J Periodont Res 2008; 43: 391–399

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01052.x

Oral malodorous compound causes apoptosis and genomic DNA damage in human gingival fibroblasts

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Yaegaki K, Qian W, Murata T, Imai T, Sato T, Tanaka T, Kamoda T. Oral malodorous compound causes apoptosis and genomic DNA damage in human gingival fibroblasts. J Periodont Res 2008; 43: 391–399. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: Volatile sulfur compounds are the main cause of halitosis. Hydrogen sulfide is one of these volatile sulfur compounds and the principal malodorous compound in physiological halitosis. Periodontally pathogenic activities of hydrogen sulfide have been previously reported. Hydrogen sulfide induces apoptotic cell death in aorta smooth muscle cells and in other tissues. Apoptosis plays an important role in the onset and progress of periodontitis. The objective of this study was to determine whether hydrogen sulfide causes apoptosis in human gingival fibroblasts.

Material and methods: Necrotic cells were detected using a lactate dehydrogenase assay. Apoptosis was ascertained using a histone-complexed DNA fragment assay and flow cytometry. The level of caspase 3, a key enzyme in apoptotic signaling, was also measured, and the effects of hydrogen sulfide on reactive oxygen species and superoxide dismutase were assessed. DNA damage caused by hydrogen sulfide was examined by means of single-cell gel electrophoresis.

Results: After 72 h of incubation with 100 ng/mL of hydrogen sulfide, necrosis was found in less than 10% of human gingival fibroblasts, whereas apoptosis was significantly increased ($p < 0.05$). Superoxide dismutase activity was strongly inhibited, and reactive oxygen species production was enhanced, after 48 and 72 h of incubation. Caspase 3 activity was also increased after 72 h of incubation ($p < 0.01$). Tail length, percentage of DNA in tail, and tail moment, measured by single-cell gel electrophoresis, were also intensified after 72 h of incubation ($p < 0.001$).

Conclusion: Hydrogen sulfide caused apoptosis and DNA damage in human gingival fibroblasts. An increased level of reactive oxygen species stimulated by hydrogen sulfide may induce apoptosis and DNA strand breaks.

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Key words: apoptosis; halitosis; hydrogen sulfide; periodontitis

Accepted for publication September 26, 2007

Halitosis is classified into three main categories: genuine halitosis, pseudo-halitosis and halitophobia. Genuine halitosis is subdivided into physiological halitosis and oral or extra-oral pathological halitosis (1). Pseudo-halitosis and halitophobia patients stubbornly complain of the existence of halitosis, but their malodor is not perceived by others and therefore these conditions are considered to be psychogenic. Therefore, most cases of halitosis in ordinary subjects without any condition causing halitosis may be categorized as physiological halitosis. Many odorous compounds are found in human mouth air or lung air, but only for volatile sulfur compounds does the concentration correlate with the odor strength of halitosis; volatile sulfur compounds are the main cause

of oral malodor (2). Volatile sulfur compounds in mouth air are composed principally of hydrogen sulfide and methyl mercaptan (3). Yaegaki & Sanada (3) have reported that hydrogen sulfide is the main component in physiological halitosis.

It is known that hydrogen sulfide and other volatile sulfur compounds are involved in periodontal pathogenic activities (1–4). Gingival epithelial tissues play a key role in periodontal pathogenesis by forming a barrier against penetration by periodontally pathogenic microorganisms and the detrimental products of microorganisms. Volatile sulfur compounds have been reported to increase the permeability of the tissue and the penetration of lipopolysaccharide and prostaglandins in a crevicular epithelia model (4–6). Volatile sulfur compounds were also reported to increase the production of interleukin-1 and prostaglandin E_2, activate matrix metalloproteinase (7), then increase collagen degradation and reduce collagen synthesis in human gingival fibroblasts (4,6–12). In addition, volatile sulfur compounds suppress wound healing, as shown by inhibition of the synthesis of basal membranes (12) and inhibition of the proliferation of epithelial cells (13). This leads to the conclusion that volatile sulfur compounds are periodontally pathogenic.

Recent studies have reported that hydrogen sulfide causes apoptotic cell death in aorta smooth muscle cells, leucocytes, pancreatic cells and other cells (14) Therefore, volatile sulfur compounds, especially hydrogen sulfide, might be a factor contributing to apoptosis in periodontal tissues, but whether hydrogen sulfide induces apoptosis in human gingival fibroblasts has not yet been reported. Apoptosis plays an important role in the onset and progress of periodontal conditions (15–21). Activation of caspase-3, p53 or Bcl-2 was found in human gingival tissues with periodontitis (18,21), and periodontal pathogenic microorganisms have been reported to cause apoptosis in periodontal tissues (15,16,20). Ekuni *et al.* (17) reported that the initial apical migration of junctional epithelium in lipopolysaccharide-induced experimental periodontitis appeared to occur simultaneously with the apoptosis of periodontal ligament fibroblasts. It was therefore suggested that the apoptosis-related detachment of connective tissue may cause the migration of junctional epithelium (17). Taken together, these observations suggest that apoptosis may have an important role in periodontal pathogenic processes. In this study, we investigated whether hydrogen sulfide causes apoptosis in human gingival fibroblasts and determined how hydrogen sulfide initiates the apoptotic process in human gingival fibroblasts.

Material and methods

Cell culture

Human gingival fibroblasts, reserved cell lines in the Department of Oral Biological and Medical Sciences, Faculty of Dentistry, University of British Columbia and in the Department of Oral Health, School of Life Dentistry at Tokyo, Nippon Dental University, were cultured in Dulbecco's modified Eagle' medium (Invitrogen, Burlington, ON, Canada), supplemented with 10% fetal bovine serum (Invitrogen), at 37°C in an atmosphere of 5% CO_2. The cells, in passages 6–10, were plated either at a density of 5×10^5 cells in 75-cm^2 flasks or at a density of 1×10^4 cells/well in 96-well plates, and allowed to attach overnight. Before incubation in the hydrogen sulfide incubation system described below, the medium was replaced with fresh medium. All protocols for obtaining and handling human oral tissues were reviewed and approved by the Institutional Review Board of the University of British Columbia and by the Ethics Committee of Nippon Dental University.

Hydrogen sulfide incubation system

The hydrogen sulfide incubation system is shown in Fig. 1. Both hydrogen sulfide and non-hydrogen sulfide (air) chambers were infused with a constant flow of prewarmed and humidified 5% CO_2 in air. Hydrogen sulfide at a concentration of 100 ng/mL was produced by a standard hydrogen sulfide gas generator (Dynacalibrator™; VICI Metronics, Poulsbo, WA, USA), which mix hydrogen sulfide with the air, prior to entry into the hydrogen sulfide chamber. The hydrogen sulfide concentration in the medium exposed to the above concentration of hydrogen sulfide was much lower than the concentration in gingival crevicular fluid reported by Persson (data not shown) (22).

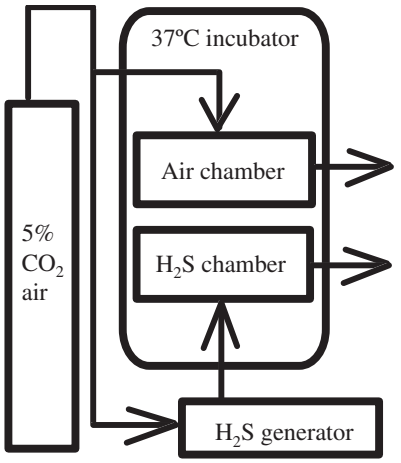

Fig. 1. Design of the hydrogen sulfide incubation system. The system allows incubation of the cells either in 5% CO_2 in air plus hydrogen sulfide (100 ng/mL) or in 5% CO_2 in air alone. The concentration of hydrogen sulfide was adjusted using a gas generator. H_2S, hydrogen sulfide.

The cells in 75-cm^2 flasks or 96-well plates were placed in the chambers and incubated in either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL) (test), or 5% CO_2 in air alone (control), for 24, 48 or 72 h (7–10).

Trypan blue staining

The effect of hydrogen sulfide on cell death was assessed by Trypan blue staining. After incubation as described above, the cells were trypsinized using 0.25% trypsin–EDTA in phosphate-buffered saline. Trypsin activity was quenched by the addition of an equal volume of medium containing serum, and the cells in the medium were centrifuged. The cells were resuspended in phosphate-buffered saline (5×10^5 cells/mL), then diluted 1:100 in 0.25% Trypan blue solution (Invitrogen) and

counted in a hemocytometer to assess the number of dead blue cells in the total number of cells counted. The percentage of viable cells was calculated (23,24).

Cell toxicity assay

The Trypan blue stain cannot identify necrotic cells that may have lysed and therefore we determined the percentage of cell lysis by measuring the release of lactate dehydrogenase from the cytosol into the supernatant using a Cytotoxicity Detection kit^{TM} (Roche Diagnostics, Laval, QC, Canada). For this assay, three controls were obtained: a background control, which provided information about the activity of the lactate dehydrogenase contained in the assay medium; and high and low controls, which provided information about the activity of the lactate dehydrogenase released from cells treated with Triton X-100 (giving the maximum releasable lactate dehydrogenase) and the untreated normal cells (giving the spontaneous lactate dehydrogenase release), respectively (24,25). After incubation in a 75-cm^2 flask and treatment with either 5% CO_2 in air with hydrogen sulfide (100 ng/mL), or 5% CO_2 in air alone, for 24, 48 or 72 h, lactate dehydrogenase activity was determined using a colorimetric assay, according to the manufacturer's instructions.

Histone-complexed DNA fragmentation assay

For quantification of apoptosis and for differentiating apoptosis from necrosis, the amount of histone-complexed DNA fragments present in the cytoplasm of cells were measured after the induction of apoptosis by using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit^{TM} (Roche Diagnostics) based on a quantitative sandwich enzyme immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones. This allows the specific detection and quantification of mononucleosomes and oligonucleosomes released into the cytoplasm from nucleus that die of apoptosis. In addition, the amount of fragments present in the medium were measured to establish the level of cell lysis caused by necrosis (24,25). Mononucleosomes and oligonucleosomes in both the cell cytoplasm and the medium were measured according to the manufacturer's instructions. Briefly, cells were grown in 96-well plates in hydrogen sulfide or air for 24, 48 or 72 h and then the plates were centrifuged at 200 *g* for 10 min. The cell pellet was suspended in 200 µL of the lysis buffer. The suspension was incubated for 30 min at 20°C and then centrifuged at 200 *g* for 10 min. Aliquots of supernatant (lysate) were transferred to a streptavidin-coated microplate supplied with the kit, then incubated with the immunoreagent containing anti-histone and anti-DNA for 2 h at room temperature, after which the wells were washed three times with incubation buffer. The substrate solution was added and incubated for 15 min at room temperature, and then the absorbance was measured at 405 nm in a Bio-Rad 3550 ELISA plate reader (Bio-Rad, Hercules, CA, USA). The results of this study were reported as colorimetric intensity.

Caspase 3 activity assay

The initiation of apoptosis mediated by mitochondria is characterized by the activation of specific proteases called caspases (cysteinyl-aspartic-acid-proteases), in particular caspase 3 (26). Caspase 3 activity was measured using a Caspase 3 Activity Assay kit^{TM} (Roche Diagnostics), which is a fluorometric immunosorbent enzyme assay for the specific, quantitative *in vitro* determination of caspase 3 activity. The amount of caspase 3 derived from the cellular lysates was measured following the manufacturer's instructions. Cells were cultured in a 75-cm^2 flask and treated with either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL), or 5% CO_2 in air alone, for 24, 48 or 72 h. After trypsinization (as described under 'Trypan blue staining') and centrifugation at 200 g for 10 min, 20°C, 2×10^6 cells were washed with ice-cold phosphate-buffered saline. The pellet was resuspended in 200 µL of the lysis buffer, which was prepared immediately before use. The lysis buffer was freshly prepared by diluting a condensed lysate buffer containing dithiothreitol, supplied with the kit, 100-fold in the incubation buffer. The suspension was incubated for 1 min on ice and then centrifuged (1 min, 20°C, 12,000 *g*). After centrifugation, 100 µL of supernatant was collected and frozen at −80°C until analysis. The caspase 3 derived from the cellular lysates was placed on the anti-caspase 3-coated 96-well plate, and nonspecific binding was blocked by adding blocking buffer. One-hundred mcrolitres of lysate was added to the well and the plate was incubated at 37°C for 1 h. After removal of the lysate using a pipette, the well was washed three times with the incubation buffer, then with 100 µL of the substrate solution supplied with the kit. After 2 h of incubation, fluorometric measurement was carried out using POLARstar OPTIMA^{TM} (BMG Labtech, Offenburg, Germany), measuring excitation at 405 nm and emission at 510 nm. The results in this study were reported as fluorescence intensity.

Annexin V and propidium iodide staining

Apoptotic cells were detected by flow cytometry using a FACScan^{TM} flow cytometer (Becton Dickinson, San Jose, CA, USA), after staining with fluorescein isothiocyanate-conjugated annexin V and propidium iodide using the Annexin V-FITC kit^{TM} (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. The cells were incubated in a 75-cm^2 flask and treated in either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL), or 5% CO_2 in air alone, for 24, 48 or 72 h. The cells were harvested by washing with phosphate-buffered saline followed by treatment with the trypsin–EDTA solution and the cell pellet was resuspended in the binding buffer at a concentration of 1×10^6 cells/mL (26). After adding annexin V–fluorescein isothiocyanate and/or propidium iodide and incubating for 15 min at room temperature in the dark, the cells were run on a

FACScan™ Flow cytometer (Becton Dickinson). For each experiment, 10,000 cells were analyzed using the software FLOWJO™ (Tree Star, Ashland, OR, USA). Cells were considered apoptotic, in particular in early apoptosis, when they were annexin V positive and propidium iodide negative (26). Staining of cells by propidium iodide is an indicator of the loss of plasma-membrane integrity and therefore of necrosis or late apoptosis.

Detecting reactive oxygen species

Intracellular reactive oxygen species were detected using the MitoSOX™ Red Mitochondrial Superoxide Indicator (Invitrogen) (27). Cells were cultured in a 75-cm^2 flask with either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL), or 5% CO_2 in air alone, for 24, 48 or 72 h. After trypsinization, the cells were collected by centrifugation at 200 g for 5 min. The precipitate was resuspended in phosphate-buffered saline, containing 1 mM Ca and 0.1 mM Mg, to a final concentration of 1×10^6 cells/mL, then a 100-μL aliquot was mixed with 1 μL of 5 mM Mitosox™ stock and 900 μL of phosphate-buffered saline in a 5-mL test tube and incubated at 37°C for 10 min. Mitosox™-positive cells were counted using a FACScan™ flow cytometer (Becton Dickinson) and their ratio (%) to the total number of cells was obtained.

Inhibition of superoxide dismutase by hydrogen sulfide

CuZn-superoxide dismutase from *Escherichia coli* (E.C.1.15.1.1; Sigma-Aldrich, Oakville, ON, Canada) and Mn-superoxide dismutase from *Bacillus* sp. (E.C.1.15.1.1; Wako Pure Chemical, Tokyo, Japan) were added to human gingival fibroblast homogenate, derived from 2.5×10^6 cells, in 2 mL of phosphate-buffered saline. Hydrogen sulfide, generated by the Dynacalibrator™ (VICI Metronics) using air as a carrier gas, was applied to the enzymes via gas infusion directly into the solution in the incubation chamber (Fig. 1) during incubation at 37°C. For the control samples, hydrogen sulfide was replaced with air. Superoxide dismutase activity for both the control samples and the hydrogen sulfide-treated samples was determined using the SOD Assay kit-WST (Dojindo Molecular Technologies, Kumamoto, Japan), according to the manufacturer's instructions, and the percentage inhibition was calculated (28).

Single-cell gel electrophoresis assay

DNA damage was detected by single-cell gel electrophoresis (CometAssy™; Trevigen) (29). The basis of single-cell gel electrophoresis is the migration of DNA in an agarose gel under electrophoretic conditions. Undamaged DNA migrates more slowly and remains within the confines of the nucleoid, whereas damaged DNA migrates faster. When viewed under a microscope, a cell has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments. Evaluation of the DNA 'comet' tail shape allows for assessment of DNA damage (29). The procedures used followed the manufacturer's instructions. Briefly, the cells were suspended in phosphate-buffered saline at 1×10^5 cells/mL, then mixed with LMAgarose™ (CometAssy™; Trevigen) at 37°C. Seventy-five microlitres of the mixture was put on the Cometslide™ (CometAssy™; Trevigen) and immersed in the lysis solution, alkaline solution and TBE buffer containing 89 mM Tris base, 89 mM Boric acid and 2 mM EDTA, respectively. Electrophoresis was carried out at a constant voltage of 1 V/cm for 10 min, then the Cometslide™ was immersed in 70% ethanol for 5 min. Agarose gel on the Cometslide™ was dried up in ambient condition, then the cells on the CometSlide™ were stained with SYBR Green 1™ (CometAssy™; Trevigen). DNA strand breaks were analyzed by examination using a fluorescence microscope (Zeiss Axioplane 2™; Carl Zeiss Canada, Toronto, Canada). Images were captured by digital camera, and quantitative data were generated by analysis of the results using COMETSCORE™ software (Tritek, Sumerduck, VA, USA). Comet assay parameters, such as tail length, percentage of DNA in the tail and tail moment, were determined to assess the DNA damage caused by hydrogen sulfide. Percentage of DNA in the tail is a measure of the proportion of total DNA present in the 'comet' tail. Tail length is expressed in pixels. Tail moment is defined as the percentage of DNA in the tail multiplied by the distance between the center of the head and the end of the tail (29).

Statistical analysis

Results are presented as the mean ± standard deviation. Statistical analysis was performed using the Mann–Whitney test and the Kruskal–Wallis test. STATVIEW™ version 5.0 (SAS institute Inc., Cary, NC, USA) was utilized for analyses. Statistical significance was accepted at a p-value of < 0.05.

Results

To examine hydrogen sulfide toxicity to human gingival fibroblasts, the percentage of viable cells in the hydrogen sulfide-treated groups was determined by Trypan blue staining. More than 90% of viable cells were found in hydrogen sulfide-treated groups at each time-point assessed (Fig. 2). Significant differences between test and control groups were not observed (data not shown). The cell lysis rate was evaluated by measuring lactate dehydrogenase activity from the cytosol of damaged and necrotic cells. The cell lysis rates were less than 10% at each time-point and there was no significant difference within each time-point (Fig. 2). It was estimated that the rate of cell necrosis was $< 10\%$ of the total number of cells.

In the DNA fragmentation analysis, a significant difference within the test groups in the cells was found (Kruskal–Wallis test, $p < 0.05$). At 24, 48 and 72 h of incubation, the cells treated with hydrogen sulfide (100 ng/mL) showed significantly more DNA fragmentation compared with the control $p < 0.05$, $p < 0.01$, $p < 0.01$, respectively, for the 24-, 48- and 72-h time-points; Mann–Whitney test)

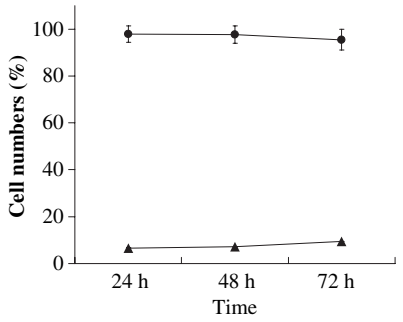

Fig. 2. Effect of hydrogen sulfide on cellular viability. The cell numbers (%) were obtained from the ratio of the cell numbers/total cell numbers. The cells were incubated with either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL), or 5% CO_2 in air alone, for the times indicated. Filled circle (Trypan blue staining): relative viable cell numbers. The cells were stained with Trypan blue and counted. Viable cells were expressed relative to the untreated control. Data were obtained from five independent experiments. Filled triangle: necrotic cells determined by the Cytotoxicity Detection kit^{TM}, which detects lactate dehydrogenase activity from the cytosol of damaged cells. Data were obtained from six independent experiments (mean ± SD).

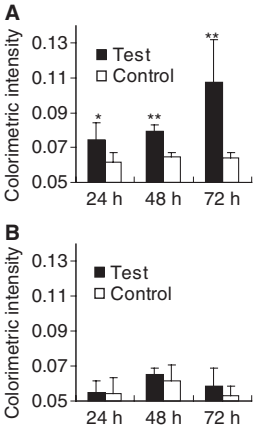

Fig. 3. DNA fragmentation and hydrogen sulfide incubation time. The cells were incubated with either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL) (Test), or 5% CO_2 in air alone (Control), for the times indicated. DNA fragmentation was assayed using the Cell Death Detection ELISA kit^{TM}. The values (mean ± SD) from six independent experiments were expressed as fluorescence intensity. A significant difference within the test groups was found using the Kruskal–Wallis test ($p < 0.05$), then the differences between the test and control groups were obtained. (A) DNA fragmentation in the cells. (B) DNA fragmentation in the medium. Single and double asterisks indicate a statistically significant difference between treatment in the test group and the control group ($p < 0.05$ and $p < 0.01$, respectively, Mann–Whitney test).

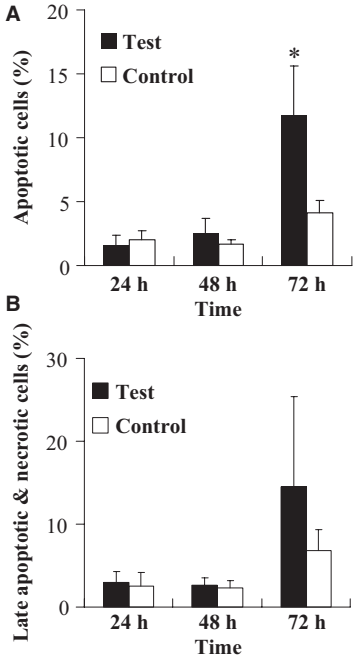

Fig. 4. Ratio of apoptotic to necrotic or late apoptotic cells induced by hydrogen sulfide. The cells were incubated with either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL) (Test), or 5% CO_2 in air alone (Control), for the times indicated. Cells were stained with annexin V and propidium iodide. Apoptotic and late apoptotic or necrotic cells were detected by flow cytometry. Data were obtained from four independent experiments (mean ± SD). In apoptotic cells (A), a significant difference was found among the test groups ($p < 0.05$, Kruskal–Wallis test). The asterisk indicates a statistically significant difference between the test and the control ($p < 0.05$, Mann–Whitney test).

(Fig. 3A), whereas the fragmentation did not increase in the culture medium (i.e. outside the cells) (Fig. 3B). A statistically significant difference was found between the test groups at 24- and 72 h of incubation ($p < 0.05$, Mann–Whitney test).

The percentages of apoptotic cells, detected by flow cytometry, are shown in Fig. 4A. In apoptotic cells (Fig. 4A), a significant difference was found between the test groups at 24 and 48 h and the test group at 72 h (Kruskal–Wallis test, $p < 0.05$). The percentage of apoptotic cells was significantly increased by exposure to hydrogen sulfide for 72 h (12% in the test group vs. 4% in the control group; $p < 0.05$, Mann–Whitney test). The number of late apoptotic and/or necrotic cells was also increased at 72 h of incubation; however, no significant difference in the number of late apoptotic and/or necrotic cells was found between the control and test groups, perhaps because of a large deviation in the hydrogen sulfide exposure group. (Fig. 4B).

The values (mean ± standard deviation) of caspase 3 activity in six independent experiments were obtained; a significant difference was found among the test groups (Kruskal–Wallis test, $p < 0.001$). Moreover, significant differences between the activities of the test and control groups were found at 48 and 72 h of incubation ($p < 0.01$ and $p < 0.01$ at 48 h and 72 h; Mann–Whitney test). (Fig. 5).

The number of Mitosox^{TM}-positive cells, which demonstrates a certain amount of mitochondrial reactive oxygen species in the cell, was obtained from six independent experiments and a significant difference was found among the test groups (Kruskal–Wallis test, $p < 0.05$). Also, the number of Mitosox^{TM}-positive cells was significantly higher at 48 and 72 h of incubation with hydrogen sulfide compared with controls ($p < 0.01$, Mann–Whitney test) (Fig. 6).

For the assay of superoxide dismutase inhibition by hydrogen sulfide, we determined the appropriate concentrations of CuZn-superoxide dismutase and Mn-superoxide dismutase for use in this study as 0.01 and 50 U/mL, respectively. At these concentrations, it was possible to determine the inhibition of superoxide dismutase by certain concentrations of hydrogen sulfide using the SOD Assay Kit-WST (Dojindo Molecular Technologies). The percentage inhibitions of CuZn-superoxide dismutase ($n = 6$) and Mn-superoxide dismutase ($n = 6$) by

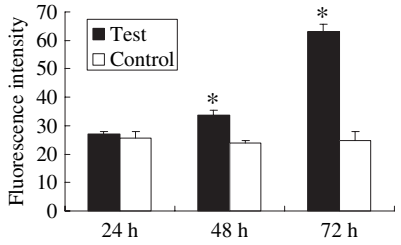

Fig. 5. Activation of caspase 3 caused by hydrogen sulfide incubation. The cells were incubated with either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL) (Test), or 5% CO_2 in air alone (Control), for the times indicated. Caspase 3 activity was measured using the Caspase 3 Activity Assay kit™. Caspase 3 activity was expressed as the ratio of test/control × 100 (%) and is shown as fluorescence intensity. Data were obtained from six independent experiments (mean ± SD). A significant difference was found among the test groups ($p < 0.001$; Kruskal–Wallis test). The asterisks indicate a statistically significant difference between the test and the control ($p < 0.01$, Mann–Whitney test).

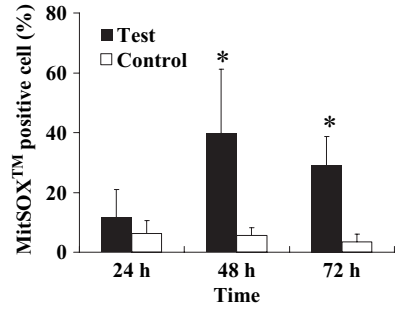

Fig. 6. Reactive oxygen species resulting from incubation with hydrogen sulfide. The cells were incubated with either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL) (Test), or 5% CO_2 in air alone (Control), for the times indicated. The amount of reactive oxygen species was determined using MitoSOX™ Red Mitochondrial Superoxide Indicator ($n = 6$, mean ± SD). A significant difference was found among the test groups ($p < 0.05$, Kruskal–Wallis test). The asterisks indicate a statistically significant difference between the test and the control ($p < 0.01$, Mann–Whitney test).

5 ng/mL of hydrogen sulfide were obtained from the ratio with the control, and found to be 82.5 ± 10.1 and 72.7 ± 1.3, respectively, during 60 min of incubation. The percentage inhibitions of CuZn-superoxide dismutase and Mn-superoxide dismutase were increased in proportion to incubation time or hydrogen sulfide concentration (data not shown). The percentage inhibition of human gingival fibroblast-superoxide dismutase by 5 ng/mL of hydrogen sulfide was 79.6 ± 5.1 ($n = 6$).

Tail length, percentage DNA in tail and tail moment, which increase in proportion to the number of DNA strand breaks, were calculated from the results of single-cell gel electrophoresis (CometAssy™). A significant difference was found in the test groups of tail length and tail moment (Kruskal–Wallis test, $p < 0.0001$) as well as for DNA in tail ($p < 0.05$). Compared with the control, tail length was significantly increased in the cells exposed to hydrogen sulfide for 48 and 72 h ($p < 0.0001$, Mann–Whitney test) (Fig. 7). Percentage DNA in tail, and tail moment, were also significantly amplified in the cells exposed to hydrogen sulfide for 72 h compared with the control ($p < 0.0001$, Mann–Whitney test) (Fig. 7).

Discussion

In the process of cell death, there are two fundamental modes: apoptosis and necrosis. Apoptosis is programmed cell death, the mechanism by which organisms eliminate unnecessary cells, and is the common form of physiological cell death during tissue remodeling, embryonic development, etc. (30). Necrosis is associated with toxic or traumatic events causing injury to cytoplasmic organelles and with rapid collapse of homeostasis, leading to cytoplasmic rupture and inflammation (30). In contrast to necrosis, apoptosis prevents leakage of catastrophic enzymes, reactive oxygen species, oxidized lipids and other toxic compounds into surrounding tissues and keeps them within the apoptotic cells until phagocytosis takes place (30).

Thus, apoptosis is a physiological process of organisms, but on occasion, apoptosis can become a cause of pathological changes such as periodontitis. Probing of periodontal

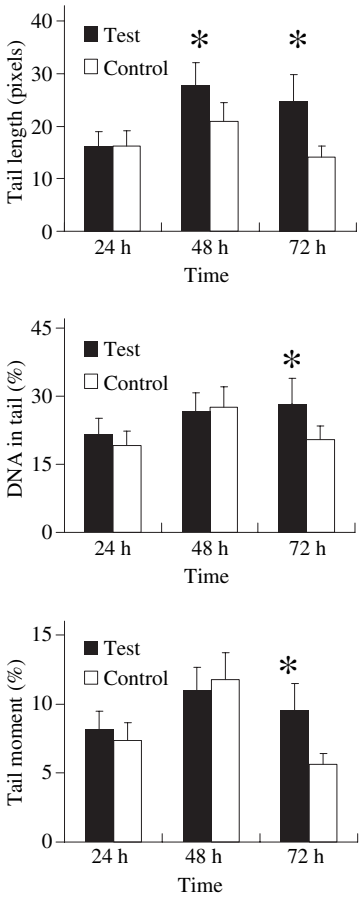

Fig. 7. Quantification of DNA strand breaks caused by incubation with hydrogen sulfide. The cells were incubated with either 5% CO_2 in air plus hydrogen sulfide (100 ng/mL) (Test) or 5% CO_2 in air alone (Control) for the times indicated. DNA strand breaks were detected using the CometAssay™. Data were obtained from six independent experiments (mean ± SD), 75 nuclei per experiment. A significant difference was found in the test groups of tail length and tail moment (respectively $p < 0.0001$, Kruskal–Wallis test) as well as in DNA in tail ($p < 0.01$). The asterisks indicate a statistically significant difference between treatment in the test and the control ($p < 0.0001$, Mann–Whitney test).

pockets is one of the essential measures for detecting periodontitis, because attachment loss and/or the existence of periodontal pockets can confirm the diagnosis of periodontitis. It is assumed that the apical migration of the junctional epithelium of periodontal tissue starts at the onset of the condition, as described previously (17). In the early stages of periodontitis, the

number of apoptotic cells among periodontal ligament fibroblasts increases significantly (17). Apoptosis has also been found in these tissues in aggressive or severe periodontitis in humans (18–21). Periodontal pathogenic microorganisms, such as *Aggregatibacter actinomycetemcomitans*, *Bacteroides forsythus* or *Porphyromonas gingivalis*, play etiological roles in the apoptotic process of periodontal tissues (15–19,21). Apoptosis was found in human gingival keratinocytes after *A. actinomycetemcomitans* infection (15). Lipoprotein of *B. forsythus* induced apoptotic cell death in human gingival fibroblasts, human gingival keratinocytes and other cell lines (16). It has also been reported that signaling of this apoptosis process caused by lipoprotein was mediated by Toll-like receptor 2 (30). The pro-inflammatory factor, nuclear factor-kappa B, was partly activated via the phosphatidylinositol (PI3) kinase/proteinkinase B (AKT) pathway in human gingival fibroblasts after *P. gingivalis* infection (21). At the later stages of infection (24–36 h) the anti-apoptotic genes largely shut down and the pro-apoptotic genes, such as Nip3, Hrk, Bak, Bik, Bok, Bax, Bad, Bim and Moap-1, were activated, causing apoptosis (30). Thus, it is postulated that apoptosis occurring in periodontal tissues may be one of the etiologic processes of periodontitis.

Lipopolysaccharide, which is a typical periodontal pathogenic compound, is one of the most effective inducers of apoptosis (31). It has been reported that volatile sulfur compounds involve biological activities similar to those of lipopolysaccharide; for example, like lipopolysaccharide, volatile sulfur compounds have been reported to stimulate the production of interleukin-1, cAMP, prostaglandin E and matrix metalloproteinase 1 in human gingival fibroblasts (7). Furthermore, volatile sulfur compounds, especially hydrogen sulfide, have been reported to induce apoptosis in several tissues (14). In the present study, we demonstrated that hydrogen sulfide induced apoptosis in human gingival fibroblasts. Histone-complexed DNA fragmentation was significantly increased by incubation with hydrogen sulfide for 72 h. Furthermore, a flow cytometric assay showed that apoptosis was significantly amplified by incubation with hydrogen sulfide for 72 h. However, a cytotoxity assay, a cell vitality assay and a DNA fragmentation ELISA assay for culture supernatant demonstrated $< 10\%$ necrosis. The flow cytometric assay also demonstrated that necrosis or late apoptosis occurred at a level of $\approx 15\%$ after incubation with hydrogen sulfide for 72 h; this implies that hydrogen sulfide also caused a certain amount of late apoptosis as well as early apoptosis.

The hydrogen sulfide cytotoxicity mechanism involves reactive oxygen species formation and mitochondrial depolarization (32). Signaling of mitochondrial-mediated apoptosis involves caspase-3. In this study, caspase-3 activity was increased upon incubation of human gingival fibroblasts with hydrogen sulfide for 72 h; therefore, we suggest that a signaling pathway involving caspase-3 from mitochondria may be activated by hydrogen sulfide. In further study, the process of this signaling pathway will be investigated. It has also been reported that extracellular signal-regulated kinase, p38 mitogen-activated protein kinase and Ras/Raf signaling were activated by hydrogen sulfide in cell lines other than human gingival fibroblasts (33–35).

We found that hydrogen sulfide increased the amount of reactive oxygen species. An increased amount of mitochondrial reactive oxygen species might suggest that necrosis is underway. On the other hand, as the reactive oxygen species originated most probably from mitochondria, reactive oxygen species might initiate mitochondrial damage followed by the apoptosis cascade. In further study, it will be determined if the cascades originating from mitochondrial damage and causing apoptosis are activated by volatile sulfur compounds.

Hydrogen sulfide also strongly inhibited superoxide dismutase activity in human gingival fibroblasts. Hydrogen sulfide binds at the catalytic Cu center of CuZn-superoxide dismutase (36), and a similar binding mechanism may also be present in Mn-superoxide dismutase, as the manganese ion is charged at $^{2+}$ like the copper ion. Sulfide oxidation increases superoxide production; however, inhibition of superoxide dismutase by hydrogen sulfide might cause a further increase of reactive oxygen species production in human gingival fibroblasts. Hydrogen sulfide exposure causes mitochondrial depolarization, but this effect is not the same as the actions of other cytochrome *c* oxidase inhibitors, in that the depolarization is not reversible (37). Such a mitochondrial injury starting apoptosis may be caused not only by cytochrome *c* oxidase-inhibitory activity but also by free-radical damage from an increased level of reactive oxygen species production (37). Moreover, superoxide dismutase, especially mitochondrial Mn-superoxide dismutase, suppresses apoptosis (38–40): superoxide dismutase prevents apoptosis indirectly by down-regulating the expression of Bax, a pro-oxidant member of the Bcl-2 protein family (39). Therefore, blocking superoxide dismutase activity with hydrogen sulfide might also suppress regulation by Bax.

The reason why human gingival fibroblasts exposed to hydrogen sulfide employ apoptotic processes leading to their own death is not clear. Skulachev (41) suggested a hypothesis that apoptosis is a selective mechanism for eliminating superoxide-producing cells, which was the primary function of apoptosis during evolution, to avoid further damage to surrounding cells or tissues. This hypothesis might be an answer to the above question.

Martis *et al.* (28) showed genotoxicity of hydrogen sulfide in ovary cells and colonocytes using single-cell gel electrophoresis, and suggested that hydrogen sulfide-induced DNA damage might be mutagenic or carcinogenic, or involve a role in chromosomal instability formation. In the present study we also observed the genotoxicity of hydrogen sulfide to human gingival fibroblasts – tail length, percentage DNA in tail and tail moment were significantly increased as a result of an increased number of DNA strand breaks, as determined by single-cell gel

electrophoresis. Yang & Wong (14) have shown that the effects of hydrogen sulfide on cell function have two aspects: apoptosis and cell proliferation. Pathological effects of hydrogen sulfide-induced cell proliferation, or its carcinogenicity, should be examined more rigorously and thoroughly in further study.

Acknowledgements

The authors thank Dean Choi, Camellia Parsa, Wendy Lee and Ali Khakbaznejad. This research was supported by the grant-in-aid no. 173905685001 from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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