Cytotoxicity and Type of Cell Death Induced by Local Anesthetics in Human Oral Normal and Tumor cells

KATSUE KOBAYASHI[1], SEIKA OHNO[1], SHIGENORI UCHIDA[1], OSAMU AMANO[2], HIROSHI SAKAGAMI[3] and HIROSHI NAGASAKA[1]

Divisions of [1]Anesthesiology, [2]Anatomy and [3]Pharmacology, Meikai University School of Dentistry, Sakado, Saitama, Japan

Abstract. *Background: Local anesthetics are often administered to tumors and surrounding tissues during the surgery of the head and neck area, however their effects on oral tissues is not well understood. In the present study, the cytotoxicity of a total of seven local anesthetics towards oral tumor and normal cells was compared. Materials and Methods: Tumor-specificity index was determined by the ratio of the mean 50% cytotoxic concentration against normal cells to that for tumor cells. Apoptosis induction was monitored by internucleosomal DNA fragmentation and caspase-3, -8, and -9 activation. Fine cell structure was observed under transmission electron microscopy. Results: All local anesthetics showed slightly higher cytotoxicity towards oral squamous cell carcinoma (OSCC) cell lines than towards normal oral cells. Dibucaine, with a log p-value of approximately 3, was the most cytotoxic, followed by tetracaine, bupivacaine or ethyl-aminobenzoate, whereas lidocaine, procaine and mepivacain were much less cytotoxic. When the tumor-specificity was evaluated between OSCC and human skin keratinocytes, the index was 6.6. Dibucaine did not induce apoptosis of OSCC cells. On the other hand, dibucaine did induce mitochondrial injury and swelling, formation of secondary lysosomes, and at high concentrations, rupture of the cell membrane. Autophagy inhibitors did not reduce the cytotoxicity of dibucaine. Conclusion: Necrosis may be involved in the induction of antitumor activity by dibucaine.*

Local anesthetics irreversibly block Na^+ channels from the inside of the cell surface membrane, and are used for spinal and epidural anesthesia and nerve blocking (1). Local anesthetics exert their action not only against neurons but also against surrounding tissues, and damage the muscle and other tissues (2, 3). Topical application of local anesthetics has been reported to produce good pain control in patients with head and neck tumors (4), to reverse surgical stress-induced inhibition of natural killer (NK) cell activity, and to inhibit the metastasis and relapse of tumors (5, 6). However, no reliable evidence has been obtained, possibly due to the limited information about the mechanism of antitumor action of local anesthetics (7).

Local anesthetics exhibited *in vitro* cytotoxicity towards various cultured cells (8-12). Apoptosis-inducing activity has been suggested to be involved in the neurotoxicity of local anesthetics (13-18). Not only general anesthesia but also local anesthesia is administered to tumors and surrounding tissues during surgery of the head and neck area. However, the effect of local anesthetics on oral cells is not well understood, possibly due to the scarcity of tissue injury by local anesthetics. Based on this, here we investigated the cytotoxicity of seven local anesthetics (Figure 1) towards human normal oral cells, oral squamous cell carcinoma (OSCC), glioblastoma cell lines, and skin keratinocytes. We also investigated the type of cell death induced by the most cytotoxic local anesthetic dibucaine, in OSCC.

Correspondence to: Professor Hiroshi Nagasaka, Division of Anesthesiology, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: +81 492855511, ext. 336, Fax: +81 492855171, e-mail: hnagasak@dent.meikai.ac.jp

Key Words: Local anaesthetics, oral squamous cell carcinoma, type of cell death, necrosis, mitochondrial injury, dibucaine.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: RPMI1640, Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum, (FBS), 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT), mepivacaine, bupivacaine (Sigma Chem. Ind., St. Louis, MO, USA); dimethylsulfoxide (DMSO), lidocaine, dibucaine, procaine, tetracaine, ethyl-*o*-aminobenzoate (Wako Pure Chemical, Osaka, Japan).

Cell culture. HL-60 (RIKEN, Tsukuba, Japan) cells were cultured at 37˚C in RPMI 1640, supplemented with 10% heat-inactivated FBS. Human OSCC cell lines (HSC-2, HSC-3, HSC-4, NA, Ca9-22) and human glioblastoma cell lines (T98G, U87MG) were

obtained from Professor Nagumo, Showa University, Japan. These adherent cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. Normal human oral cells, gingival fibroblast (HGF), pulp cells (HPC) and periodontal ligament fibroblast (HPLF), were prepared from periodontal tissues, according to the guideline of the Intramural Ethic Committee (no. A0808), after obtaining informed consent from the 12-years-old patient at the Meikai University Hospital. Since normal oral cells have a limited lifespan of 43-47 population doubling levels (PDL) (19), they were used at 8-15 PDL. Human skin keratinocytes (HEKa, HEKn) (purchased from KURABO, Osaka, Japan) were cultured in HuMedia-KG2, supplemented with insulin, human recombinant EGF (hEGF), hydrocortisone, gentamicin, amphotericin B, bovine pituitary gland extract (BPE).

Assay for cytotoxic activity. All of the cells were inoculated at 5×10^3 cells/well in 96-microwell plates (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 48 h, the medium was removed by suction with an aspirator, and replaced with 0.1 ml of fresh medium containing different concentrations of the test compounds. The cells were incubated for another 48 h, and the relative viable cell number was then determined by the MTT method (20). In brief, the cells were washed once with phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(–)], and replaced with fresh culture medium containing 0.2 mg/ml MTT. After incubation for 4 h, the cells were lysed with 0.1 mL of DMSO, and the absorbance of the cell lysate was determined at 540 nm, using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). From the dose-response curve, the 50% cytotoxic concentration (CC_{50}) was determined. Tumor-specificity (TS) was determined by the ratio of the mean CC_{50} for three normal cells to that for tumor cell lines.

Assay for DNA fragmentation. Cells were lysed by lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsalcosinate]. The lysate was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 1-2 h 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 100 μl of ethanol. After centrifugation for 20 min at 20,000 ×*g*, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 3-5). The sample (10-20 μl) was then applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0) (20). A DNA molecular marker (TAKARA, Japan) and the DNA from apoptotic HL-60 cells induced by ultraviolet (UV) irradiation were used for calibration. The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Assay for caspase activation. Cells were washed with PBS(–) and lysed in lysis solution (MBL, Nagoya, Japan). After resting of cells for 10 min on ice and centrifugation for 5 min at 10,000 ×*g*, the supernatant was collected. The lysate (50 μl, equivalent to 200 μg protein) was mixed with 50 μl 2× reaction buffer (MBL) containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)), caspase-8 (IETD-pNA) or caspase-9 (LEHD-*p*NA). After incubation for 4 h at 37˚C, the absorbance of the liberated chromophore pNA was measured at 405 nm by a microplate reader (20).

Electron microscopy. Dibucaine-treated cells were harvested by trypsin-EDTA and were pelleted by centrifugation at 1000 ×*g* for 5 min. The cells were fixed for 1 h with 2% gultaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated and then embedded in Araldite 502 (CIBAGEIGY Swiss; NISSHIN EN Co., Ltd., Tokyo Japan). Fine sections were stained with uranyl acetate and lead citrate, and were then observed under a JEM-1210 transmission electron microscope (JEOL), at an accelerating voltage of 80 kV (21).

Results

Tumor-specificity of local anesthetics. Dibucaine dose-dependently reduced the viability of all human normal and tumor cells investigated (Figure 1). The minimum lethal concentration of dibucaine against skin keratinocytes (HEKa, HEKn) (500 μM) (Figure 1F) was approximately two-fold that toward human normal oral cells (HGF, HPC, HPLF) (Figure 1E). Cell killing activity of dibucaine toward human OSCC cell lines (HSC-2, -3, -4, NA, Ca9-22) was observed at concentrations above 125 μM (Figure 1A, B). Dibucaine at 125 μM was lethal toward both glioblastoma (T98G, U87MG) (Figure 1C) and promyelocytic leukemia (HL-60) cells (Figure 1D).

Similar experiments were performed with another six local anesthetics to yield the CC_{50} values (listed in Table I). Dibucaine exhibited the highest cytotoxicity toward OSCC cell lines (mean CC_{50}=34.8 μM), followed by tetracaine (199.5 μM), bupivacaine (429.2 μM), ethylaminobenzoate (519.4 μM), lidocaine (676.6 μM), procaine (725.8 μM) and mepivacaine (820.8 μM).

Glioblastoma cells (T98G, U87MG) were slightly resistant to the cytotoxicity of local anesthetics as compared with OSCC cell lines. However, the relative cytotoxicity of these local anesthetics was in the same order: dibucaine (mean CC_{50}=109.7 μM) > tetracaine (201.5 μM) > bupivacaine (534.5 μM) > ethylaminobenzoate (559.5 μM) > lidocaine (735.5 μM), procaine (807 μM) > mepivacaine (950 μM).

Normal cells were the least affected. Dibucaine (mean CC_{50}=78.0 μM) again was the most cytotoxic, followed by tetracaine (216.0 μM) > ethylaminobenzoate (673.7 μM) > bupivacaine (753 μM) > lidocaine (811.6 μM) > procaine (>963.0 μM) and mepivacaine (>988.0 μM).

Skin keratinocytes (HEKa, HEKn) were highly resistant to dibucaine (CC_{50}=229.7 μM) (Table I). When the cytotoxicity of dibucaine toward five OSCC cells lines and human keratinocytes (both epithelial lineages) was compared, dibucaine had a much higher tumor-specificity (TS=6.6).

Amido- and ester-type local anesthetics had comparable cytotoxicity toward the cells investigated (Table I).

Type of cell death induced by dibucaine in OSCC. We first determined the minimum exposure time required to induce irreversible cell death using HSC-2 and HSC-4 cells (Figure 2). The cytoxocity of dibucaine towards both of these cell lines was increased with increasing exposure time up to 24 h. Further exposure did not apparently enhance the cytotoxicity

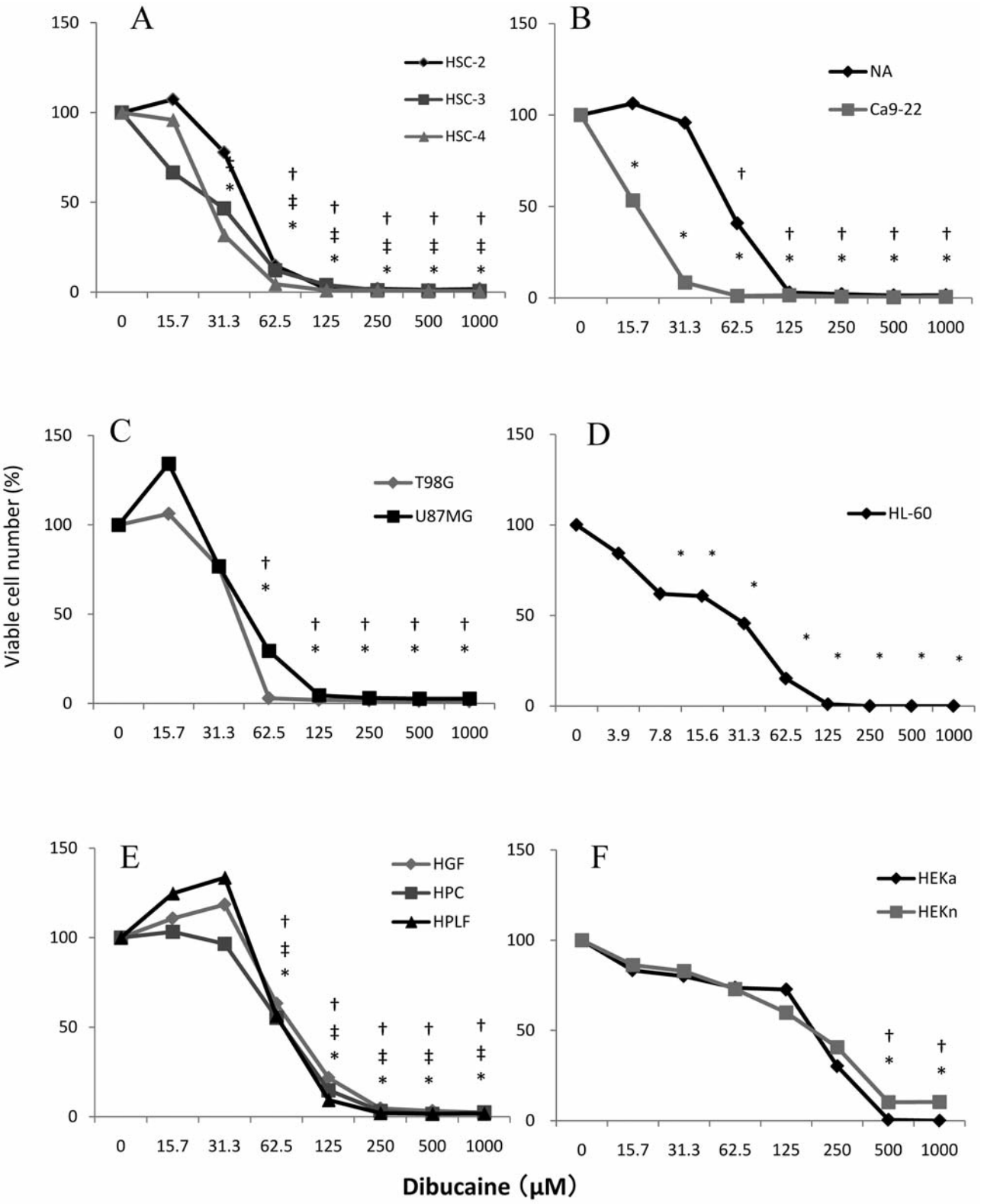

Figure 1. *Cytotoxic activity of dibucaine against human oral squamous cell carcinoma cells (A, B), glioblastoma cells (C), leukemia cells (D), and human normal oral cells (E), and human keratinocytes (F), treated for 48 h without (control), or with the indicated concentrations of dibucaine. The viable cell number was then determined by the MTT method, and expressed as control. A percentage of each value represents the mean±S.D. from 4 or 5 independent experiments. *p<0.01 †p<0.01 ‡p<0.01 compared to control (no reagent) of each cells.*

Table I. *Cytotoxic activity of local anesthetics against human normal and tumor cells.*

	CC_{50} (μM)						
	Lidocaine	Mepivacaine	Dibucaine	Bupivacaine	Procaine	Tetracaine	Ethyl aminobenzoate
Log *p*-value	2.36	2.04	3.03	3.64	2.36	3.65	1.95
Tumor cell lines							
HSC-2	660±159	>788	39.6±6.0	392±157	655±39.7	145±32.6	501±82.2
HSC-3	835±93.2	>1000	29.3±17.3	625±163	>931	203±45.2	664±106
HSC-4	673±70.7	756±40.4	28.8±7.7	430±27.9	759±47.6	263±266	555±108
NA	680±88.2	>921	58±15.9	457±126	>758	297±311	514±151
Ca9-22	535±131	639±80.7	17.8±3.6	242±76.2	>526	89.4±60.8	363±137
(Mean)	(676.6)	(820.8)	(34.8)	(429.2)	(>725.8)	(199.5)	(519.4)
Glioblastoma cell Lines							
T98G	764±112	>928	176±305	640±145	>905	241±186	654±56.3
U87MG	707±81.1	>972	43.4±9.5	429±75.7	>709	162±41.8	465±241
(Mean)	(735.5)	(>950)	(109.7)	(534.5)	(807)	(201.5)	(559.5)
Normal cell lines							
HGF	804±128	>987	101±74.9	763±73.4	>1000	250±57.8	661±70.3
HPC	840±84.3	>979	70.3±41.1	765±65.4	>950	236±131	703±94.2
HPLF	791±61.6	>1000	62.8±3.2	731±68.2	>939	162±117	657±25.4
(Mean)	(811.6)	(>988)	(78)	(753)	(>963)	(216)	(673.7)
HEKa			213.9±5.0				
HEKn			245.5±6.4				
(Mean)			(229.7)				

The CC_{50} values were calculated from the dose response curve. Each value represents the mean±S.D. from 4 or 5 independent experiments. The log *p*-value is cited from Chem Spider (http://www.chemspider.com/Chemical-Structure).

of dibucaine (Figure 2). Based on these data, HSC-2 and HSC-4 cells were treated for 24 h with different concentrations of dibucaine in the subsequent experiments. Since dibucaine had cytotoxic, but not cytostatic effects on the cells (Figure 1), we investigated which type of cell death dibucaine induced in OSCC cells. Dibucaine induced internucleosomal DNA fragmentation in HL-60 cells (used as positive control due to its high sensitivity to various apoptosis inducers), but not in OSCC cell lines (HSC-2, HSC-4) (Figure 3A). Similarly, dibucaine activated caspase-3 in HL-60 cells, but not in OSCC cell lines (Figure 3B). These data suggest that dibucain does not induce apoptosis in OSCC cells.

Next, the possibility of autophagy induction was tested using autophagy inhibitors. Pretreatment of OSCC cells with either 3-methyladenine (Figure 4A) or bafilomycin A1 (Figure 4B) failed to inhibit the cell death of OSCC, induced by dibucaine. These data suggest that autophagy is not involved in dubucaine-induced cell death of OSCC cell lines.

To assess the possibility of induction of other types of cell death, the fine cell structure after dibucaine treatment was observed under transmission electron microscopy (Figure 5). When HSC-2 and HSC-4 cells were treated for 24 h with different concentrations of dibucaine, cellular swelling accompanied by dysfunction of mitochondria and endoplasmic reticulum and formation of secondary lysosome were observed already at the half CC_{50} concentration (HSC-2: 19.8 μM, HSC-4:14.4 μM) (Figure 5). Higher concentrations of dibucaine (>CC_{50}) induced disruption of the plasma membrane and leakage of cellular substrates, and rapid cell breakage. These data indicate the possible occurrence of necrosis.

Discussion

Local anesthetics have been used as spinal anesthesia, dermenchysis and topical anesthesia to relieve pain in patients with cancer. The concentration of dibucaine used clinically as spinal anesthesia ranges from 0.3 to 0.75% (7.9 to 19.7 mM). The exact tissue concentration of local anesthetics used in pain relief is estimated to be 400 μM, approximately 1/100 of the initially administered concentration (8). However, dibucaine has been reported to be destructive towards the myelinated nerve Aβ fiber [at 0.003% (69 μM)] and unmyelinated nerve C fiber at 0.03% (690 μM) (22), suggesting a narrow safety margin.

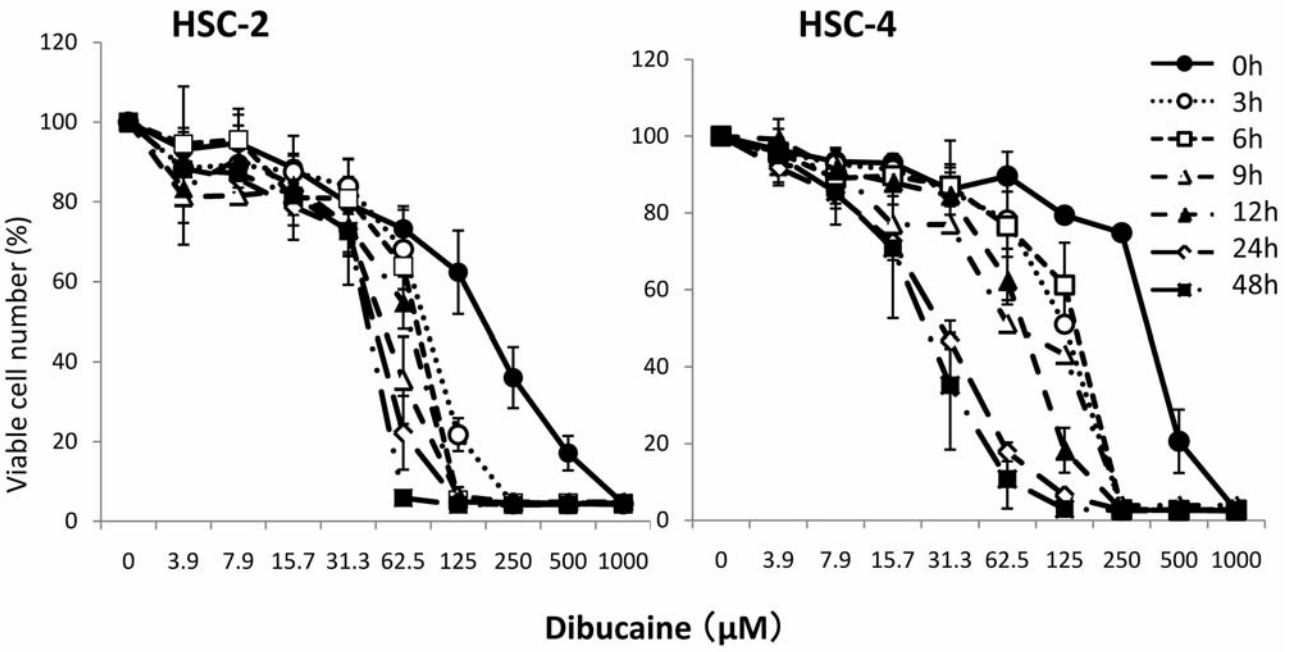

Figure 2. *Effect of exposure time of dibucaine on the induction of cytotoxicity in HSC-2 and HSC-4 cells. Cells were treated for the indicated times without (control) or with the indicated concentrations of dibucaine. The viable cell number was then determined by the MTT method, and expressed as a percentage of the control. Each value represents the mean±S.D from 3 independent experiments.*

Dibucaine has been reported to show neurotoxicity even at as low as 20 μM (23). These data indicate that dibucaine can induce neurotoxicity at a concentration lower than its clinically used concentration (22, 23). Due to neurotoxicity of dibucaine as spinal anesthesia (24, 25), the opportunities for its clinical use have become fewer as compared with bupivacaine or ropivacaine. However, despite the reporting of many cases of neurotoxicity of dibucaine, including cauda equine syndrome, and its infiltration into surrounding tissues, there are no reports of this agent in inducing necrosis.

The present study demonstrated that both amido- and ester-type local anesthetics have comparable cytotoxicity towards both normal oral and tumor cells, however, the tumor cell lines were slightly more sensitive to these anesthetics. Dibucaine (15-100 μM) had the highest cytotoxicity and tumor-specificity among the seven anesthetics investigated. When the cytotoxicity of dibucaine against OSCC and skin keratinocytes was compared, tumor specificity index was 6.6. Dibucaine did not induce apoptosis (characterized by DNA fragmentation and caspase activation). In addition, dibucaine-induced cell death was not inhibited by autophagy inhibitors. The electron microscopic observation demonstrated the swelling of organelles. These data suggest the possible induction of necrosis, especially at higher concentrations of dibucaine. The dibucaine used at concentrations below 15 μM was not cytotoxic.

Higher concentrations (100 μM, 150 μM) of dibucaine have been reported to induce apoptosis of neuroblastoma and leukemia cell lines (9, 10). We also found that higher concentrations (30-125 μM) of dibucaine induced apoptosis of HL-60 cells, used as control cells that are easily committed to apoptosis. The present study demonstrated, to our knowledge for the first time that dibucaine failed to induce apoptosis of human OSCC cell lines, suggesting that the type of cell death may depend on the target cells. This possibility is supported by a previous report that local anesthetics such as lidocaine, bupivacaine and tetracaine induced apoptosis of renal proximal tubule cells, but not in trachea smooth muscle cells (10). Lidocaine at 400 and 4000 μM was cytotostatic and cytotoxic, respectively, towards tongue cancer cell lines (8). Since these tongue cancer cell line expresses a higher tyrosine kinase activity of epidermal growth factor receptor (EGFR), the cytotoxic action of local anesthetics may be due to its inhibition of tyrosine kinase activity (26). Kamiya *et al*. have reported that lidocaine induced apoptosis at relatively low millimolar concentrations (12 mM), but necrosis at much higher millimolar concentrations (above 15 mM) in human histiocytic lymphoma cells (16). Our findings are in agreement with these data.

It has been reported that many drugs, toxicants and radiation have biphasic effects, that is a growth-stimulatory effect (hormesis) at lower concentration and a cytotoxic effect at higher concentration (27). We found that when normal cells (HGF, HPC, HPLF) were incubated for 24 h with low

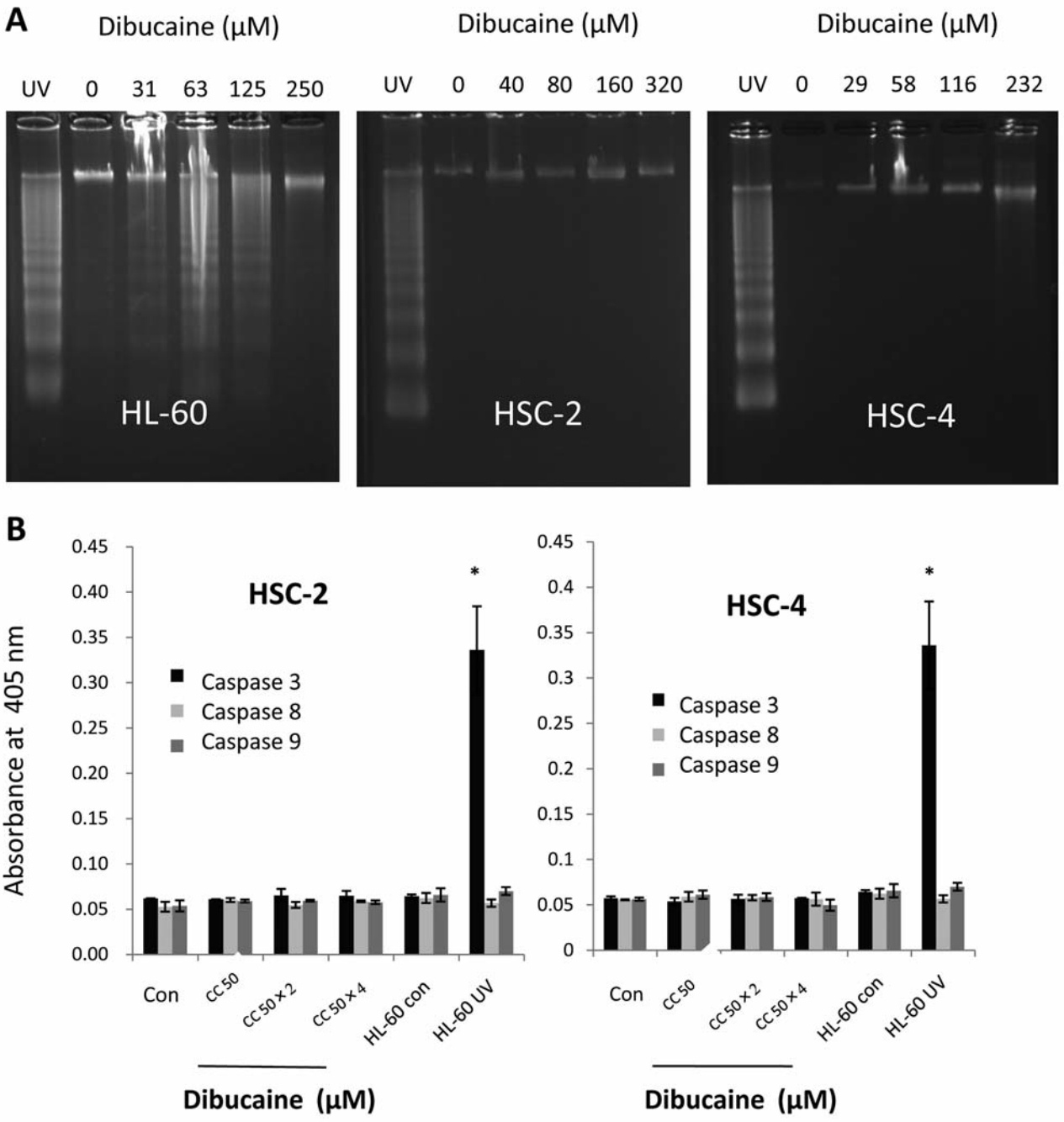

Figure 3. *Effect of dibucaine on the DNA fragmentation and caspase activity. HL-60, HSC-2 and HSC-4 cells were incubated for 24 h with 0 (control), CC_{50}, CC_{50}×2 or CC_{50}×4 of dibucaine. (A): DNA was then extracted and subjected to agarose gel electrophoresis. UV; DNA from apoptotic HL-60 cells induced by UV-irradiation. (B): Caspase activity (expressed as 405 nm of each cleaved product) was determined. Apoptotic HL-60 cells induced by UV were used as positive control. *p<0.01 compared to control of each cell line.*

concentrations (0.4-6.3 μM) of dibucaine, their growth was stimulated by up to 238% compared to that of untreated cells; such a hormetic effect has not been observed in any of the OSCC cell lines (data not shown). The biological significance of this finding remains to be investigated.

Local anesthetics stimulated the membrane fluidity and permeability of artificial membranes (8, 28-30) and this stimulation may be involved in apoptosis induction in a neuroblastoma cell and HL-60 cells by dibucaine (9, 10). These findings were confirmed by the report that local

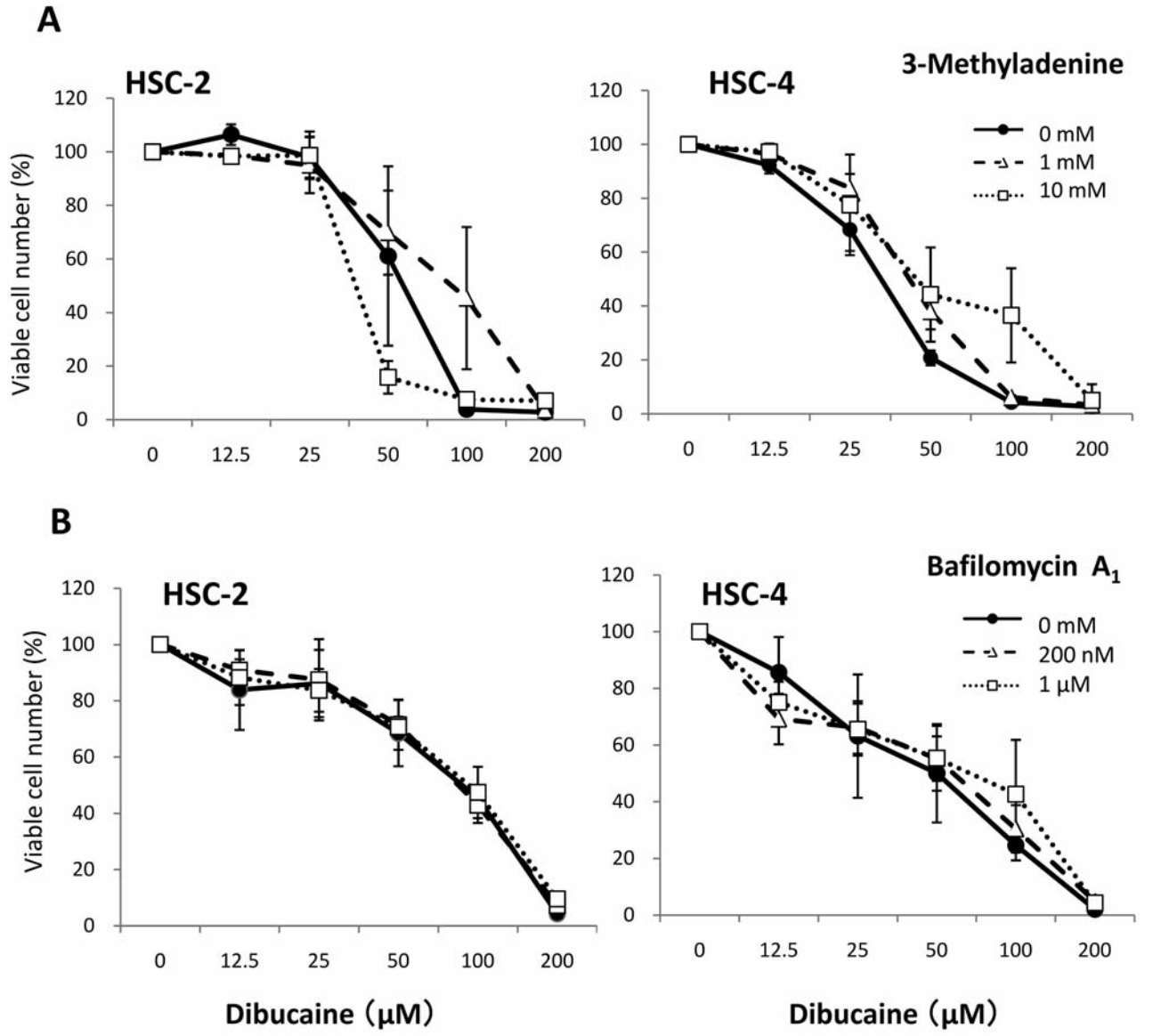

Figure 4. *Effect of autophagy inhibitors (3-methyladenine and bafilomycin A1) on dibucaine-induced HSC-2 and HSC-4 cytotoxicity. HSC-2 or HSC-4 cells were pre-treated for 1 h with the indicated concentration of 3-methyladenine (A), or bafilomycin A1 (B) and then incubated for 24 h with 0 (control), or with the indicated concentrations of dibucaine. The viable cell number was then determined by the MTT method, and expressed as a percentage of the control. Each value represents the mean±S.D. from triplicate determinations.*

anesthetics, including dibucaine, changed the membrane fluidity of hepatoma cells (31).

It has been reported that membrane permeability and cytotoxicity became maximum when the lipophilicity, as determined by the octanol-water partition coefficient (log p), approached 3 (32, 33). In accordance with this hypothesis, we found that dibucaine (log p=3.03) had the highest cytotoxicity against OSCC, followed by tetracaine (log p=3.65) and, bupivacaine (log p=3.64). Procaine (log p=2.36), lidocaine (log p=2.36), mepivacaine (log p=2.04), ethyl aminobenzoate (log p=1.95) were much less cytotoxic (Table I). This suggests that the cytotoxicity of local anesthetics is indeed related to their membrane permeability. Kitagawa *et al*. has reported that local anesthetics directly damaged the model membranes composed of phospholipid (34).

Dibucaine has been frequently used for spinal and lumbar anesthesia. Moreover, dibucaine is also most commonly used for topical anesthesia (1). However, the use of dibucaine has been limited due to its neurotoxicity, despite its potent

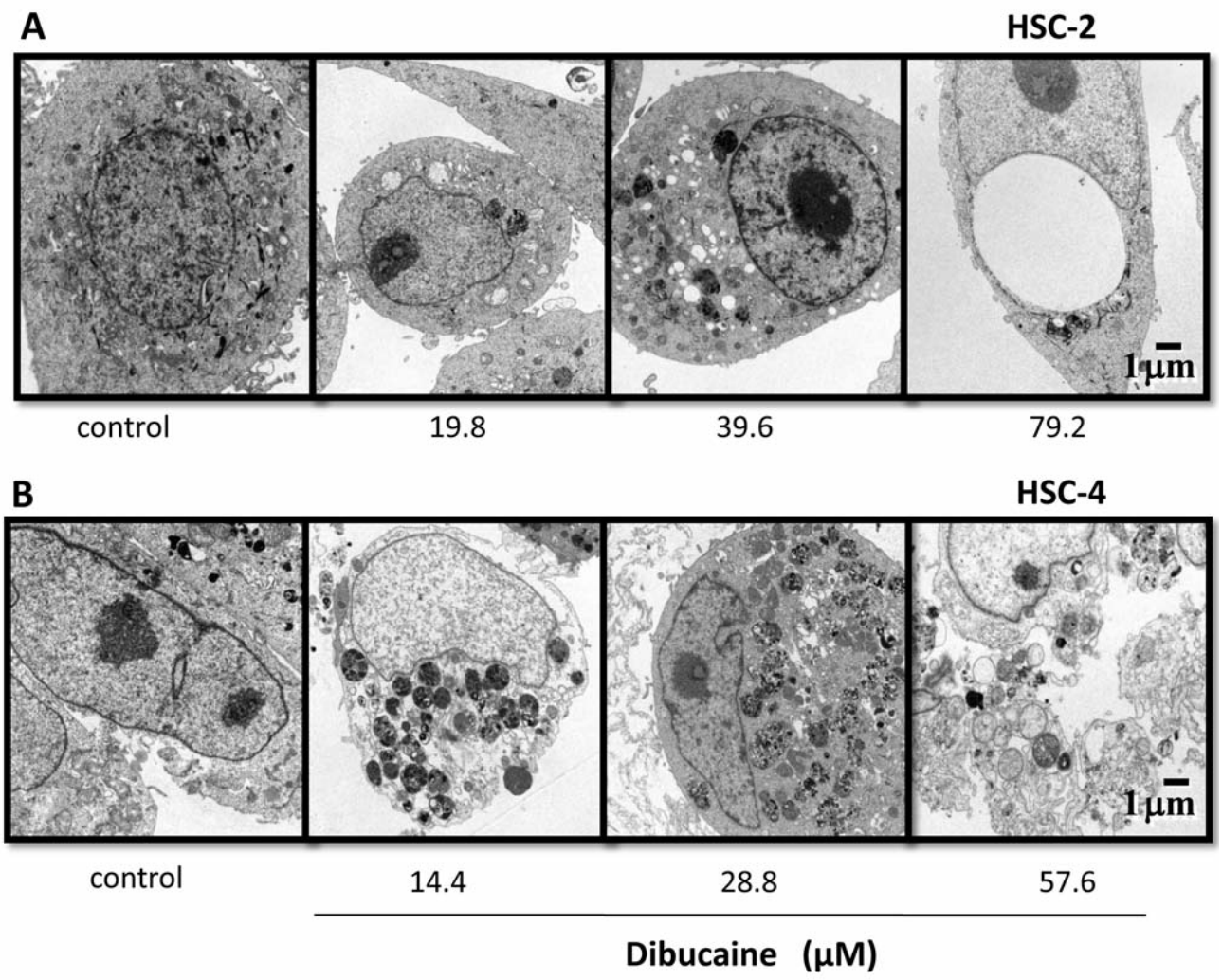

Figure 5. *Change in the fine cell structure induced by dibucaine. HSC-2 and HSC-4 cells were treated for 24 h without (control), or with the indicated concentrations of dibucaine [0 (control), CC_{50}×1/2, CC_{50} or CC_{50}×2], and then processed for electron microscopy. Bar=1 μm.*

anesthetic potency. Furthermore, lidocaine, but not dibucaine, has been extensively used in dentistry due its the pharmacological merits. However, dibucaine has the most potent antitumor activity among the local anesthetics investigated and should thus be re-considered for its possible application in the surgery of head and neck cancer.

Acknowledgements

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