-L-ascorbate (SBA) against eight human cancer cell lines and three human normal cells was investigated. SBA showed slightly higher cytotoxicity against human tumor cell lines, as compared with normal cells, with a tumor-specificity index of 2.0. The human myelogenous leukemia cell lines (HL-60, ML-1, KG-1) were the most sensitive to SBA, followed by human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4) and human glioblastoma (T98G, U87MG). Human oral normal cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast) were the most resistant. In contrast to actinomycin D, SBA induced little or no activation of caspase-3, caspase-8 and caspase-9 in the HSC-2, HSC-4, T98G and HL-60 cells, regardless of incubation time (either 6 or 24 h). SBA induced little or no internucleosomal DNA fragmentation after 6 h in all of these cells. However, prolonged treatment with SBA (24 h) induced a smear pattern of DNA fragmentation in the HSC-2, HSC-4 and T98G cells and a low level of internucleosomal DNA fragmentation in the HL-60 cells. Electron microscopy demonstrated the destruction of mitochondrial structure and autophagocytosis of broken organelles by SBA in the HSC-2, HSC-4 and HL-60 cells. At higher concentrations of SBA, necrotic cell death was observed in the HSC-2 cells, but not in the T98G cells, where the production of acidic organelles (detected by acridine orange staining) was much lower than that attained by nutritional starvation, a well-defined method of inducing autophagy. The present study suggests that SBA induces various degrees of autophagic cell death, followed by either necrosis or apoptosis at laters stage, depending on the cell type.*

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Benzaldhyde, a volatile fraction of figs (1) and its derivatives have shown antitumor activity against Ehrlich carcinoma, adenocarcinoma 755 and colon 38 in mice, but were inactive against several other implanted tumors in mice (2-4). Although benzaldehyde derivatives such as β-cyclodextrin benzaldehyde inclusion compound (CDBA), 4,6- benzaldehyde-α-D-glucose and sodium 5,6-benzylidene-L-ascorbate (SBA) (Figure 1) have shown weak antitumor activity against tumors implanted in mice, the administration of these compounds to patients with advanced, inoperable carcinomas has induced remarkable antitumor activity (5-7). We have also reported that the intravenous administration of SBA induced the degeneration of rat chemically induced hepatocellular carcinomas (8) and colon tumors (9) and the antitumor activity of SBA was not induced *via* a host-mediated mechanism (8). It has been reported that the cytotoxicity and protein synthesis inhibition of the 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 (10). We also confirmed by peroxyoxalate chemiluminescence that SBA produced H_2O_2 in quantities necessary for cell death induction (11). We previously reported that SBA induced rapid changes in mitochondria, such as the disassembly of cristae and a decrease in the electron density, at non-cytotoxic concentrations and it induced the swelling and vacuolization of the mitochondria at cytotoxic concentrations, while the nuclear architecture (the profile and the ratio of heterochromatin and euchromatin and thickness of the nuclear membrane) was intact in a human submandibular gland carcinoma cell line (12). This suggested that the mitochondria, not the nucleus, may be the target organelle of SBA. However, the mechanism inducing this antitumor activity by SBA is unclear.

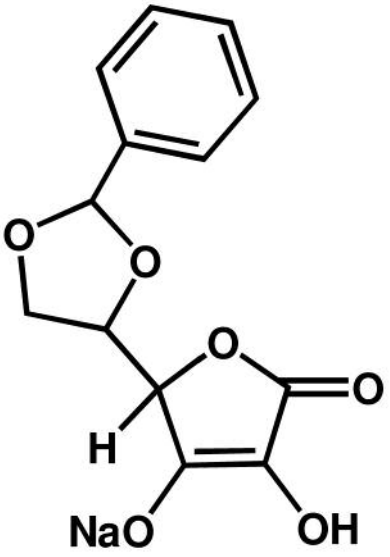

Figure 1. *Chemical structure of SBA.*

We recently found that CDBA showed slightly higher cytotoxicity against human tumor cell lines, as compared with normal cells (13). There are at least three types of cell death, apoptosis (type I programmed cell death characterized by blebbing, chromatin condensation, internucleosomal DNA fragmentation and the loss of cell surface microvilli), autophagy (type II programmed cell death characterized by the formation of autophagosomes and autophagolysosomes engulfing the cytosol organelles) and necrosis (14, 15). As a part of the continuing program studying the antitumor activity of benzaldehyde and its derivatives, whether or not SBA similarly induced specific cytotoxic activity was investigated in three human normal oral cells (gingival fibrobast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF), three human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4), two human glioblastoma (T98G, U87MG) and three human myelogenous leukemia (promyelocytic leukemia HL-60, myeloblastic leukemia ML-1, myeloid leukemia KG-1) cell lines. The type of cell death induced by SBA in the tumor cell lines was also investigated.

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), Hank's buffer (Sigma Chemical Co., St. Louis, MO, USA). The SBA was provided by Ichijokai Hospital, Chiba, Japan and was dissolved directly in culture medium at a concentration of 64 mM.

Cell culture. The HL-60 cells were provided by Prof. K. Nakaya, Showa University. The ML-1 and KG-1 cells were provided by Prof. K. Takeda, Tokyo University of Science. The HSC-2, HSC-3 and HSC-4 cells were obtained from Prof. M. Nagumo, Showa University, and the T98G and U87MG cells were provided by Dr. M. Iida, Showa University, Japan. The normal oral cells (HGF, HPC, HPLF) were prepared from periodontal tissues, according to the guideline of The Intramural Ethic Committee (No. 0206), after obtaining informed consent from the patients. Since normal oral cells have a limited lifespan of about 20 population doubling levels (PDL) (16), they were used at 5-9 PDL in the present study. The HL-60, ML-1 and KG-1 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. The other eight adherent cells (three normal cells and five tumor cell lines) 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^{2+} and Ca^{2+} (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 cytotoxic activity. Near-confluent cells were treated for 48 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 SBA-treated 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 reaction product, formazan, was extracted with DMSO and the absorbance (the relative viable cell number) was measured at 540 nm by a microplate reader (Multiskan Bichromatic Labsystems, Helsinki, Finland). The viability of the cell suspension, *i.e.* HL-60, ML-1 and KG-1, was determined by cell counting with a hemocytometer after staining with 0.15% trypan blue in the culture medium. The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve and the mean value of CC_{50} against each cell type was calculated from 3-6 independent experiments. The tumor-specificity index (TS) was measured by the following equation: $TS=(CC_{50}[HGF]+CC_{50}[HPC]+CC_{50}[HPLF]) / (CC_{50}[HSC\text{-}2]+CC_{50}[HSC\text{-}3]+CC_{50}[HSC\text{-}4]+CC_{50}[T98G]+CC_{50}[U87MG]+CC_{50}[HL\text{-}60]+CC_{50}[ML\text{-}1]+CC_{50}[KG\text{-}1])\times (8/3)$.

Assay for DNA fragmentation. The HSC-2, HSC-4 and T98G cells, collected by scraping with a rubber policeman on ice, and the 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. The DNA was extracted with 50 μl NaI solution (40 mM Tris-HCl [pH 8.0], 7.6 M NaI, 20 mM EDTA-2Na) and then 250 μl of ethanol. After centrifugation for 20 min at 20,000 xg, the precipitate was washed 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 cells induced by UV irradiation (6 J/m^2/min, 1 min), followed by 6 h incubation in regular culture medium, was run in parallel as a positive control (17). After staining with ethidium bromide, the DNA was visualized by UV irradiation, and photographed by a 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 (50 mM Tris-HCl [pH 7.5], 0.3% NP-40, 1 mM DTT). After standing for 10 min on ice and centrifugation for 20 min at 15,000×g, the supernatant was collected. Lysate (50 μl, equivalent to 100 μg protein) was incubated with 50 μl lysis solution containing substrates for caspase-3 (DEVD-*p*-nitroanilide [*p*NA], caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA) for 4 h at 37˚C. The absorbance of the liberated chromophore *p*NA was measured at 405 nm using a microplate reader (18). HL-60 cells were incubated for 6 h without or with 1 μg/ml actinomycin D to prepare the positive control of apoptotic cells.

Detection of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were stained with acridine orange (Sigma Chemical Co.) as described previously (18). The T98G cells were treated for 6 h without (control), or with test sample, and then stained with 1 μg/ml acridine orange for 20 min. After washing once with PBS(–), the samples were then examined under a laser scanning microscope (LSM510; Carl Zeiss Inc., Gottingen, Germany), using the following filter: excitation filter 488 nm, emission filter 505-530 nm and >650 nm. Autophagy was induced by culturing for 6 h in Hank's buffer (starvation condition) as a positive control (19).

Electron microscopy. After incubation for 6 h without (control) or with various concentrations of SBA, the cells were washed once with PBS(–), and fixed for 1 h with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C. The fixed cells were postfixed in 1% osmium tetraoxide-0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated and embedded in Araldite 502 (CIBA-GEIGY, Basel, Switzerland). Fine sections were stained with uranylacetate and lead citrate, prior to being analyzed under a JEM-1210 transmission electron microscope (JEOL) at an accelerating voltage of 100 kV (18).

Results

Tumor-specific cytotoxic action of SBA. SBA showed slightly higher cytotoxicity against tumor cells as compared to normal cells, with a tumor specificity index of 2.0 (Table I). There was a considerable variation of sensitivity to SBA among the cells used (Table I). The sensitivity to SBA was roughly in the following order: myelogenous leukemia (mean CC_{50}=1.37 mM) (the most sensitive) > oral squamous cell carcinoma (mean CC_{50}=2.33 mM) > glioblastoma (mean CC_{50}=4.25 mM) > normal cells (mean CC_{50}=4.80 mM) (the most resistant).

Type of cell death. Caspase activation and DNA fragmentation were used as markers of apoptosis induction. SBA induced little or no activation of caspase-3, caspase-8 and caspase-9 in the HSC-2, HSC-4, T98G and HL-60 cells during the first 6 h of incubation (Figure 2, A-D). Prolonged incubation for 24 h still did not activate the caspases in the HSC-2, HSC-4 and T98G cells (Figure 2, E-G), but a 2-fold increase in caspase activity was observed in the HL-60 cells (Figure 2H), while a more than 10-fold increase was attained by actinomycin D (1 μg/ml).

Table I. *Cytotoxic activity of SBA against human normal cells and tumor cell lines*.

	CC_{50} of SBA (mM)
Human normal oral cells	
HGF	3.9±1.4 (n=4)
HPC	4.6±1.5 (n=5)
HPLF	5.9±2.4 (n=5)
	Mean=4.80 mM
Human oral squamous cell carcinoma	
HSC-2	2.1±0.8 (n=5)
HSC-3	3.1±2.1 (n=4)
HSC-4	1.8±1.8 (n=5)
	Mean=2.33 mM
Human glioblastoma	
T98G	5.5±2.1 (n=3)
U87MG	3.0±2.8 (n=3)
	Mean=4.25 mM
Human myelogenous leukemia	
HL-60	2.0±1.0 (n=5)
ML-1	1.1±0.43 (n=3)
KG-1	1.0±1.2 (n=3)
	Mean=1.37 mM
Tumor specificity index	2.0

Each value represents the mean±S.D. from 3-5 independent experiments (n=3-5).

SBA induced little or no internucleosomal DNA fragmentation in all the cells tested after 6 h (Figure 3, A-D). However, prolonged treatment with SBA (24 h) induced a smear pattern of DNA fragmentation in the HSC-2, HSC-4 and T98G cells (Figure 3, E-G), and slight internucleosomal DNA fragmentation in the HL-60 cells (Figure 3H).

Electron microscopy (Figure 4) revealed structural changes including the swelling of mitochondria, increase of lucency of the mitochondrial matrices and enlargement of the endoplasmic reticulum in the HSC-2 cells at 4 mM SBA (Figure 4C), and the rupture of the cell membrane and the discharge of damaged organelles with increasing SBA concentration (8 mM) (D). Overly autophagic cell death was observed in the HSC-2 cells, except for higher concentrations of SBA that induced necrotic cell death. In the HSC-4 cells, SBA induced slight or aggravated mitochondrial damage at 2 (Figure 4F), 4 and 8 (Figure 4, G, H) mM SBA, respectively. In the HL-60 cells cultured with 2 mM SBA, slight swelling of the mitochondria and enlargement of the endoplasmic reticulum were observed (Figure 4J). At 4 mM SBA, many lysosomes with electron-dense matrices and vacuolated endoplasmic reticulum were noticeable (K), suggesting the autophagocytic activity of damaged organelles in these cells. At 8 mM, vacuoles of various sizes in the cytoplasm as well as elongation of the nuclear membrane and condensation of peripheral

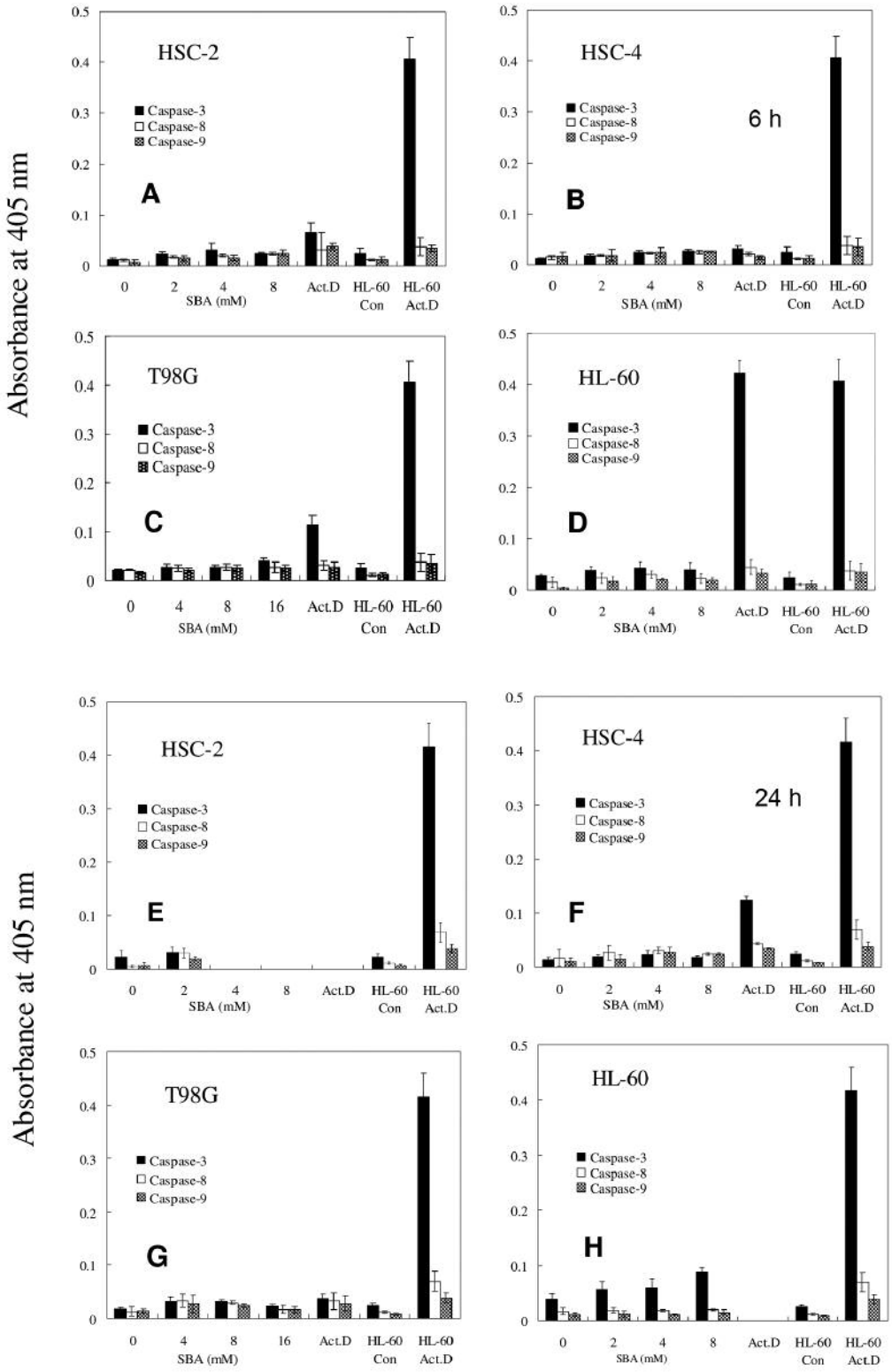

Figure 2. *Effect of SBA on the caspase activity. HSC-2, HSC-4, T98G and HL-60 cells were incubated for 6 (A-D) or 24 h (E-H) with the indicated concentrations of SBA, and caspase activity (expressed as absorbance at 405 nm of the cleaved product for each substrate) was then determined. Each value represents the mean±S.D. from 3 independent experiments. HL-60 cells were incubated for 6 h without (Con) or with 1 μg/mL actinomycin D (Act. D) to prepare the positive control of apoptotic cells. Three bars at each SBA concentration (from left to right) show caspase-3, caspase-8 and caspase-9, respectively.*

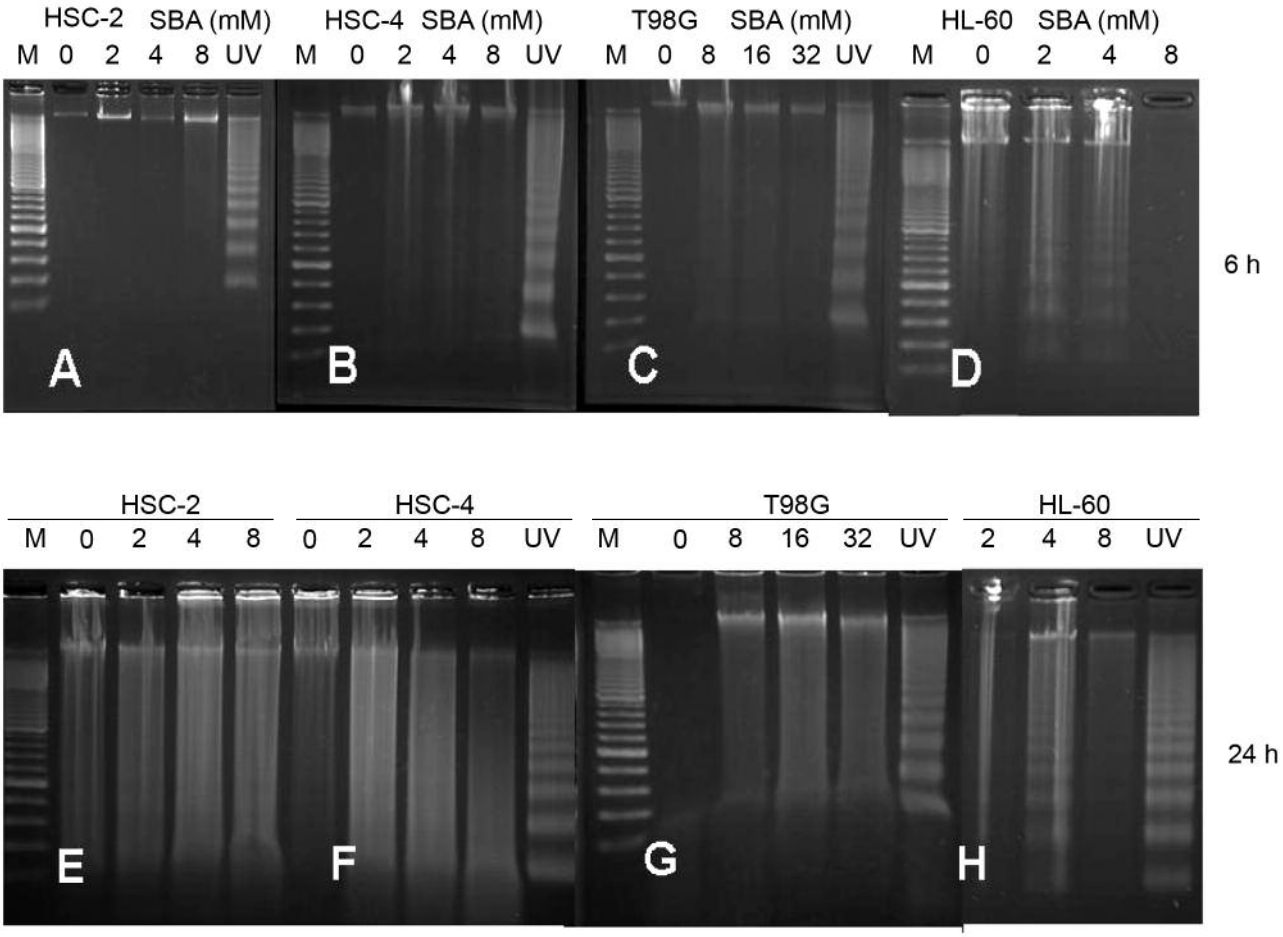

Figure 3. *Effect of SBA on the induction of DNA fragmentation. HSC-2, HSC-4, T98G and HL-60 cells were incubated for 6 or 24 h with the indicated concentrations of SBA. DNA was then extracted and applied to agarose gel electrophoresis. M denotes DNA marker. UV denotes DNA from apoptotic HL-60 cells induced by UV irradiation. Similar reproducible data were obtained in another two independent experiments.*

heterochromatin in the nucleus were observed (Figure 4L). In the T98G cells, the fine structure of the cell organelles including the mitochondria and endoplasmic reticulum seemed healthy and did not change when cells were cultured with 4-16 mM SBA (Figure 4, N-P). The T98G cells were insensitive up to 16 mM of SBA. The ultrastructural observation was largely consistent with the acridine orange staining experiment which showed that production of acidic organelles induced by SBA was much lower than that induced by nutritional starvation (Figure 5).

Discussion

The present study demonstrated that SBA showed slightly higher cytotoxicity against human oral squamous cell carcinoma, human glioblastoma and human myelogenous leukemia cell lines than against human normal oral cells, further demonstrating the antitumor potential of SBA against human carcinoma cell lines. SBA induced autophagic cell death in the early stages (6 h after treatment) (Figure 5), followed by necrotic changes in the HSC-2, HSC-4 and T98G cells (based on the smear pattern of DNA fragmentation in Figure 3, E-G), or apoptotic cell death in the HL-60 cells (based on the internucleosomal DNA fragmentation in Figure 3H) at the later stages (24 h). The transient appearance of an autophagic phenotype may have been a cellular response to protect the cells from cell death (20) and the disappearance of the autophagic phenotype may have indicated a switch to the cell death pathway, either necrotic or apoptotic, depending on the type of cell. This possibility was supported by recent findings that autophagy inhibitors such as 3-methyladenine and bafilomycin A1 induced apoptotic cell death in mouse macrophage-like RAW 264.7 cells (21) and that oridonin induced apoptosis with reduced autophagy in human breast cancer MCF-7 cells (22). However, it should be noted that the apoptosis-inducing activity of SBA (assessed by the extent of

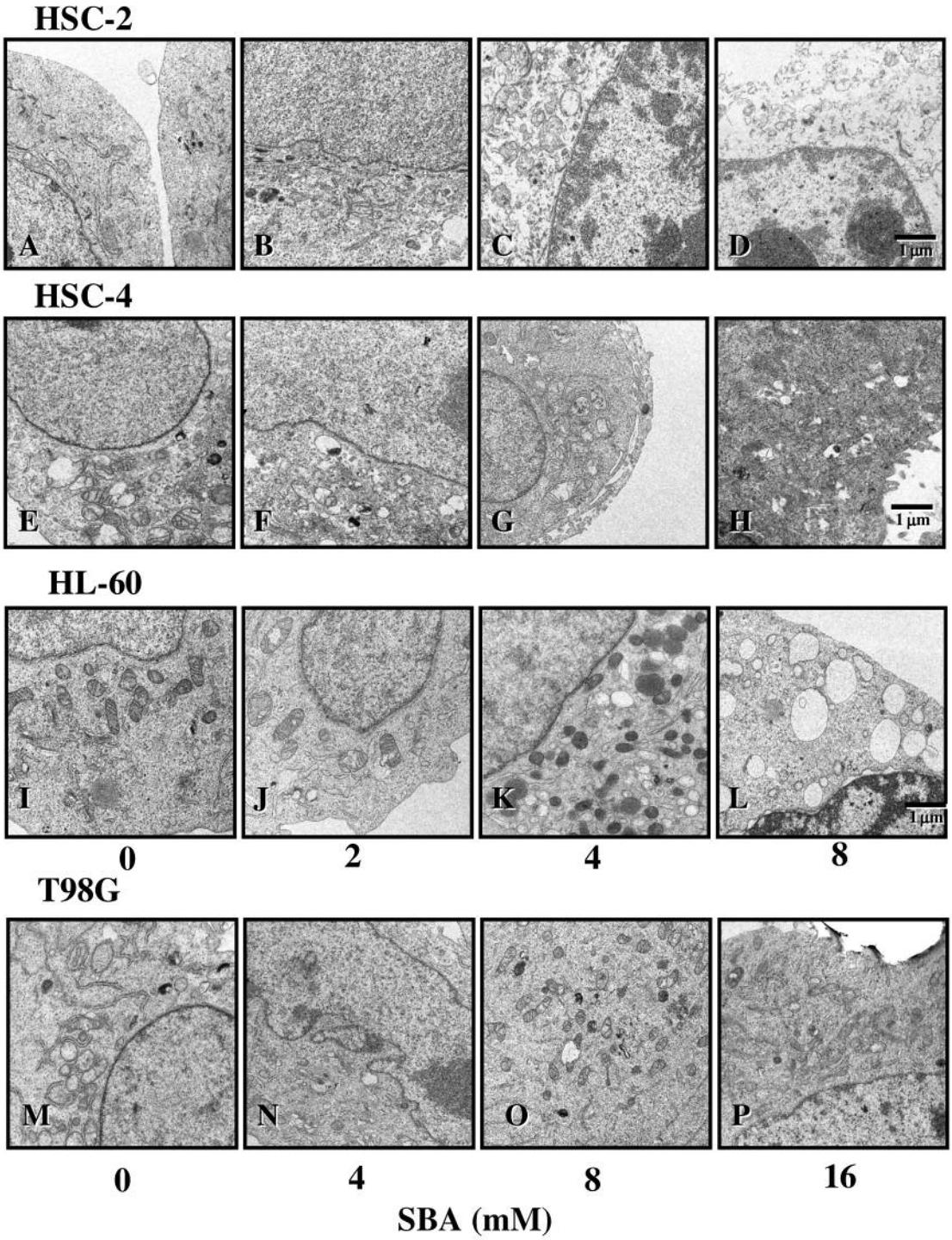

Figure 4. *Changes in the fine structure of cells induced by SBA. HSC-2 (A-D), HSC-4 (E-H), HL-60 (I-L) and T98G (M-P) cells were treated for 6 h without (A, E, I, M) (control) or with 2 (B, F, J), 4 (C, G, K, N), 8 (D, H, L, O) or 16 (P) mM SBA and then processed for electron microscopy.*

caspase activation, a much more reliable parameter than the visual evaluation of DNA fragmentation pattern) was much lower than that of actinomycin D. This was unexpected, since hundreds of papers have demonstrated the apoptosis induction of HL-60 cell lines by various chemotherapeutic agents. The present finding further supported our hypothesis that the type of cell death is determined not only by the type of cell, but also by the unique structure of the inducer (23).

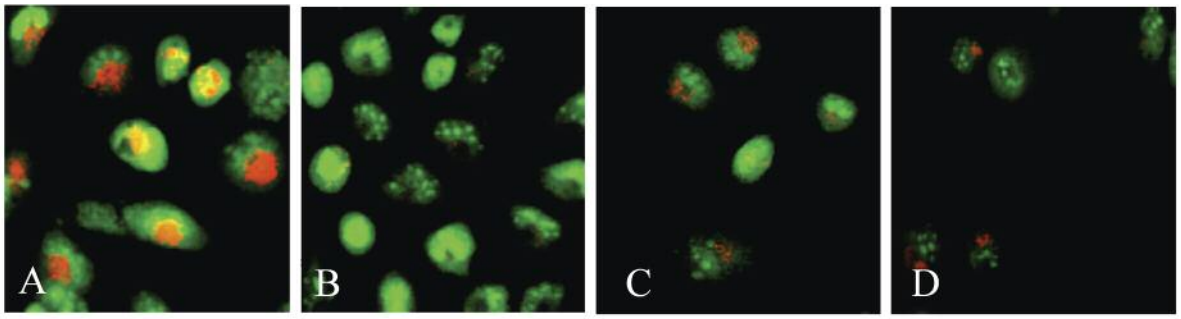

Figure 5. *Production of acidic vesicular organelles by SBA in T98G cells. T98G cells were induced to autophagy by culturing for 6 h in Hank's buffer (under starved condition) (A), or treated for 6 h without (control) (B) or with 4 (C) or 16 (D) mM SBA, and then stained with acridine orange.*

It was unexpectedly found that the tumor specificity index of SBA (TS=2.0) was much lower than that of conventional antitumor chemotherapeutics such as doxorubicin (TS=255) (24), mitomycin C (TS>22.7) (25) and 3-(3,4,5-trimethoxyphenyl)-1-oxo-2-propene peplomycin (TS>176) (26), in contrast to its dramatic antitumor action in cancer patients (5-7). This suggested that SBA administered to cancer patients may be metabolized to more active forms. We have previously reported that SBA is unstable, especially under acidic conditions, producing ascorbic acid and benzaldehyde by cleavage of the acetal linkage in the SBA molecule (27, 28). Our preliminary study demonstrated that benzaldehyde possessed much higher tumor-specific cytotoxicity (TS=24-39) (unpublished data) and may be easily oxidized and the oxidized compound binds to many cellular components. A comparative study of the *in vitro* antitumor action between SBA and benzaldehyde is underway and will be reported elsewhere.

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