Abstract. Aim: The cytotoxicity of four dental compounds, hydroquinone, benzoquinone, eugenol and phtharal towards human oral squamous cell carcinoma (OSCC) cell lines, normal human oral cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast) and skin keratinocytes was investigated. Materials and Methods: Viable cell number was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. The concentration that reduced the viable cells by 50% (CC50) and the concentration that increased the viability of UV-irradiated cells to 50% (EC50) were determined from the dose-response curves. The tumor-specificity index (TS) was determined by the ratio of the mean CC50 for normal cells to the one for tumor cells. Apoptosis induction was monitored by assay of internucleosomal DNA fragmentation and caspase-3/-7 activation. Results: When both oral OSCC and normal oral cells were incubated for 4 h with any of hydroquinone, benzoquinone, eugenol and phtharal, irreversible cell growth inhibition, accompanied by cell death occurred without induction of apoptotic markers, although caspase-3/-7 activation was observed at 6 h or later. These compounds exhibited very low tumor-specificity (TS=0.4-1.3), as compared with anticancer drugs (5-fluorouracil, melphalan, peplomycin) (TS=4.1-9.7). Human skin keratinocytes were the most resistant to these drugs, and a long incubation time was required to induce irreversible growth inhibition. However, all dental compounds exhibited very low tumor-specificity (TS=0.4-2.4), compared to human skin keratinocytes and OSCC cell lines. None of the dental compounds exhibited any hormetic growth stimulation, nor protected the cells from UV-induced damage. Conclusion: These results suggest that apoptosis is not involved in the early stage of growth inhibition induced by dental compounds.

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Cytotoxicity of Dental Compounds towards Human Oral Squamous Cell Carcinoma and Normal Oral Cells

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Using a newly-established evaluation system for UV-induced cellular damage (15, 16), we have reported that several natural polyphenols protect cells from UV-induced cytotoxicity (19). We investigated here whether these dental compounds also exhibit such an anti-UV activity.

Materials and Methods

Materials. The following chemicals and materials were obtained from the indicated companies: RPMI-1640, Dulbecco's Modified Eagle's Medium (DMEM) from Gibco BRL, Grand Island, NY, USA; fetal bovine serum (FBS), phthalal, melphanal, 3,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phenylmethysulfonyl fluoride (PMSF) from Sigma Chemical Ind., St. Louis, MO, USA; hydroquinone (MW=110), benzoquinone (MW=108), eugenol (MW=164), dimethylsulfoxide (DMSO) from Wako Pure Chemical, Osaka, Japan; peplomyacin sulfate from Santa Cruz Biotechnology, Santa Cruz, CA, USA; 5-fluorouracil (5-FU) from Kyowa, Tokyo, Japan; RNase A, Proteinase K, ethidium bromide, agaroše from Nippon Gene Co., Ltd., Toyama, Japan; DNA molecular marker from Bayou Biolabs, Harahan, LA, USA; 6-well plates, 24-well plates, 96-microwell plates from Becton Dickinson, Franklin Lakes, NJ, USA; substrate of caspase-3, DEVD-pNA (p-nitroanilide) from MBL, Aichi Prefecture, Japan; HuMedia-KG2 from Kurabo, Osaka, Japan; Hydroquinone and benzoquinone were dissolved in DMSO at 100 mM, whereas eugenol and phthalal were dissolved in DMSO at 200 mM before use, and diluted with medium.

Cell culture. HL-60 cells (Riken, Tsukuba, Japan) were cultured at 37°C in RPMI-1640 supplemented with 10% heat-inactivated FBS. Human OSCC cell lines (HSC-2, HSC-4, Ca9-22) were kindly provided by Professor Nagumo, Showa University, Japan. These adherent cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. Normal human oral cells, HGF, HPC and HPLF were prepared from periodontal tissues, as previously reported (15), and used at 8-15 population doubling levels (PDL). Human OSCC cell lines (HSC-2, HSC-4, Ca9-22) were kindly provided by Professor Nagumo, Showa University, Japan. These adherent cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. Normal human oral cells, HGF, HPC and HPLF were prepared from periodontal tissues, as previously reported (15), and used at 8-15 population doubling levels (PDL). HEKn (purchased from Kurabo, Osaka, Japan) were cultured in HuMedia-KG2 supplemented with insulin, human recombinant epidermal growth factor, (hEGF), hydrocortisone, gentamicin, amphotericin B and bovine pituitary gland extract (BPE).

Assay for cytotoxic activity. All cells were inoculated at 3×10^3 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 (0, 1.85, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 μM for hydroquinone and benzoquinone, 0, 62.5, 125, 250, 500 μM for eugenol and phthalal). Cells were washed once with phosphate-buffered saline without Ca^2+ and Mg^2+ [PBS(–)] and lysed with lysis buffer [50 mM Tris-HCl (pH 8.0), and 100 μl of ethanol. After centrifugation for 20 min at 20,000 x g, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). 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). DNA molecular marker (Takara, Shiga, 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 incubated for 4 h without or with CC50, CC50×2, or CC50×4 of each compound. Cells were then washed with PBS(–) and lysed with lysis solution (MBL, Nagoya, Japan). After resting cells for 10 min on ice and centrifugation for 5 min at 10,000 x g, the supernatant was collected. The lysate (50 μl, equivalent to 100 μg protein) was mixed with 50 μl 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-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 (20).

Assay for western blotting. HSC-2 cells were treated for 1, 3, 6, 12 or 24 h with CC50×2 of each dental compound. The cleavage of poly ADP-ribose polymerase (PARP) was measured using a Promega PARP (Asp 214) human-specific antibody (Cell Signaling Technology, Inc., Boston, MA, USA). In brief, cells were washed in ice-cold PBS(–), scraped, collected in lysis buffer (20 mM HEPES [pH 7.4], 1% Triton X-100, 150 mM NaCl, 1.5 mM MgCl₂, 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol (DTT), 1 mM Na₂VO₃, 1 mM PMSF, 1x protease inhibitor). The cell lysates (equivalent to 30 μg protein) were applied to a 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands in the gels were transferred onto polyvinylidene difluoride membranes. The membranes blocked with 5% (w/v) non-fat dry milk, incubated with primary antibody [anti-cleaved PARP1 (Cell Signaling Technology, Beverly, MA, USA) (dilution, 1:1000)], anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (dilution, 1:10000)], and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (21).
Assay for UV protection. HSC-2 cells were incubated for 48 h to attach to the 96-microwell plate. The medium was replaced with PBS(−) containing 0, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, 1, 2 or 4 mM hydroquinone, benzoquinone or eugenol, or 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 or 32 mM sodium ascorbate. The plate was immediately placed at 21 cm of distance from a UV lamp (wavelength 253.7 nm) and cells were then exposed to UV irradiation (6 J/m²/min) for 1 min. The media were replaced with fresh DMEM plus 10% FBS and cells were cultured for a further 48 h to determine the viable cell number (15, 16, 19).

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

Results

Tumor–specificity. The four dental compounds exhibited a cytotoxic effect, but not a cytostatic effect on human OSCC cell lines (HSC-2, HSC-4, Ca9-22) (Figure 2A) and human oral normal cells (HPC, HGF, HPLF) (Figure 2B). From the dose-response curves, the CC₅₀ value was determined (Table I). Benzoquinone exhibited the highest cytotoxicity, followed by hydroquinone, phtharal and then eugenol. These compounds had comparable magnitudes of cytotoxicity, regardless of the treatment time (4, 24 or 48 h) (Figure 1, Table I), indicating that the irreversible growth inhibition accompanied by cell death had already occurred during the first 4 h.

The tumor–specificity of these compounds was next investigated. Hydroquinone and benzoquinone were slightly more cytotoxic towards normal oral cells (HGF, HPC, HPLF), as compared with OSCC cell lines (HSC-2, HSC-4, Ca9-22) (Table I), yielding TS values of 0.39-0.76. Eugenol and phtharal had comparable cytotoxicity towards normal oral cells and OSCC cell lines, yielding TS values of 1.02-1.28. On the other hand, 5-fluorouracil, melphalan and peplomycin had much higher TS values (TS=3.43, 4.09 and 9.73, respectively) (Table I).

HEKn were the most resistant to these drugs, and therefore a long incubation period was needed to induce irreversible cell death. When comparing cytotoxicity towards HEKn and towards OSCC cell lines, all dental compounds had a very low level of tumor–specificity (TS=0.4-2.4), as compared with the anticancer drugs (TS=7.3-23.5) (Table I).

Hormetic response. All four dental compounds, at their lowest concentration, induced only slight hormetic growth-stimulatory effects on all three OSCC cell lines (hormetic response=0-9.7%), three oral normal cell types (hormetic response=0-26.2%) and skin keratinocytes (hormetic response=0-57.4%), regardless of incubation time (4, 24 or 48 h) (Table II).

Type of cell death. Since the growth inhibition or cell death signal was triggered within 4 h after treatment with any of the four dental compounds, next, whether 4 h-treatment induces apoptosis markers (i.e. internucleosomal DNA fragmentation by activated DNase(s) and caspase-3) (22) in both tumor and normal cells was investigated. It was unexpected that these compounds did not induce internucleosomal DNA fragmentation in the three OSCC cell lines (Figure 3A) and three normal oral cell types (Figure 3B), in contrast to the DNA laddering pattern observed in apoptotic HL-60 cells, induced by UV irradiation (indicated by UV in Figure 3A and B). Similarly, these compounds at the concentrations of CC₅₀, CC₅₀×2 or CC₅₀×4 did not induce caspase-3 activation, in contrast to higher caspase-3 activation (p<0.01) induced in apoptotic HL-60 cells (Figure 4). Western blot analysis demonstrated that caspase-3/-7 activation, detected by the
Figure 2. Continued
Figure 2. Cytotoxicity of the four dental compounds studied towards human oral squamous cell carcinoma cell lines (A) and normal oral cells (B). Cells were treated for 4, 24, or 48 h with the indicated concentrations of hydroquinone (HQ), benzoquinone (BQ), eugenol (EUG) and phtharal (PHA). The viable cell number was then determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method, and expressed as a percentage to that of the control. Each value represents the mean±S.D. from three independent experiments.
production of cleaved PARP, was observed only at later stages (6-24 h after treatment) (Figure 5).

**Anti-UV activity.** Exposure of HSC-2 cells to UV irradiation for 1 min killed nearly all cells after culture in regular medium for 48 h (Figure 6). Although the addition of sodium ascorbate (positive control) at the time of UV irradiation protected the cells from UV-induced damage (EC$_{50}$=0.38 mM, CC$_{50}$>32 mM, SI=84.2), neither hydroquinone, benzoquinone nor eugenol exhibited such an anti-UV activity (SI<1.0) (Figure 6).

**Discussion**

The present study demonstrated, to our knowledge for the first time, that these four dental compounds induced rapid irreversible growth inhibition and cell death on both oral OSCC cell lines and normal oral cells. The concentration of eugenol and phtharal used in the present study (CC$_{50}$=696-796 μM and 144-415 μM, respectively) was close to that used in clinical dentistry (600 and 400 μM, respectively). The minimum treatment time required for cell death induction was found to be 4 h or less. During 4 h after treatment, apoptosis markers such as internucleosomal DNA fragmentation and caspase-3 activation were not observed. Caspase-3/-7 activation was observed only at 6 h or later. These data suggest that apoptosis may not be involved in the early stage of growth inhibition and cell death induction by dental compounds. This result is not consistent with previous finding that hydroquinone induced apoptotic cell death via mitochondrial intrinsic pathway in HL-60 cells (7), suggesting that the type of cell death induced may depend on the cell types as well as the chemical structure of inducers (23).
We found that these four dental compounds, especially hydroquinone and benzoquinone, exhibited potent cytotoxicity towards both oral normal cells below 50 μM (Table I). Hydroquinone, benzoquinone and phthalal (log \( p = 0.394 \), 0.62 and 0.395, respectively) have higher hydrophilicity than eugenol (log \( p = 2.403 \)). Judging from these log \( p \) values, eugenol may be more easily incorporated into the cells. Hydroquinone has been reported to increase reactive oxygen species (ROS) generation and dysfunction of mitochondria [inhibition of manganese superoxide dismutase (SOD) induction] in the HL-60 promyelocytic leukemia cells, which was inhibited by N-acetyl-L-cysteine, a popular antioxidant (7). The apoptotic effect of EUG is also accompanied by the elevation of ROS (24, 25). Hydroquinone and benzoquinone induced oxidative stress such as glutathione depletion, and the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and antioxidant response element (ARE) pathways are essential for protection against hydroquinone, and benzoquinone-induced toxicity (26). Cytotoxicity of phthalal has not yet been reported. Identification of early metabolic changes after exposure to these compounds may contribute to our understanding of how to combat their cytotoxicity.

We used HEKn due to the difficulty of establishing oral keratinocytes in our laboratory. To maintain HEKn, the use

![Figure 3. Effect of the four dental compounds studied on DNA fragmentation in human oral squamous cell carcinoma cell lines (A) and normal oral cells (B). Cells were incubated for 4 h with the indicated concentrations of hydroquinone (HQ), benzoquinone (BQ), eugenol (EUG) and phthalal (PHA). DNA was then extracted and subjected to agarose gel electrophoresis. MA: DNA marker; UV: DNA from HL-60 cells induced to apoptosis by UV irradiation.](image)
of nutritionally enriched HuMedia-KG2 medium, which contains insulin, human recombinant EGF (hEGF) and hydrocortisone and bovine pituitary gland extract (BPE), was inevitable (27). The presence of such growth factors may have worked to counteract apoptosis and thus delayed the onset of cytotoxic action.
We investigated whether dental compounds have any beneficial effects on these cells. However, we found that the four dental compounds had marginal hormetic effects on OSCC and normal cells, regardless of the treatment time (0-48 h) (Table II). Taken together with our previous data, oral cells seem to have very weak responsiveness to hormetic stimuli. These compounds did not protect cells from UV-induced cellular injury. We are also investigating their possible anti-inflammatory action, since eugenol exhibited potent anti-inflammatory action (28, 29).

In conclusion, the present study demonstrated the rapid cytotoxic action of eugenol at the concentration used for the topical application in the dentistry, suggesting the importance of careful use of this drug. Further investigation is necessary to elucidate the target molecule of eugenol.

Figure 4. Effect of the four dental compounds studied on caspase-3 activity in human oral squamous cell carcinoma cell lines and normal oral cells. Cells were incubated for 4 h without (control), or with 50% cytotoxic concentration (CC50), CC50×2, or CC50×4 of dental compounds and then assayed for caspase-3 activity (expressed as 405 nm of cleaved product for each substrate). Data are expressed as the mean±S.D. **Significantly different from the HL-60 control value (p<0.01). UV: HL-60 cells were exposed to 1 min UV irradiation, followed by 3 h of incubation.

References
Figure 5. Activation of caspase-3/-7 by dental compounds. HSC-2 cells were incubated for the indicated times with a 50% cytotoxic concentration (CC50)×2 of dental compounds, and the production of cleaved product of poly ADP-ribose polymerase (PARP) was detected by western blot analysis.

Figure 6. Effect of UV irradiation on the viability of cultured HSC-2 cells. HSC-2 cells were exposed to UV irradiation (6 J/m²/min, 1 min) for 1 min in PBS (−) containing the indicated concentrations of hydroquinone (HQ), benzoquinone (BQ), eugenol (EUG) or sodium ascorbate. After removing the medium or PBS(−), the cells were incubated for 48 h in fresh culture medium. The viable cells were then determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method, and the absorbance (relative viable cell number) was determined at 540 nm. Each value represents the mean of triplicate assays.


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