Abstract. We investigated six endodontic agents for their ability to induce apoptosis and modify the cytotoxic activity of NaF against human squamous cell carcinoma (HSC-2) and human promyelocytic leukemia (HL-60) cell lines. Four Group I agents (Form Cresol, Cam Phenic, Eucaly Soft, GC Fuji Varnish), but not two Group II agents (Caviton, Canals-N), induced internucleosomal DNA fragmentation and activated caspases 3, 8 and 9 in HL-60 cells. Only Cam Phenic among these agents additively enhanced the cytotoxic activity of NaF in HSC-2 and HL-60 cells. Form Cresol and Cam Phenic reduced the glucose consumption at early stage, possibly due to their toxic effect. Amino acid analysis suggests that the higher cytotoxicity of Form Cresol may be derived, at least in part, from its oxidizing action.

Sodium fluoride (NaF), used for dental cleaning, mouth rinsing and added to the drinking water, is thought to prevent caries (1). Higher concentrations of fluoride have shown cytotoxic activity against established aneuploid cell lines (2-4) and human diploid cells (5-9). NaF showed slightly higher cytotoxic activity against human tumor cell lines than against normal human cells, possibly by its apoptosis-inducing activity (10). These findings urged us to investigate various substances for their ability to modify the apoptosis-inducing activity of NaF. We found that several antioxidants (such as sodium ascorbate, gallic acid, epigallocatechin gallate, chlorogenic acid and curcumin), oxidants (such as hydrogen peroxide and sodium hypochlorite) (11) and antitumor agents (such as cisplatin, etoposide, doxorubicin and pepsinmycin) (12) additively enhanced the cytotoxic activity of NaF against human promyelocytic leukemia (HL-60) and human oral squamous cell carcinoma (HSC-2) cell lines. From the point of view of dentistry, endodontic agents should be tested for their effect on the oral environment. Therefore, we investigated here whether six endodontic agents (Form Cresol, Cam Phenic, Eucaly Soft, Fuji Varnish, Caviton, Canals-N) can induce apoptosis in HL-60 cells and modify the cytotoxic activity of NaF against HL-60 and HSC-2 cell lines. NaF, an inhibitor of enolase (one of the glycolytic enzymes) (13), reduced the extracellular concentration of glucose at early stage of cell death induction (11). We therefore investigated here whether these endodontic agents similarly affect the glucose consumption.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium (Gibco BRL, Grand Island, NY, USA); 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, USA); fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA); NaF, dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); Form Cresol (formalin: 50:50), Cam Phenic (phenol: camphor: ethanol = 30:60:10) (Neo Dental Chemical Products Co., Ltd., Tokyo, Japan); Eucaly Soft (composed of Eucalyptus oil and alcohol) (Toyo Chemical Laboratories Inc., Tokyo, Japan); GC Fuji Varnish (composed of vinyl acetate and ethyl acetate) (GC Corporation, Tokyo, Japan); Cavition (composed of menthol, ethyl alcohol, zinc oxide, zinc sulfate, calcium sulfate, plaster and vinyl acetate) (G-C Dental Industrial Corp., Tokyo, Japan); Canals-N (composed of zinc oxide, barium sulfate, bismuth subcarbonate, fatty acid and propylene glycol) (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan).

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Effect of Endodontic Agents on Cytotoxicity Induction by Sodium Fluoride

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Cell culture. HSC-2 and HL-60 cells were cultured at 37 °C in DMEM or RPMI1640 medium, respectively, supplemented with 10% heat-inactivated FBS under a humidified 5% CO₂ atmosphere.

Assay for cytotoxic activity. HSC-2 cells were inoculated at 12 x 10⁴ cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 24 hours, the medium was removed by suction with aspirator and replaced with 0.1 mL of fresh medium containing various concentrations of test samples. Cells were incubated for another 24 hours and the relative viable cell number was then determined by MTT method. In brief, cells were replaced with fresh culture medium containing 0.2 mg/mL MTT and incubated for another 4 hours. The cells were lysed with 0.1 mL of DMSO and the absorbance at 540 nm of the cell lysate was determined from the dose-response curve (14).

The viability of HL-60 cells was determined by cell counting kit (Dojindo, Kumamoto, Japan). HL-60 cells were inoculated at 5x10⁴/0.1 mL in 96-microwell and various concentrations of NaF and endodontic agents added. After incubation for 24 hours, the viable cell number was determined as described previously (15). The cell density of control cells at cell harvest was in the range of 8-9 x 10⁵/ml.

Assay for DNA fragmentation. Cells were lysed with 50 μL lystate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium N-lauroyl-sarcosinate solution]. The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1-2 hours at 37 °C. After incubation, lystate was mixed with 50 μL of NaCl solution [7.6 M NaCl, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0]. Lysate was mixed with 100 μL of ethanol and centrifuged for 20 minutes at 20,000 xg. The precipitate was washed with 1 mL of 70% ethanol and dissolved in 20 μL of water. Lysis solution (MBL, Nagoya, Japan). After standing for 10 minutes on ice and centrifugation for 5 minutes at 10,000 xg, the supernatant was collected. Lysate (50 μL, equivalent to 200 μg protein) was mixed with 50 μL 2x reaction buffer (MBL) containing substrates for caspase 3 (DEVD-pNA (p-nitroanilide)), caspase 8 (IETD-pNA) or caspase 9 (LEHD-pNA). After incubation for 2 hours at 37 °C, the absorbance at 405 nm of chromophore pNA, liberated by cleavage of substrate by activated caspases, was measured by plate reader.

Determination of free amino acids. Culture supernatant (medium fraction) of control and treated cells was collected by centrifugation at 4,800 rpm, 3 minutes in appendix tube to remove the cell debris, mixed with an equal volume of 10% TCA and stood on ice for 30 minutes. After centrifugation for 5 minutes at 10,000 xg, the deproteinized supernatant was collected and stored at -40 °C. The supernatants (20 μL) were analyzed by a JEOI LC-300 amino acid analyzer and amino acids were detected by the ninhydrin reaction (17).

Results

Induction of apoptosis. Figure 1 shows that four endodontic agents, namely Form Cresol (A), Cam Phenic (B), Eucaly Soft (C) and GC Fuji Varnish (D) (tentatively classified as Group I agents), induced internucleosomal DNA fragmentation in HL-60 cells. There was an optimal concentration for each of them (for example, 0.003-0.01% for Form Cresol (A); 0.03-0.1% for Cam Phenic (B); 0.1-1% for Eucaly Soft (C); 1-3% for Fuji Varnish (D)). Higher concentrations of these agents significantly reduced the extent of DNA fragmentation. Figure 2A-D shows that these four agents also activated caspase 3, to a comparable level attained by 1 μg/mL actinomycin D. The optimal concentration for caspase 3 activation was similar to that for DNA fragmentation (for example, 0.01% for Form Cresol (A); 0.1% for Cam Phenic (B); 0.3% for Eucaly Soft (C); 3% for GC Fuji Varnish (D)). These agents also activated both caspase 8 and caspase 9, but to a lesser extent (Figure 2).

On the other hand, two other endodontic agents, namely Cavilon (0.001-1 mg/mL) and Canals-N (0.03-3 mg/mL) (tentatively classified as Group II agents), did not induce any clear-cut DNA fragmentation (Figure 1E, F) nor activate any of caspases 3, 8 and 9 (Figure 2E, F).

Cytotoxic activity of Group I endodontic agents. Among the four Group I agents, Form Cresol showed the highest cytotoxic activity against HSC-2 cells (Figure 3A). The viable cell number declined dose-dependently by increasing concentrations of Form Cresol (from 0.0049 to 0.156%). HL-60 cells showed similar sensitivity to Form Cresol (Figure 4A). Addition of NaF did not enhance the cytotoxicity of Form Cresol (Figures 3A, 4A).

Cam Phenic showed slightly lower cytotoxic activity against HSC-2 cells. The viable cell number declined by increasing concentrations of Cam Phenic (from 0.0195 to 2.5%) (Figure 3B). NaF additively enhanced the cytotoxicity of Cam Phenic (Figure 3B). The viability of HL-60 cells declined above 0.0195% of Cam Phenic. Cam Phenic at 0.039% resulted in extensive cell death, suggesting the narrow range of optimal concentration for cell death induction. NaF also additively enhanced the cytotoxicity of Cam Phenic against HL-60 cells (Figure 4B).

Eucaly Soft showed much lower cytotoxicity against both HSC-2 (CC₅₀>5%) (Figure 3C) and HL-60 cells (CC₅₀>5%) (Figure 4C). The addition of NaF did not further enhance the cytotoxicity of Eucaly Soft (Figures 3C, 4C).
Figure 1. Induction of DNA fragmentation by endodontic agents in HL-60 cells. (A) HL-60 cells were inoculated at 5 x 10⁵ cells/1 mL in 24-well plate, in fresh culture medium (RPMI1640 +10% FBS) with the indicated concentrations of Form Cresol (A), Cam Phenic (B), Eucaly Soft (C), Fuji Varnish (D), Cavinton (E) or Canals-N (F). After incubation for 6 hours, DNA was extracted and applied to agarose gel electrophoresis. UV, DNA from apoptotic HL-60 cells induced by UV irradiation (Ref. 16).
Figure 2. Activation of caspases 3, 8 and 9 by endodontic agents in HL-60 cells. HL-60 cells were incubated for 4 hours with the indicated concentrations of Form Cresol (A), Cam Phenic (B), Eucaly Soft (C), Fuji Varnish (D), Cavilon (E) or Canals-N (F). Act. D, positive control (1 μg/mL actinomycin D-treated HL-60 cells).
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**HSC-2 cells**

Figure 3. Effect of endodontic agents on NaF-induced cytotoxicity against HSC-2 cells. HSC-2 cells were inoculated at 12 x 10^3 cells/well/0.1 mL in 96-microwell plate. After incubation for 24 hours, cells were replaced with fresh culture medium (DMEM + 10% FBS) without (control) or with the indicated concentrations of Form Cresol (A), Cam Phenic (B), Eucaly Soft (C), Fuji Varnish (D), Caviton (E) or Canals-N (F) in the presence of various concentrations (0-10 mM) of NaF. After incubation for a further 24 hours, the cell survival (relative viable cell number) was determined by MTT method and expressed as % of control. Each value represents mean from 4 determinations.
**Figure 4. Effect of endodontic agents on NaF-induced cytotoxicity against HL-60 cells.** HL-60 cells were inoculated at 5 x 10⁴ cells/well/0.1 mL in 96-microwell plate, in fresh culture medium (RPMI 1640 + 10% FBS) without (control) or with the indicated concentrations of Form Cresol (A), Cam Phenic (B), Eucaly Soft (C), Fuji Varnish (D), Caviton (E) or Canals-N (F) in the presence of various concentrations (0-10 mM) of NaF. After incubation for a further 24 hours, the cell survival (relative viable cell number) was determined by cell counting kit and expressed as % of control. Each value represents mean from 3 determinations.
Figure 5. Effect of endodontic agents on glucose utilization in HL-60 cells. HL-60 cells (5 x 10⁵/mL) were incubated for 6 or 24 hours with the indicated concentrations of Form Cresol or Cam Phenic. The changes in the extracellular concentration of glucose (μg/mL) during the first 6 hours (○) or the following 18 hours (6-24 hours) (●) was then determined, after subtraction of that observed without cells. Each value represents mean from 3 determinations.

Discussion

We demonstrated that four Group I endodontic agents (Form Cresol, Cam Phenic, Eucaly Soft, GC Fuji Varnish) induced apoptotic cell death (characterized by internucleosomal DNA fragmentation and caspase activation) in HL-60 cells. These endodontic agents activated caspase 8 (mitochondria-independent extrinsic pathway) and caspase 9 (mitochondria-dependent intrinsic pathway) (18), suggesting the activation of both pathways. On the other hand, the highly insoluble Group II agents (Caviton, Canals-N) did not induce apoptotic cell death in HL-60 cells. The study of apoptosis induction by endodontic agents has been limited. 4-META/MMA-TBB resin (4MMT), one of the capping agents, has been reported to induce secondary apoptosis of pulp cells throughout the mesial coronal pulp (19). The present investigation suggests the possible application of Group I agents for the treatment of oral carcinoma. The mechanism of apoptosis induction by Group I agents is unclear.

There was a possibility that these agents may have oxidized the essential amino acids such as methionine, as has been observed after treatment with ascorbic acid, gallic acid or hydrogen peroxide (20), and may indirectly affect the cell growth. We tested this possibility, using amino acid analyzer. We found that the extracellular concentration of methionine was reduced to approximately one-half and its oxidation product, methionine sulfoxide (shown in inset), was accumulated by Form Cresol treatment (Figure 6). The effect of Form Cresol was observable within 6 hours after treatment. We also found that the concentration of cysteine, which is also susceptible to oxidation, slightly declined by Form Cresol (Figure 6). On the other hand, Cam Phenic did not significantly affect the extracellular concentration of methionine and cysteine (data not shown). The higher cytotoxic activity of Form Cresol may result at least in part from its oxidizing action. Form Cresol has a very strong smell, possibly releasing cytotoxic volatile substances. The disappearance of ornithine and asparagine by Form Cresol treatment may reflect the loss of growth potential (Figure 6).
Figure 6. Stimulation of methionine oxidation by Form Cresol. RPMI1640 medium (+10% FBS) was supplemented without (control) or with 1% Form Cresol. After incubation for 6 hours at 37°C in a humidified 5% CO₂ atmosphere, 5% TCA-soluble fraction of the medium was applied to amino acid analyzer. The positions of methionine and methionine sulfoxide are indicated by arrows.
NaF has been shown to induce apoptosis, perhaps by G-protein-linked systems involving PKC, PKA, tyrosine kinase and Ca\(^{2+}\)-linked systems (21-23) or MAK kinase p38 and possibly JNK (24). It has also been reported that the NaF cytotoxicity was enhanced at acidic pH (25). The present study demonstrates that only Cam Phnic, but not the other endodonic agents investigated here, enhanced the cytotoxic activity of NaF. The combination effect of Cam Phnic and NaF should be further pursued for future clinical application of these agents to oral carcinoma. Since the clearance of NaF in the mouth is very rapid, the elevation of the effective concentration of NaF is crucial. In this respect, the potentiation of the apoptosis-inducing activity of NaF by endodontic agents may be one choice to recommend.

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