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Induction of Non-apoptotic Cell Death by *Odontioda* Marie Noel 'Velano' Extracts in Human Oral Squamous Cell Carcinoma Cell Lines

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Abstract. *Background: We recently reported that the MeOH extract from bulbs of Odontioda Marie Noel 'Velano' exhibited diverse biological activities but most of the activity was concentrated into the EtOAc layer separated by sequential organic solvent extractions. In the present study, the EtOAc layer was subjected to silica-gel column chromatography for further separation into five fractions, and the cytotoxicity and apoptosis-inducing activity of the fractions against human normal oral and tumor cells was further investigated. Materials and Methods: Cytotoxic activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The 50% cytotoxic concentration (CC_{50}) was determined by the dose–response curve. Tumor specificity (TS) was determined by the ratio of the mean CC_{50} for normal cells to the one of tumor cell lines. DNA fragmentation was assayed by agarose gel electrophoresis, caspase-3/-7 activation was monitored by cleavage of substrates either spectrophotometrically or by western blot analysis. Results: Among five fractions, the most hydrophobic fraction (Fr. 1) showed the highest cytotoxicity against all cell lines tested, followed by Fr. 2 >Fr. 3 >Fr. 4 >Fr. 5, in order of increasing polarity. Fr. 2 had the highest tumor-specificity, followed by Fr. 3, Fr. 4, Fr. 1 and Fr. 5. Fr. 1 induced caspase-3 activation more potently in promyelocytic leukemia HL-60 cells, than in oral squamous cell carcinoma (OSCC) HSC-2 cells, whereas it did not induce internucleosomal DNA fragmentation in either of these cell lines. Conclusion: The present study suggests that hydrophobic substances in the EtOAc extract of Odontioda Marie Noel 'Velano' exhibit tumor-specific cytotoxicity without inducing apoptosis in the HSC-2 OSCC cell line.*

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Various pharmacologically active substances have been isolated from the stalks of certain orchid species (*Prosthechea michuacana, Dendrobium nobile, Bletilla striata, Dendrobium densiflorum, Cremastra appendiculata*). These compounds have shown radical-scavenging, antioxidant (1), antifibrosis (2), antitumor (3), antimicrobial (3, 4), antiplatelet aggregation (5) and antimuscarinic (6) activities, and exert growth modulation of mouse T and B lymphocytes (7) We also recently reported the diverse biological activity of extracts from another species of orchid, *Odontioda* Marie Noel 'Velano' (8). Among four extract fractions of this species separated by successive organic solvent extractions, the EtOAc extract exhibited the highest tumor-specific cytotoxicity, followed by the *n*-hexane, *n*-BuOH and H_2O extracts. The EtOAc and *n*-BuOH extracts protected HSC-2 cells against the cytotoxicity induced by UV irradiation. The EtOAc and *n*-hexane extracts inhibited NO production by lipopolysaccharide-stimulated mouse macrophage-like cells. The EtOAc extract most strongly inhibited receptor activator for nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis, followed by the *n*-BuOH, *n*-hexane and H_2O extracts (8). In the present study, we further separated the most active EtOAc extract into five fractions (Fr. 1-5), with silica-gel column chromatography, and investigated their tumor-specific cytotoxicity and apoptosis-inducing activity.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA); fetal bovine serum (FBS) (SAFC Biosciences, St. Louis, MO, USA); RPMI1640 mediun, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) (Wako Pure Chemical, Osaka, Japan); melphalan (Sigma-Aldrich Inc., St. Louis, MO, USA).

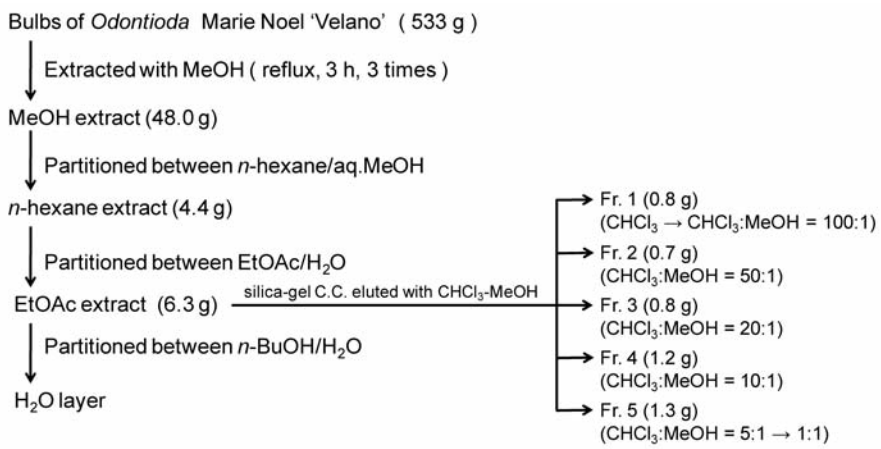

Figure 1. *The EtOAc layer of Odontioda Marie Noel 'Velano' extract (6.3 g) was chromatographed on a silica-gel column with different solvent ratios of $CHCl_3$-MeOH (100:1, 50:1, 20:1, 10:1, 5:1, 1:1) to yield five fractions.*

Sequential fractionation with organic solvents. Bulbs of *O*. Marie Noel 'Velano' (Orchidaceae) were supplied from Orchid Garden Co., Ltd., Nagano Prefecture, Japan, during April of 2009. This specimen was proved and identified by Mr. H. Sumiyoshi (Orchid Garden Co., Ltd.) and also a voucher specimen (#20090617) was deposited in the Medicinal Plant Garden of Josai University.

The air-dried bulbs (533 g) of *O*. Marie Noel 'Velano' were extracted three times with MeOH under reflux for 3 hours. The MeOH extract (48.0 g) was fractionated by sequential organic solvent extraction as described previously (8). The EtOAc layer (6.3 g) was fractionated into five fractions, Fr. 1-5, by silica-gel column chromatography (Figure 1).

Cell culture. Human oral squamous cell carcinoma (OSCC) cells (HSC-2, HSC-3, HSC-4), human promyelocytic leukemia HL-60 cells, and normal human oral cells [gingival fibroblast (HGF), pulp cells (HPC) and periodontal ligament fibroblast (HPLF)] were prepared and cultured as described previously (8).

Assay for cytotoxic activity. The cells (3×10^3 cells/well, 0.1 ml/well) were seeded in 96-microwell plates (Becton Dickenson, Flanklin Lakes, NJ, USA) and incubated for 48 hours to allow cell attachment. Near-confluent cells were treated for 48 hours with different concentrations (7.8-250 μg/ml) of extract fractions in fresh medium. The relative viable cell number of adherent cells (except for HL-60 cells) was then determined by the MTT method (8). The viability of the suspended cells, namely, HL-60, was determined by cell counting with a hemocytometer after staining with 0.15% trypan blue (8). The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve. The tumor-specificity index (TS) was calculated by the following equation: TS=mean CC_{50} (normal cells)/mean CC_{50} (all tumor cell lines).

Assay for DNA fragmentation. The cells were lysed with 50 μl lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate]. The lysate was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 1 hour 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 250 μl of ethanol. After centrifugation for 20 min at 15,000 ×*g*, the precipitate was washed with 1 ml of 70% ethanol. After centrifugation for 5 min at 15,000 ×*g*, the precipitate was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 3-5). The samples (10-20 μl) were applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA molecular marker (Takara, Bio, Siga, Japan) and DNA from apoptotic HL-60 cells induced by UV irradiation (6 J/m^2/min), followed by 3 hours incubation, were used for calibration. The DNA fragmentation pattern was examined in a photograph taken under UV illumination.

Assay for caspase activation. The cells were washed with phosphate-buffered saline without magnesium and calcium [PBS(−)] and lysed in lysis solution. After resting on ice for 10 min and centrifugation at 4000 ×*g* at 4˚C for 3 min, the supernatant was collected. The lysate (50 μl, equivalent to 100 μg protein) was mixed with 50 μl reaction buffer (MBL) containing substrate for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)). After incubation for 3 hours at 37˚C, the absorbance at 405 nm of *p*NA produced by the cleavage of substrates was measured with microplate reader.

Assay for western blotting. HSC-2 cells were treated with 0, 25 or 50 μg/ml of Fr. 1, respectively. The cleavage of Poly ADP ribose polymerase (PARP) was measured using a Promega PARP (Asp 214) human specific antibody (Distributed by Cell Signaling Technology, Inc. Boston, MA, USA). In brief, cells were washed in ice-cold PBS, scraped, collected in lysis buffer [20 mM HEPES pH7.4, 1% Triton-X 100, 150 mM NaCl, 1.5mM $MgCl_2$, 12.5 mM β-glycerophosphate, 2 mM EDTA, 10 mM NaF, 2 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) plus 1× protease inhibitors]. The cell lysates were applied to a 8% sodium dodecyl sulfate-polyactylamide gel electrophoresis (SDS-PAGE) and the protein bands in the gels were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dry milk, incubated with primary antibody [anti-cleaved PARP1 (Cell Signaling Technology), anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)], followed by incubation then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies.

Table I. *Cytotoxic activity of Odontioda Marie Noel 'Velano' EtOAc extracts towards cultured human normal and tumor cells. Cells were incubated for 48 hours with different concentrations of extract fractions. means±S.D. of three independent experiments.*

	CC_{50} (μg/ml)							
	Tumor cell lines				Normal cells			
	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	TS
Fr.1	21.1±2.4	23.1±0.7	17.6±4.7	9.5±0.7	28.7±10.8	36.8±7.5	49.3±1.0	2.2
Fr.2	65.4±18.2	92.3±18.0	48.1±10.1	11.8±1.5	178.3±50.6	208.1±13.5	216.4±12.3	3.7
Fr.3	159.8±15.9	149.0±18.0	72.9±4.0	13.4±5.1	242.8±4.2	227.8±12.8	>250	>2.4
Fr.4	161.3±15.7	172.7±29.5	82.8±3.3	21.0±2.8	240.7±6.3	248.1±1.1	>250	>2.3
Fr.5	236.4±14.6	>250	196.8±16.0	66.9±15.0	>250	>250	>250	>1.3

CC_{50}, 50% Cytotoxic concentration; TS, tumor specificity; HGF: human gingival fibroblast; HPC: human pulp cells; HPLF: human periodontal ligament fibroblast.

Statistical analysis. The difference between two groups was evaluated by Student's *t*-test.

Results

Cytotoxicity. Among the five fractions prepared from the EtOAc layer, Fr. 1 exhibited the highest cytotoxicity against all human tumor cell lines (mean CC_{50} against tumor cells=17.8 μg/ml), followed by Fr. 2 (54.4 μg/ml), Fr. 3 (98.8 μg/ml), Fr. 4 (109.5 μg/ml) and Fr. 5 (>187.4 μg/ml) (Table I). HL-60 cells were the most sensitive to Fr. 1 (CC_{50}=9.5 μg/ml), followed by HSC-4 (CC_{50}=17.6 μg/ml) >HSC-2 (CC_{50}=21.1 μg/ml) >HSC-3 (CC_{50}=23.1 μg/ml) (Table I). On the other hand, human normal oral cells (HGF, HPC, HPLF) exhibited lower sensitivity to Fr. 1 (CC_{50}=28.7~49.3 μg/ml), yielding the tumor-specificity index (TS) of 2.2 (Table I).

Fr. 2 had the highest tumor specificity (TS=3.7), followed by Fr. 3 (TS>2.4) >Fr. 4 (TS>2.3) >Fr. 1 (TS=2.2) >Fr. 5 (TS>1.3) (Table I). Since Fr. 1 exhibited the highest cytotoxicity among the five fractions, subsequent experiments were performed with Fr. 1.

Assay for DNA fragmentation. Fr. 1 (20, 40 or 80 μg/ml, corresponding to 2.1-, 4.2- and 8.4-fold of CC_{50}) did not induce internucleosomal DNA fragmentation in HL-60 cells (Figure 2A). Similarly, Fr. 1 (50, 100 or 200 μg/ml, corresponding to 2.4-, 4.7- and 9.5-fold of CC_{50}) did not induce internucleosomal DNA fragmentation in HSC-2 cells (Figure 2B). It should be noted that Fr. 1 produced a large DNA fragment in both cell lines (indicated by arrows in Figure 2A and B).

Assay for caspase activation. Fr. 1 (10 μg/ml, corresponding to 1× CC_{50}) induced caspase-3 activation significantly (p<0.001) in HL-60 cells, but to an extent considerably lower than the one attained by UV irradiation (Figure 3A). On the other hand, Fr. 1 (25 μg/ml, corresponding to 1.2-fold of CC_{50}) induced caspase-3 activation only slightly but not significantly in HSC-2 cells. Furthermore, UV irradiation induced a much lower level of caspase-3 activation in HSC-2 cells (absorbance at 405 nm=0.097) (Figure 3A) than that observed in HL-60 cells (absorbance at 405 nm=0.23) (Figure 3B). Western blot analysis revealed that Fr. 1 (25 μg/ml) induced the cleavage of PARP, but to an extent less than that attained by UV irradiation of HSC-2 cells (Figure 4).

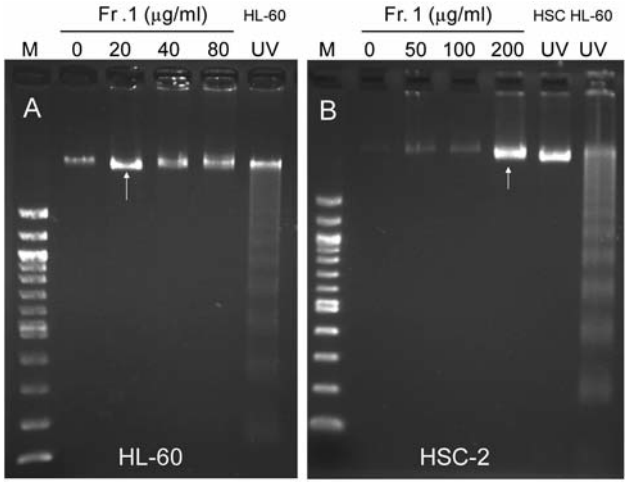

Figure 2. *Effect of fraction 1 (Fr. 1) on induction of DNA fragmentation. HL-60 (A) and HSC-2 (B) cells were incubated for 6 h with the indicated concentrations of Fr. 1, and DNA fragmentation was assayed by agarose gel electrophoresis. M, DNA marker; UV, DNA from HL-60 and HSC-2 cells that had been exposed to UV irradiation (6 J/m^2/min, 1 min) and then incubated for 3 h in regular culture medium. Arrows indicate the large DNA fragment.*

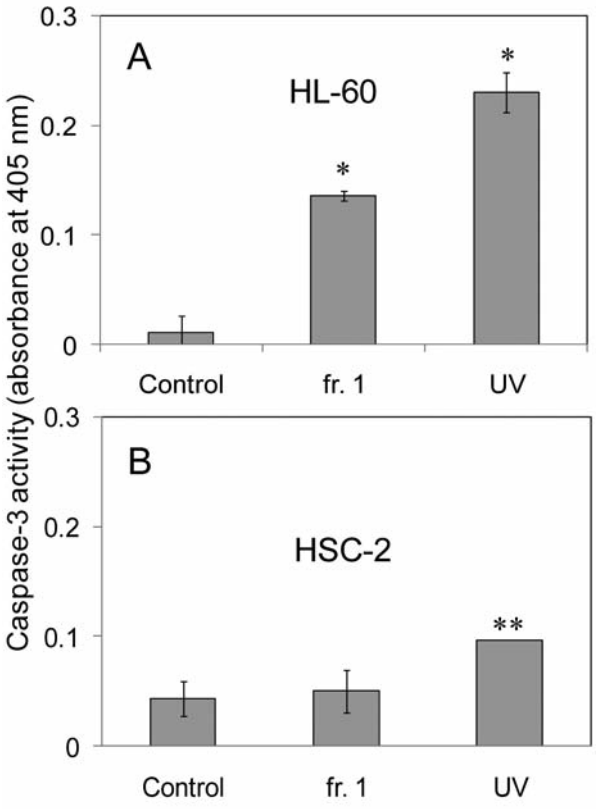

Figure 3. *Effect of fraction 1 (Fr. 1) on caspase-3 activity. HL-60 (A) and HSC-2 (B) cells were incubated for 6 hours with the indicated concentrations of Fr. 1, and the caspase-3 activity was determined by the cleavage of (DEVD-pNA). UV, HL-60 and HSC-2 cells were exposed to UV irradiation (6 J/m^2/min, 1 min) and cultured for 3 hours. Each value represents the mean±S.D. of triplicate assays. *p<0.001, **p<0.05 related to the control.*

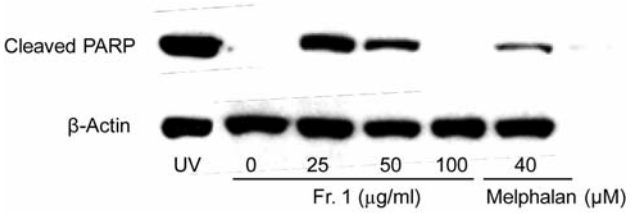

Figure 4. *Activation of caspase-3/-7 by fraction 1 (Fr. 1). HSC-2 cells were incubated for 6 hours with the indicated concentrations of Fr. 1, and the production of cleaved product of Poly ADP ribose polymerase (PARP) was detected by western blot analysis. UV, HSC-2 cells were exposed to UV irradiation (6 J/m^2/min, 3 min) and cultured for 3 hours. Each value represents the mean±S.D. of triplicate assays.*

Discussion

The present study demonstrated that among the five fractions of EtOAc extract, Fr. 1 exhibited the highest cytotoxicity against all cell lines tested, and the cytoxicity declined with increasing polarity (Fr. 2 >Fr. 3 >Fr. 4 >Fr. 5). The further purification of cytotoxic principle is under way.

The present study also demonstrated that Fr. 1 did not induce internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis, in HL-60 or HSC-2 cells (Figure 2), suggesting that Fr. 1 does not induce apoptotic cell death. However, we found that Fr. 1 significantly activated caspase-3 in HL-60 cells, although the extent of caspase-3 activation was much lower than the one induced by UV irradiation (Figure 3A). This suggests that Fr. 1 may change the chromatin structure in HL-60 cells so as it is not attacked by caspase-3-activated DNases (9). There is an alternative possibility that Fr. 1 induces the activation of nuclease(s) distinct from caspase-activated DNase (CAD) that cleaves the DNA at the oligonucleosomal units (10). We found that Fr. 1 cleaved the DNA to produce a large fragment (indicated by arrows in Figure 2), favoring the latter possibility.

We found that Fr. 1 activated caspase-3 only slightly but not significantly in HSC-2 cells (Figure 3B). Western blot analysis demonstrated that Fr. 1 activated caspase-3/-7 to a level slightly lower than the one achieved by UV irradiation (Figure 4). Melphalan, an alkylating agent (11), induced a very low level of PARP cleavage (Figure 4). Since the level of caspase-3 activation induced by UV irradiation in HSC-2 cells was much lower than the one induced in HL-60 cells, activation of caspase-3 above some threshold level may be necessary to induce internucleosomal DNA fragmentation.

In conclusion, the present study suggests that the hydrophobic substances present in the EtOAc extract of *Odontioda* Marie Noel 'Velano' exhibit tumor-specific cytotoxicity without inducing apoptosis in the OSCC cell line HSC-2.

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