Tumor-specificity and Type of Cell Death Induced by Vitamin K_2 Derivatives and Prenylalcohols

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Abstract. *Fourteen vitamin K_2 (menaquinone (MK)-n, n=1~14) and ten prenylalcohol derivatives (n=1~10) with different numbers (n) of isoprenyl groups in the side chains were investigated for their cytotoxicity against nine human tumor cell lines and three human normal oral cells. Among the vitamin K_2 derivatives, MK-2 (n=2) showed the greatest cytotoxicity, followed by MK-1 (n=1) and MK-3 (n=3). MK-1, MK-2 and MK-3 showed the highest tumor-specific index (TS= >2.0, 2.0 and >1.7, respectively). Among the prenylalcohols, geranylgeraniol (GG) (n=4) showed the highest cytotoxicity, followed by farnesol (n=3) and geranylfarnesol (GF) (n=3). GG showed the highest tumor-specificity (TS=1.8), followed by farnesol (TS=>1.4), GF (TS=><1.3). However, the tumor-specificity of MK-2 and GG was much lower than that of conventional chemotherapeutic agents. The human leukemic cell lines were the most sensitive, whereas the human glioblastoma cell lines were the most resistant to MK-2 and GG. MK-2 did not induce internucleosomal DNA fragmentation in either the human promyelocytic leukemia HL-60 or the human squamous cell carcinoma HSC-4 cell lines. GG induced marginal internucleosomal DNA fragmentation in the HL-60 cells, but not in the HSC-4 cells. Both MK-2 and GG did not induce the formation of autophagosomes, nor did they clearly change the intracellular concentration of three polyamines. Electron spin resonance (ESR) spectroscopy showed that only MK-1 (n=1), as well as GGF (n=7) and GFF (n=8) which had lower cytotoxicity, produced radicals, suggesting the lack of connection between cytotoxicity and radical production. The present study demonstrates that the presence of 1,4-naphtoquinone structure (including α,β-unsaturated ketones) in vitamin K_2 derivatives confers on them the ability to induce non-apoptotic cell death.*

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Key Words: Vitamin K_2, prenylalcohol, cytotoxicity, apoptosis, autophagy, type of cell death.

Hundreds of natural and synthetic compounds have been investigated for their cytotoxicity against both human oral squamous cell carcinoma cell lines and human normal cells. Based upon the tumor-specificity index (TS), determined by the ratio of the mean value of 50% cytotoxic concentration (CC_{50}) against normal cells to that against the tumor cell lines, anthracycline antibiotics, cyclic α,β-unsaturated ketone and nocobactins showed much higher tumor-specificity than that of tannin- and flavonoid-related compounds, and several antioxidants (reviewed in ref. 1). There was no clear-cut relationship between the tumor-specific cytotoxicity and apoptosis-inducing activity (1). There are at least three types of cell death, apoptosis (type I programmed cell death), autophagy (type II programmed cell death) and necrosis. The type of cell death induced by chemicals may be determined by both the chemical structure of the inducers and the type of target cells (2).

Vitamin K is a dietary component essential for the normal biosynthesis of several factors required for clotting of blood. Vitamin K_1 (phylloquinone, phytonadione) is a 2-methyl-3-phytyl-1,4-naphthoquinone, and is the only natural vitamin available for therapeutic use. Vitamin K_2 represents a series of compounds (the menaquinones, MK) in which the phytyl side chain of phytonadione has been replaced by a side chain built up of 1 to 14 isoprenyl units (MK-1 ~ MK-14, Figure 1). Vitamin K_3 (menadione, 2-methylnaphtoquinone) is a synthetic compound with the highest activity. It has been reported that vitamin K_2 (MK-4) with four isoprenyl units induced differentiation (expression of Naphthol AS-D chloroacetate esterase activity, α-naphtylacetate esterase activity, Fc receptor and phagocytosis) in human myeloid leukemia cell lines (HL-60, ML-1, U-937) (3), or apoptosis

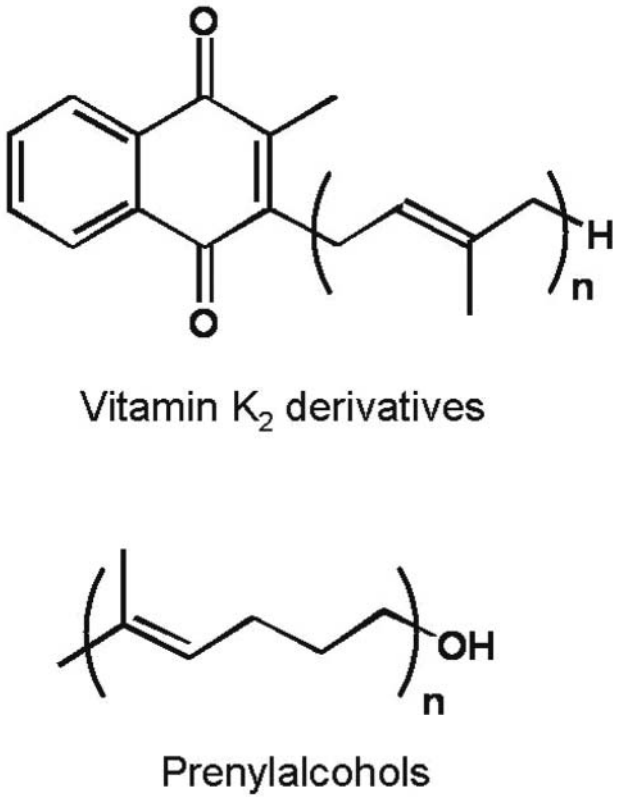

Figure 1. *Structure of vitamin K_2 derivatives and prenylalcohols.*

(characterized by DNA fragmentation and caspase activation) in isolated osteoclast (4) and human ovary cancer cells (5). Prenylalcohols are components of the side chain of vitamin K_2. All-trans geranylgeraniol (GG), one of such prenylalcohols with four isoprenyl units, induced apoptotic cell death in human leukemic cell lines (6, 7). We have recently reported that the cytotoxic activity and radical intensity of vitamin K was increased in the order of $K_1<K_2<K_3$ (8-10). Vitamin K_3 induced the cell death in a synergistic fashion with vitamin C (11, 12), possibly inducing the autoschizis characterized by exaggerated membrane damage and progressive loss of organelle-free cytoplasm through a series of self-excisions. However, the study of the relative cytotoxicity of the vitamin K series against both normal and tumor cells is limited.

Therefore, fourteen vitamin K_2 derivatives (MK-n, $n=1\sim14$) and ten prenylalcohols ($n=1\sim10$) with different numbers (n) of isoprenyl groups in the side chains (Figure 1) were investigated for their cytotoxicity against nine human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, HSC-4, hepatoma HepG2, glioblastoma multiform tumor T98G, U87MG, myelogenous leukemia HL-60, ML-1, KG-1, erythroleukemia K-562) and three human normal oral cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF). The type of cell death induced by MK-2 (with two isoprenyl units) and GG, that were found in this study to express the highest tumor-specificity and cytotoxicity was also investigated. Since the intracellular concentration of putrescine, but not that of sperimidine and spermine, declined during the apoptotic cell death of HL-60 cells (13, 14), we also investigated whether MK-2 and GG induce similar changes in polyamine levels. Since there is some relationship between cytotoxic activity and radical generation/scavenging activity in several antioxidants (15), we lastly investigated the radical intensity of MK-2 and prenylalcohol derivatives in relation to their cytotoxicity, using electron spin resonance (ESR) spectroscopy.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: vitamin K_2 derivatives and prenylalcohols (listed in Tables I and II) (provided by Eisai Co., Ltd., Tokyo); Dulbecco's modified Eagle's 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) (Sigma Chemical Co., Ind., St. Louis, MO, USA) and dimethylsulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan).

Cell culture. Normal human 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 normal volunteers. Since normal oral cells have the limited lifespan, all of them ceasing proliferation at the 20 population doubling level (PDL), these cells were used at 7-12 PDL in the present study. The HSC-2 and HSC-4 (supplied by Prof. M. Nagumo, Showa University), HSC-3 (supplied by Prof. Y. Ohmori, Meikai University), HepG2 (supplied by Prof. T. Tobe, Showa University), and T98G and U87MG cells (supplied by Dr. M. Iida, Showa University) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. The HL-60 and K-562 cells (supplied by Prof. K. Nakaya, Showa University), and the ML-1 and KG-1 cells (supplied by Prof. K. Takeda, Tokyo University of Science) were cultured in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. The normal cells were harvested by detaching with 0.25% trypsin-0.025% EDTA-2NA in phosphate-buffered saline without Mg^{2+} and Ca^{2+} (PBS(–)) and subcultured at the 1:4 split ratio once a week, with one medium change in between. The adherent tumor cell lines were similarly trypsinized and subcultured twice a week.

Assay for cytotoxic activity. Near-confluent cells were treated for the indicated times 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 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

Table I. *Cytotoxic activity of vitamin K_2 derivatives against human tumor and normal cells.*

		CC_{50} (μM)								
		Tumor cell lines					Normal cells			
	MW	HSC-2	HSC-3	HSC-4	HepG2	HL-60	HGF	HPC	HPLF	TS
MK-1 (n=1)	240	130±27	104±13	129±55	55±15	46±18	>200	>200	159±40	>2.0
MK-2 (n=2)	308	114±24	76±3	79±38	104±36	30±5	169±21	166±23	142±13	2.0
MK-3 (n=3)	377	159±40	137±26	93±76	193±8	13±6	>200	>200	>200	>1.7
MK-4 (n=4)	445	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-5 (n=5)	513	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-6 (n=6)	581	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-7 (n=7)	649	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-8 (n=8)	717	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-9 (n=9)	785	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-10 (n=10)	853	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-11 (n=11)	922	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-12 (n=12)	990	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-13 (n=13)	1058	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
MK-14 (n=14)	1126	>200	>200	>200	>200	>200	>200	>200	>200	><1.0

n, number of isoprenyl units. Each value represents mean±S.D. from 4 independent experiments.

Table II. *Cytotoxic activity of prenylalcohols against human tumor and normal cells.*

		CC_{50} (μM)								
		Tumor cell lines					Normal cells			
	MW	HSC-2	HSC-3	HSC-4	HepG2	HL-60	HGF	HPC	HPLF	TS
Prenol (n=1)	86	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
Geraniol (n=2)	154	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
Farnesol (n=3)	222	131±11	166±34	139±11	173±28	61±10	209±63	221±59	179±27	>1.4
GG (n=4)	290	67±12	94±20	72±12	148±7	9±2	143±9	150±8	137±9	1.8
GF (n=5)	359	167±24	190±19	171±31	>200	19±7	>200	>200	>200	><1.3
FF (n=6)	427	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
GGF (n=7)	495	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
GFF (n=8)	563	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
Solanesol (n=9)	631	>200	>200	>200	>200	>200	>200	>200	>200	><1.0
Decapr. (n=10)	699	>200	>200	>200	>200	>200	>200	>200	>200	><1.0

n, number of isoprenyl units. Each value represents mean±S.D. from 4 independent experiments. GG, geranylgeraniol; GF; geranylfarnesol; FF, farnesylfarnesol; GGF, geranylgeranylfarnesol; GFF, geranylfarnesylfarnesol; Decapr., decaprenol.

nm of the cell lysate (the relative viable number) was measured by a microplate reader (Labsystems Multiskan, Biochromatic Labsystem, Osaka, Japan). The viable cell number of the HL-60, ML-1, KG-1 and K-562 cells 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 was calculated from four independent experiments. The TS was calculated by 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} [$HepG_2$] + CC_{50} [HL-60]) x (5/3) (Tables I and II).

Assay for DNA fragmentation. The HSC-4 cells, collected by scraping with a rubber policeman, and the HL-60 cells in suspension, were pelleted and washed once with PBS(–). They were then lysed with 50 μL lysate 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. DNA was extracted with 50 μL NaI sodium [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 treatment with actinomycin D (1 μg/mL, 6 hours) were 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-It, UVP, Inc., Upland, CA, USA).

Detection of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were stained with acridine orange (Sigma Chemical Co.) as described previously (16). HSC-4 cells were treated without (control), or with the test sample and then stained with 1 μg/mL acridine orange for 15 minutes. Samples were then examined under a Laser Scanning Microscope LSM510, using the following filter: excitation filter 488 nm, emission filter 505-530 nm and >650 nm.

Determination of polyamines. The cells were harvested by 0.25% trypsin-0.025%EDTA in PBS(–), washed twice with PBS(–), and extracted with 10% trichloroacetic acid (TCA). After centrifugation for 5 minutes at 10,000 xg, the deproteinized supernatant was collected and stored at –40˚C. The polyamines in the supernatant were determined by high-performance liquid chromatography (HPLC), after dansyl-derivatization, as described previously (17).

Assay for radical intensity. The radical intensity of sample (2 mM) was determined at 25˚C in 0.1 M $NaHCO_3/Na_2CO_3$ (pH 9.0 or 11.0) 40 seconds after mixing in each buffer, using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (9-11). Instrument settings; center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT: gain, 500, time constant, 0.03 seconds; scanning time, 4 minutes.

Results

Cytotoxicity. Among the fourteen vitamin K_2 derivatives, MK-2 (with two isoprenyl groups) showed the greatest cytotoxicity, followed by MK-1 (with one isoprenyl group), MK-3 (with three isoprenyl groups) (Table I). MK-1, MK-2 and MK-3 showed the highest tumor-specific cytotoxicity (TS=>2.0, 2.0 and >1.7, respectively) (Table I). Among the ten isoprenyl alcohols, geranylgeraniol (GG) (with 4 units of isoprenyl groups) showed the highest cytotoxicity, followed by farnesol (with 3 units of isoprenyl groups) and geranylfarnesol (GF) (with 5 isoprenyl groups) (Table II). GG showed the highest tumor-specificity (TS=1.8), followed by farnesol (TS=>1.4), GF (TS=><1.3) (Table II).

Drug sensitivity. Considerable difference in drug-sensitivity was found between these cell lines. The sensitivity to MK-2 was in the order: HL-60 (CC_{50}=30 μM) (most sensitive) >KG-1 (52 μM)>HSC-3 (76 μM)>K-562, HSC-4 (79 μM)>ML-1 (80 μM)>HepG2 (104 μM)>HSC-2 (114 μM)>U87MG (115 μM)>HPLF (142 μM)> HPC (166 μM)>HGF (169 μM)>T98G (>200 μM) (most resistant) (Tables I and III). Similarly, the sensitivity to GG was in the order: HL-60 (CC_{50}=9 μM) (most sensitive)>KG-1 (61 μM)>ML-1 (62 μM)>K-562, HSC-2 (67 μM)>HSC-4 (72 μM)>HSC-3 (94 μM)> U87MG (95 μM)>HPLF (137 μM)>HGF (143 μM) >HepG2 (148 μM)>HPC (150 μM)>T98G (152 μM) (most resistant) (Table II, III).

Table III. *Cytotoxic activity of vitamin K_2 derivatives against human leukemia and glioblastoma cell lines.*

	CC_{50} (μM)					
	Human leukemia			Human glioblastoma		
	HL-60	ML-1	KG-1	K-562	T98G	U87MG
Vitamin K_2 derivatives						
MK-1 (n=1)	46	111	98	172	177	91
MK-2 (n=2)	30	80	52	79	>200	115
MK-3 (n=3)	13	36	36	29	>200	>200
MK-4 (n=4)	>200	>200	>200	>200	>200	>200
Prenylalcohols						
Farnesol (n=3)	61	62	41	72	148	122
GG (n=4)	9	62	61	67	152	95

Each values represents mean from duplicate determinations.

Type of cell death. MK-2 did not induce internucleosomal DNA fragmentation in the human promyelocytic leukemia HL-60 and squamous cell carcinoma HSC-4 cell lines. GG induced internucleosomal DNA fragmentation marginally in the HL-60 cells, but to a lower extent than that achieved by actinomycin D (positive control). However, GG did not induce DNA fragmentation in the HSC-4 cells (Figure 2). Actinomycin D induced the formation of autophagosomes, as judged by the granular distribution of acridine orange in the HSC-4 cells, whereas MK-2 and GG did not induce such morphological changes (Figure 3).

Change in the polyamine level. Neither MK-2 (7.5-200 μM [up to 7 times CC_{50}])) nor GG (2.5-40 μM [4 times CC_{50}]) affected the intracellular concentration of putrescine, spermidine and spermin in the HL-60 cells (Figure 4A, B). Similarly, MK-2 (2.5-200 μM [1.6 times CC_{50}]) and GG (2.5-100 μM [1.6 times CC_{50}]) did not show any apparent effect on the polyamine level in the HSC-2 cells (Figure 4C, D).

Relationship beween the radical intensity and cytotoxicity. ESR spectroscopy showed that only MK-1 (n=1), geranylgera-nylfarnesol (n=7) and geranylfarnesylfarnesol (n=8), which had lower cytotoxicity, produced strong quintuplet signals under alkaline conditions, whereas the other compounds did not produce detectable ESR signals (Figure 5).

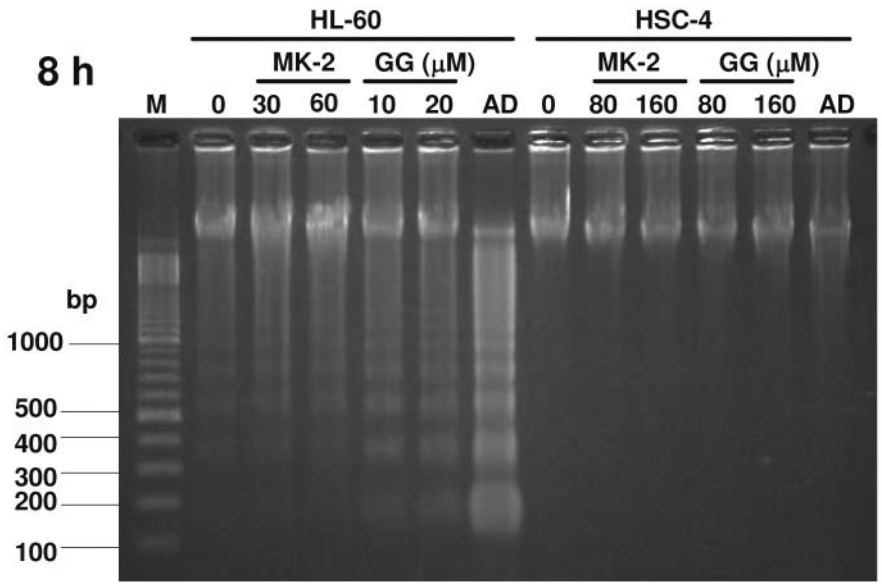

Figure 2. *Effect of MK-2 and GG on DNA fragmentation in HL-60 and HSC-2 cells. Cells were incubated for 8 hours with the indicated concentrations of MK-2 or GG, or 1 μg/mL actinomycin D (AD), and DNA fragmentation was assayed by agarose gel electrophoresis. M, Marker DNA.*

Discussion

The present study demonstrated for the first time that both vitamin K_2 derivatives and prenylalcohols with various lengths of isoprenyl units attached showed surprisingly low tumor-specific cytotoxicity. Among them, MK-2 (with two isoprenyl groups) and GG (with 4 isoprenyl groups) showed the highest tumor-specificity and cytotoxicity against various tumor cell lines with TS values of as little as 2, possibly due to their optimal hydrophobicity. With increasing length of the isoprenyl units, the solubility of the compounds declines below the level needed to express biological activity.

Considerable difference in the sensitivity to MK-2 and GG was found among the tumor cell lines used, with human leukemic cell lines (HL-60, ML-1, KG-1, K-562) the most sensitive and the human glioblastoma cell lines (T98G, U87MG) the most resistant, while the sensitivity of the human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4) was intermediate. Consistent with previous reports (6, 7), GG induced apoptosis in the most sensitive HL-60 cells, but not in the less sensitive HSC-4 cells. This suggested that the type of cell death induced (either apoptosis or non-apoptosis) may depend on the target cells. It has recently been reported that human glioblastoma cell lines (M059J, M059K, U373-MG, T98G) were committed to autophagy (characterized by autophagosome formation, the accumulation of Agp8p/Aut7p and LC3 (ATG 8 homolog) in autophagosomes, and the susceptibility to 3-methyladenine, an autophagy inhibitor), upon exposure to radiation (^{137}Cs) (18), arsenic trioxide (19) ceramide (20) or temozolomide (a new alkylating agent) (21). This suggests a possible link between the autophagic cell death and drug resistance or malignancy.

The other factor that might affect the type of cell death is the chemical structure. MK-2 induced the apoptosis to a much lesser extent compared to GG. The major difference between MK-2 and GG is the α,β-unsaturated ketone structure present in MK-2 but not in GG. We have recently found that α,β-unsaturated ketones, such as 4,4-dimethyl-2-cyclopenten-1-one, α-methylene-γ-butyrolactone, 5,6-dihydro-2*H*-pyran-2-one (22), 3,3,3-trifluoro-2-hydroxy-1-phenylpropanone (23), codeinone, an oxidative product of codeine (24) and morphinone, an oxidative metabolite of morphone (25) induced vacuolization or autophagosome formation engulfing organelles, but without induction of apoptosis markers. However, the induction of autophagosome formation and cell death was not restricted to the α,β-unsaturated ketones. For example, α-hydroxylketones such as 3,3,3-trifluoro-2-hydroxy-1-phenyl-1-propanone, induced caspase-independent cell death (23), accompanied by autophagosome formation detected by acridine orange and LC3-GFP (Ideo *et al.*, manuscript in preparation).

The induction of cell death by MK-2 or GG was not coupled with the radical generation. It has recently been reported that the induction of apoptosis by MK-4 in human ovary cancer cells is mediated by oxidative stress in mitochondria (26). Since

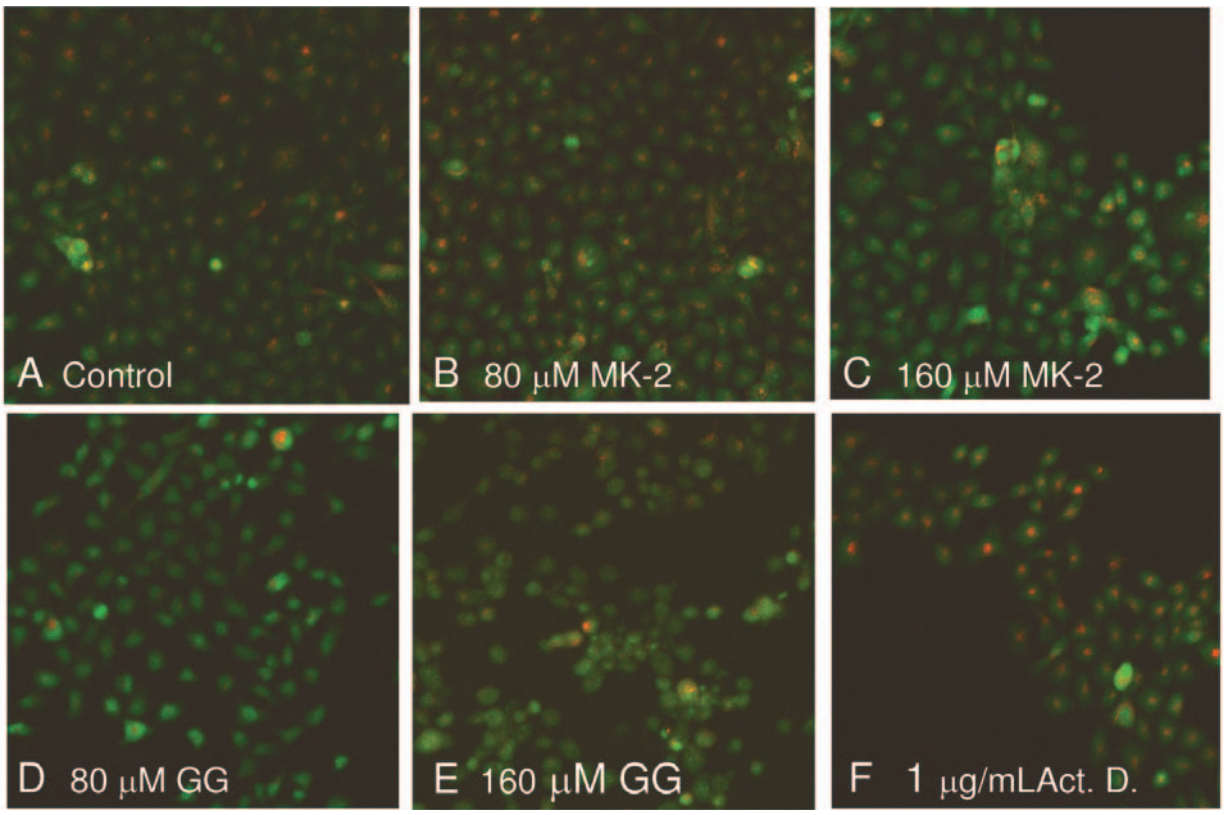

Figure 3. *Failure of MK-2 and GG to induce the formation of autophagosome in HSC-4 cells. Cells were treated for 8 hours with the indicated concentrations of MK-2, GG or actinomycin D (Act. D), and stained with acridine orange.*

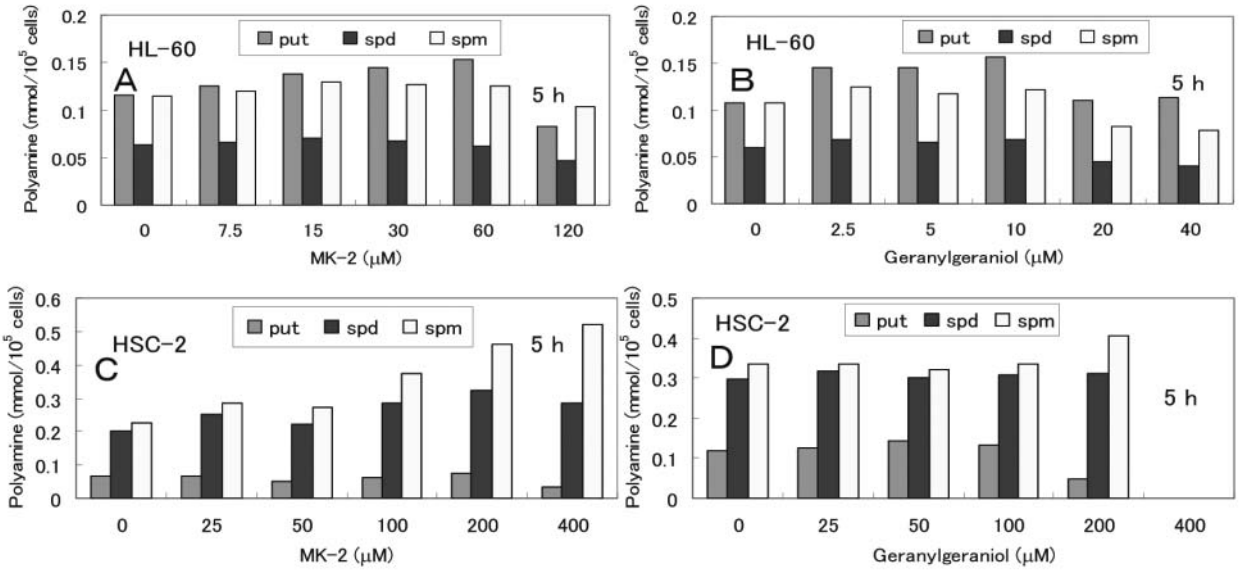

Figure 4. *Effect of MK-2 and GG on the intracellular concentration of polyamines in HL-60 and HSC-2 cells. HL-60 (A, B) or HSC-2 (C, D) cells were incubated for 5 hours with the indicated concentration of MK-2 (A, C) or GG (B, D), and the intracellular concentration of three polyamines were determined by HPLC. Put, putrescine; spd, spermidine; spm, spermin.*

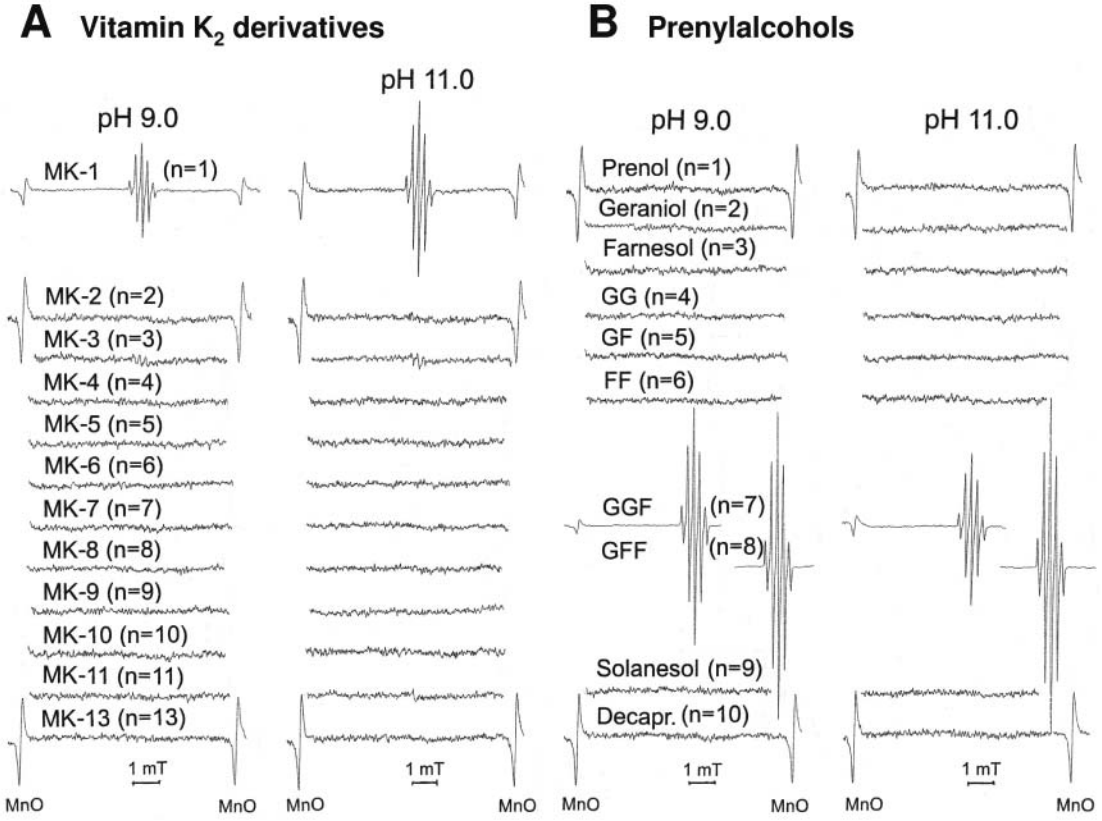

Figure 5. *Radical production by vitamin K_2 analogs and prenylalcohols. Sample (2 mM) was mixed in 0.1 M $NaHCO_3$/Na_2CO_3 (pH 9.or 11.0).*

autophagy plays an important role in reducing the mitochondrial damage and reactive oxygen species, there is a possibility that the apoptosis-inducing activity of MK-4 may be derived from the inhibition of autophagy.

MK-2 and GG did not apparently affect the putrescine level. This further supports the suggestion that these compounds induce non-apoptotic cell death, although the type of cell death has not yet been conclusively determined.

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. 14370607; Sakagami, No. 19592156).

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Received July 2, 2007
Revised October 9, 2007
Accepted October 30, 2007