Table 3 Salivary bacteria analyzed by T-RFLP

Fragment size (bases)	Group	Number of fragments (log_{10} copies/10 µl)		TRFMA database assignments
		Baseline	2 h	
162	Placebo	5.30±0.20	5.06±0.16	Not identified
	Test	5.40±0.16	5.16±0.13*	
203	Placebo	6.50±0.15	6.32±0.09	*Myxococcus coralloides*
	Test	6.44±0.20	6.25±0.16*	*Desulfobulbus* sp. oral clone CH031
				Corallococcus exiguous
				Corallococcus sp. SDU-2
249	Placebo	5.16±0.16	5.08±0.13	Not identified
	Test	5.36±0.17	5.12±0.14*	
257	Placebo	5.10±0.11	4.97±0.10	*Bifidobacterium* sp. oral strain A32ED
	Test	5.15±0.12	4.96±0.11*	*Leptotrichia* sp. oral clone EI022
262	Placebo	6.29±0.25	6.02±0.22*	*Flavobacterium*-like sp. oral clone AZ105
	Test	6.32±0.28	6.14±0.22	*Bifidobacterium* sp. oral strain H6-M4
				Bacteroides cf. *forsythus* oral clone BU063
				Prevotella sp. oral clone DO045
				TM7 phylum sp. oral clone FR058
265	Placebo	5.08±0.08	5.38±0.15	*Prevotella* spp. (10 phylotypes)
	Test	5.03±0.14	4.89±0.11**	*Porphyromonas*-like sp. oral clone DA065
				Streptococcus mitis
				Treponema spp. (5 phylotypes)
				Prevotella loescheii
				Eubacterium sp. oral clone BU014
				Clostridiales bacterium oral clone P4PA_66 P1
				Bacteroidales oral clone MCE7_20
				Bacteroides-like sp. oral clone X083
				Prevotella dentalis
				Desulfomicrobium orale
				Streptococcus sp. oral clone FP064
294	Placebo	5.31±0.20	5.29±0.19	*Oscillatoria corallinae*
	Test	5.45±0.18	5.27±0.15*	*Sphingomonas* sp. oral clone AW030
				Spiroplasma litorale
302	Placebo	5.00±0.09	4.87±0.08*	*Firmicutes* spp. (2 phylotypes)
	Test	4.98±0.08	4.96±0.09	*Eubacterium saburreum*
				Eubacterium saburreum-like sp. oral clone CK004
				Peptostreptococcus spp. (2 phylotypes)
				Eubacterium spp. (4 phylotypes)
				Human oral bacterium C73
				Lachnospiraceae oral clone MCE9_104
				Streptococcus mitis
306	Placebo	6.54±0.20	6.29±0.19	*Streptococcus mitis*
	Test	6.52±0.23	6.12±0.25*	*Streptococcus* spp. (8 phylotypes)
				Streptococcus oralis
				Streptococcus cristatus
				Streptococcus sanguinis
320	Placebo	5.37±0.17	5.32±0.13	*Streptococcus gordonii*
	Test	5.48±0.15	5.18±0.15*	*Streptococcus intermedius*
				Streptococcus constellatus
				Streptococcus mutans

Table 3 (continued)

Fragment size (bases)	Group	Number of fragments (log_{10} copies/10 μl)		TRFMA database assignments
		Baseline	2 h	
				Firmicutes oral clone CH017
				Filifactor alocis
				Clostridiales spp. (2 phylotypes)
323	Placebo	5.36±0.18	5.20±0.15	Not identified
	Test	5.51±0.13	5.26±0.12*	
329	Placebo	5.31±0.21	5.19±0.15	*Flexibacter litoralis*
	Test	5.33±0.18	5.14±0.14*	
574	Placebo	5.63±0.22	5.50±0.15	*Bacteroidales* oral clone MCE7_120
	Test	5.50±0.18	5.21±0.15*	*Porphyromonas*-like sp. oral clone DA064
913	Placebo	5.28±0.16	5.06±0.12*	*Tannerella forsythensis*
	Test	5.12±0.14	5.00±0.11	
915	Placebo	5.16±0.13	4.88±0.09*	*Tannerella forsythensis*
	Test	5.08±0.10	4.81±0.05*	*Bacteroidetes* sp. oral clone FX069
				Bacteroides-like sp. oral clone AU126
				Porphyromonas sp. oral clone HF001

T-RFs with significant intra- or inter-group differences are listed. Data are expressed as mean log_{10} copies/10 μl ± standard error. The detection limit was 4.75 log_{10} copies/10 μl for the statistical analysis.

*$P<0.05$ between baseline and 2 h; **$P<0.05$ between the test and placebo groups

whereas the concentration in the placebo group was above the threshold level during the entire experimental period. In previous reports, the H_2S concentration of 1.5 ng/10 ml was still at a detectable but not objectionable level, and that of 2.5 ng/10 ml was at a slightly objectionable level [18, 19, 30]. Our results indicate that the median concentrations of H_2S in the test group after 10 min until 2 h were not objectionable, whereas the concentration in the placebo group increased to the objectionable concentration at 2 h. The actual numbers of subjects who showed the lower concentration than the olfactory threshold in the test and placebo groups were 2 and 0 at baseline, 10 and 4 at 10 min, 8 and 4 at 1 h, and 8 and 5 at 2 h, respectively in CH_3SH concentration, and 1 and 1 at baseline, 7 and 3 at 10 min, 7 and 3 at 1 h, and 8 and 4 at 2 h, respectively in H_2S, although any statistical difference between the test and placebo groups was not found in both CH_3SH and H_2S by χ^2 test.

The suppressive effect of the test tablet on the concentration of VSCs in the mouth air was more clearly observed in the levels of CH_3SH compared to the levels of H_2S. The odor of CH_3SH has been found to be more objectionable with a lower threshold of objectionability compared to H_2S [19]. Anaerobic periodontopathic bacteria have been implicated in the production of VSCs, especially CH_3SH, in the oral cavity [31–33]. LF and LPO have an inhibitory effect on several species of periodontopathic bacteria in vitro [14, 15, 17, 34]. The suppressive effect of LF on the number of periodontopathic bacteria in the subgingival plaque was also demonstrated [16]. The in vitro experiments based on the method previously described by Shin et al. [17] showed that the test tablet reduced the number of oral bacteria such as *A. actinomycetemcomitans* by more than 4 log units after incubation for 5 min in the presence of SCN^- at a physiological concentration in saliva, whereas the placebo tablet exhibited no bactericidal activity (data not shown). It is likely that the ingestion of the test tablet suppressed metabolic activity or the living number of periodontopathic bacteria residing in the oral microbial community (microflora). LF and LPO have also been shown to inhibit acid production and the uptake of amino acids or other nutrients [12, 13, 35]. In the metabolic pathway that produces VSCs, the uptake of amino acids or peptides is an essential step in utilizing sulfur-containing substrates [2, 3, 36, 37]. LF and LPO inhibit the activity of protease, which is a key enzyme in the degradation of high molecular weight proteins into low molecular weight peptides that provide substrates for VSC production [38, 39].

T-RFLP has been applied to the analysis of microflora profiles in oral and extra-oral specimens from humans [24, 40–43]. T-RFLP is a rapid and effective molecular method for analyzing the profile of microflora in oral specimens, as information on the constituents of oral microflora has been accumulated by sequencing the clone library of bacterial 16S rRNA [44–46] and a useful database has been established from the genetic information [25]. We improved the T-RFLP profiling reported by Takeshita et al. [24], i.e., we used the new internal size standard GeneScan 1200 LIZ to improve the accuracies of the standard peaks. The base

size of each T-RF was estimated and searched for in the TRFMA database, which contains information of the theoretical T-RFs of oral bacteria using the same primer sets and restriction enzymes as those used in the current study. Because the total bacterial numbers obtained by quantitative PCR analysis were diverse among saliva samples, the copy numbers of T-RFs estimated from the percentage area of the fragments and the copy numbers of total bacteria obtained from the analysis of 16S rRNA were compared. In the T-RFLP analysis of the present study, the 265-base T-RF had a significantly lower copy number at 2 h in the test group compared to the placebo group. This fragment was assigned to bacterial species belonging to *Prevotella*, *Porphyromonas*, and *Treponema*, which demonstrate the ability to produce VSCs both in vitro and in vivo [31–33, 36, 37]. These results suggest that the ingestion of a test tablet influenced the number of oral bacteria, especially VSC-producing microorganisms, although neither culturing nor quantitative PCR results could demonstrate the effects of the test tablet on the number of oral pathogenic bacteria in saliva.

In this study, the placebo tablet showed some suppression of VSC production, although to an extent less than that of the test tablet. Since fatty acid esters of sucrose and glycerol, which were added as lubricants to the tablets, are known to have the properties of detergents with bacteriostatic activity [47, 48], the suppressive effect of the placebo tablet on oral malodor may be due to the actions of these components. Trisodium citrate dihydrate and citric acid were added as buffer salts to the test tablet to make the solvent a slightly acidic condition, which itself does not influence the number of living bacteria in vitro but provide LPO with optimal condition for showing its antibacterial activity [17]. There were also differences in the contents of sugar and sugar alcohols between the test and placebo tablets. Glucose was added in the test tablet as a substrate for GO. The test tablet contains erythritol, xylitol, and maltitol at lower percentage of total sugar alcohols compared to the placebo tablet, which contains maltitol only. These sugar alcohols are not utilized by mutans streptococci and classified as non-cariogenic [49]. Furthermore, sugar alcohols, such as xylitol and maltitol, added to chewing gum were demonstrated not to affect the production of VSCs in vivo [21]

In conclusion, the results of this clinical study indicate short-term effects on oral malodor when ingesting a test tablet containing LF and LPO. As antimicrobial effects of the tablet were found in vivo, further clinical studies for a longer experimental period of several months should be conducted to demonstrate the long-term effects of the test tablet.

Conflict of interest statement Four of the authors, K. Shin, K. Yamauchi, T. Toida, and K. Iwatsuki, are employees of Morinaga Milk Industry Co., Ltd. All other authors declare that they have no conflict of interest related to this study.

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Hydrogen Sulfide Causes Apoptosis in Human Pulp Stem Cells

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Abstract

Introduction: Untreated dental caries will eventually lead to pulpal inflammation. Although much is known regarding the interaction of microbial antigens and the immunologic defense systems of pulp, many aspects of the pathogenesis of pulpitis are not fully understood. The relationship between human pulp stem cells (HPSCs) and the pathogenesis of pulpitis remains among the poorly understood areas. Many of the invading bacteria are known to produce considerable amounts of hydrogen sulfide (H_2S), which causes apoptosis in some tissues. The aims of this study were to determine whether H_2S causes apoptosis in HPSCs and to examine its signaling pathway. **Methods:** Stem cells were isolated from human dental pulp and incubated with 50 ng/mL H_2S for 48 hours. To detect apoptosis, the cells were analyzed by using flow cytometry. The mitochondrial signaling pathway was examined by determining mitochondrial membrane depolarization. Activation of the key apoptotic enzymes caspase-9, caspase-8, and caspase-3 was assessed by using enzyme-linked immunosorbent assay. Release of cytochrome C from mitochondria was also determined. **Results:** The number of apoptotic cells increased significantly with H_2S treatment from 1.6% to 16.3% ($P < .01$). Significant increases were also measured in the amounts of caspase-9 and caspase-3 and in cytochrome C release (all $P < .01$) and in mitochondrial membrane depolarization ($P < .05$), whereas caspase-8 activity was not found. **Conclusions:** H_2S causes apoptosis in HPSCs by activating the mitochondrial pathway. It is suggested that H_2S might be one of the factors modifying the pathogenesis of pulpitis by causing loss of viability of HPSCs through apoptosis. *(J Endod 2011;37:479–484)*

Key Words

Apoptosis, dental pulp, hydrogen sulfide, pulpitis, stem cells

Pulpitis under dentin caries involves various cells and inflammatory mediators in initial pulpal immune responses (1–4). Previous reports have shown that interleukin-6 and interluekin-8 are present in lipopolysaccharide-stimulated pulp cells or inflamed pulp (5–9). Progression of pulpitis is facilitated by the immune system's response to bacteria, enhancing the production of inflammatory mediators (10). Severe inflammation in the pulp will eventually lead to pulpal necrosis (11). Infection and apoptosis are both believed to play an important role in the inflammatory process (12). Regarding the relationship between apoptosis and pulpitis, there are very few data on the occurrence of apoptosis in the cells of the dental pulp. It has been found that *Streptococcus mutans* lipoteichoic acid can induce apoptosis in pulp cells (13). Therefore, it is possible that other substances present in the disease process also can trigger apoptosis in the pulp.

The presence of hydrogen sulfide (H_2S) produced by bacteria has been demonstrated in infected root canals (14). Several studies have demonstrated that H_2S activates the apoptotic process in oral tissues (15–18). If H_2S causes apoptosis in pulp cells, it might affect the pathogenesis of pulpitis. Another situation in which dental pulp stem cells might be involved is proliferative pulpitis. Proliferation of dental pulp tissues might be caused not only by matured dental pulp cells but also by stem cells. During pulp inflammation, stem cells may start to differentiate to protect the tissue by using their ability to differentiate into a variety of cell types (19). If this process is suppressed by H_2S, differentiation might be replaced by tissue damages. The purposes of the present study were to verify that H_2S causes apoptosis in human pulp stem cells (HPSCs) and to examine its signaling pathway.

Materials and Methods

Primary Cultivation and Isolation of Stem Cells

All protocols of this study were reviewed and approved by the Research Ethics Board of Nippon Dental University and by the Research Ethics Committee of Tokyo Medical and Dental University. Informed consent was obtained from the subjects who agreed to participate voluntarily in this study. The pulp was removed by a sterile barbed broach after extraction of the deciduous teeth. The pulp tissue was then digested in a solution of 4 mg/mL collagenase type (Wako Pure Chemicals, Osaka, Japan). Cell suspensions of the pulp were seeded into 25-cm^2 flasks (TPP, Trasadingen, Switzerland) in Dulbecco modified Eagle medium (D-MEM) (Invitrogen GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, Ottawa, Ontario, Canada) and 1% antibiotic-antimycotic (Ab) (Invitrogen GIBCO) and incubated at 37°C in 5% carbon dioxide in air. Cell cultures were grown until they reached 90% confluence and then were passaged in 3:1 ratio. After culture,

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cells were trypsinized by 0.05% Trypsin-EDTA (Invitrogen GIBCO) and resuspended in phosphate-buffered saline (PBS) at a density of 1×10^8. Cells were labeled magnetically by using CD117 MicroBead Kit (Miltenyi Biotec Inc, Bergisch Gladbach, NRW, Germany). The cell suspension was loaded into a column placed in the magnetic field of a MACS Separator (Miltenyi Biotec Inc) (20). Unlabeled cells ran through the column and represented the CD117 negative (CD117 (–)) fraction, whereas the magnetically labeled cells, representing the CD117 positive (CD117(+)) cell fraction, were retained in the column. Ishkitiev et al (21) reported that the CD117(+) fraction was assumed to represent the stem cell population. Cells that had been passaged 4–17 times were used for the experiment. At every third or fourth passage, the cells were subjected to the fractionating process of separating CD117(+) cells as described above. CD117(+) HPSCs were plated in 25-cm^2 flasks at 3×10^5 cells density and allowed to attach overnight. Prepared samples were placed in an H_2S incubation system for apoptosis detection and pathway analysis.

Immunocytochemistry

Immunocytochemical staining was carried out for both CD117(–) and CD117(+) HPSCs immediately after magnetic separation. The cells were subcultured (3×10^4 cells/well) into 4-chamber slides (Nalge Nunc Int, Rochester, NY). The cells were fixed in 4% paraformaldehyde and then reacted with saturating levels of primary antibodies. The following antibodies were used to show stem cell markers: anti-Oct3/4, anti-CD44H, anti–cytokeratin19 (CK19) (R&D Systems, Minneapolis, MN), anti–alkaline phosphatase, anti-nestin, anti–cytokeratin18 (CK18) (AbCam, Cambridge, UK), anti-NANOG (Sigma-Aldrich, St Louis, MO), anti-secreted protein acidic and rich in cysteine (osteonectin), anti-p63 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), and anti-CD117 (Miltenyi Biotec Inc) (21). Goat anti-mouse Alexa fluor 568-conjugated secondary antibodies (Invitrogen GIBCO) were used. Negative controls for staining omitted the primary antibodies during the staining procedure. SybrGreen and DAPI (Invitrogen, Eugene, OR) were used for staining the nuclei.

H_2S Incubation

To produce H_2S, a permeator (PD-1B-2; Gastec, Kanagawa, Japan) and H_2S permeation tubes (Permeacal; Gastec) were used. The 25-cm^2 flasks containing HPSCs with D-MEM supplemented with 10% FBS and 1% Ab were placed inside the sealed chamber. The chamber was put in a 37°C incubator for 48 hours. Approximately 300 mL/min of 5% CO_2 air flowed into it constantly. The test cells were then exposed to H_2S at a concentration of 50 ng/mL in 5% CO_2. The H_2S concentration in the medium exposed to the above concentration was 18 ng/mL as measured by a gas chromatograph (Gas Chromatograph GC-8A; Shimadzu, Kyoto, Japan). This is similar to or lower than the concentration measured previously in infected root canals (14, 22). Negative control samples were subjected to an identical procedure but without H_2S. For a positive control, 30 μmol/L camptothecin (Sigma-Aldrich Japan, Tokyo, Japan) was used.

Cell Viability

Cell necrosis was determined by trypan blue staining. After 48-hour incubation with or without H_2S, the cells were trypsinized, centrifuged, and resuspended in PBS (1×10^5 cells/mL), diluted 1:100 in 0.25% trypan blue solution (Sigma-Aldrich), and counted in a hemocytometer. The percentage of viable cells in the total number of cells was calculated.

Apoptosis Detection

The percentage of apoptotic cells was determined by Guava Nexin Reagent (Guava Technologies, Hayward, CA) by using 2 fluorescent dyes, Annexin V-phycoerythrin conjugate (Annexin V-PE) and 7-aminoactinomycin D (7-AAD). Annexin V has high affinity for phosphatidylserine (PS), a membrane component normally localized to the internal face of the cell membrane. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane where Annexin V-PE can readily bind PS. The cell impermeant dye 7-AAD has a high DNA binding constant and is excluded from live, healthy cells as well as early apoptotic cells; however, late apoptotic or necrotic cells were detected. After incubation for 48 hours, with treatment with 50 ng/mL H_2S for the test group, the cells were trypsinized, centrifuged, and resuspended in the medium described above. Apoptosis was analyzed by employing 2000 cells and Guava EasyCyte (Guava Technologies).

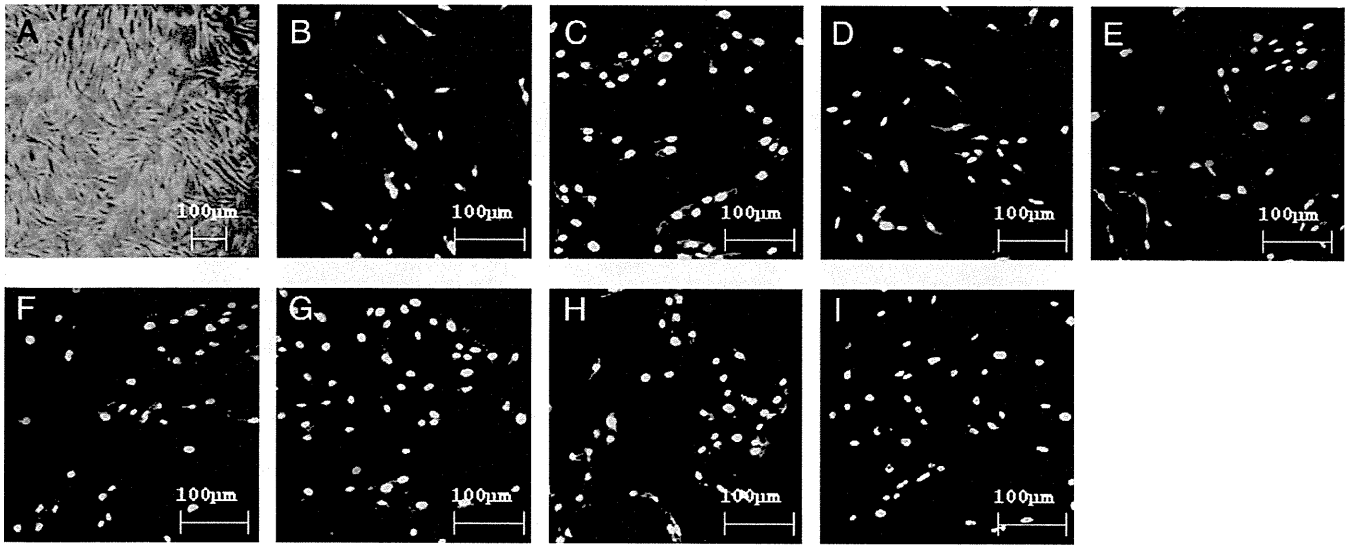

Figure 1. Photomicrograph and immunocytochemical staining of HPSCs. (*A*) Phase contrast microscopy; (*B*) CD117 (red); (*C*) CD44H (red); (*D*) alkaline phosphatase (red); (*E*) nestin (red); (*F*) osteonectin (red); (*G*) CK19 (red); (*H*) p63 (red); (*I*) CK18 (negative). Nuclei were stained by SyberGreen (yellow). Original magnification, ×10.

Measurement of Mitochondrial Membrane Potential

To assess changes in mitochondrial membrane potential, Guava EasyCyte Millipore MitoPotential kit (Guava Technologies) was used. Early stages of apoptosis often cause loss of the mitochondrial inner transmembrane potential. Guava EasyCyte Millipore MitoPotential kit includes 5,5′,6,6—tetrachloro-1,1′,3,3′-tetraethyl-benzamida-zolocarbocyanin iodide (JC-1) and 7-AAD. JC-1 is a cationic dye. In this assay, JC-1 fluoresced either green or red, depending on the mitochondrial membrane potential. The dye 7-AAD was used for observing cell membrane permeability associated with late apoptosis or cell death. After incubation for 48 hours, with treatment with 50 ng/mL H_2S for the test group, the cells were trypsinized, centrifuged, and resuspended in the medium described above at a concentration of 4×10^5 cells/mL. The cells were then stained with JC-1 and 7-AAD and incubated for 30 minutes at 37°C. Mitochondrial depolarized cells were analyzed by employing 2000 cells and Guava EasyCyte (Guava Technologies).

Released Cytochrome C Measurement

Cytochrome C released from the mitochondria to cytosol was determined. The assay human cytochrome C enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems, Vienna, Austria) was used. After incubation for 48 hours, with treatment with H_2S for the test group, the cells were trypsinized, centrifuged, and resuspended in lysis buffer at a concentration of 1.2×10^6 cells/mL according to the protocol. The amount of cytochrome C was determined by using a microplate reader (Bio-Rad Benchmark Plus; Bio-Rad Japan, Tokyo, Japan) set to 450 nm with a wavelength correction set to 620 nm.

Active Caspase-3 Detection

Active caspase-3 was measured by using Quantikine Human active Caspase-3 Immunoassay (R&D Systems). The assay uses biotin-ZVKD-fmk, biotinylated caspase inhibitor, to modify covalently the large subunit of caspase-3. After incubation for 48 hours, with treatment with H_2S for the test group, the inhibitor was added directly to the cell cultures treated with and without H_2S. The cells were trypsinized, centrifuged, and resuspended in the extraction buffer of the kit, which includes protease inhibitor. The cells were put in a microplate precoated with a monoclonal antibody specific for caspase-3. After the cultures were washed, streptavidin–horseradish peroxidase, which binds to the biotin on the inhibitor, was added to the wells. After the second washing, the substrate solution was also added to the wells. The amount of active caspase-3 is directly proportional to the amount of biotin-ZVKD-fmk–modified large subunit of caspase-3. The amount of active caspase-3 was determined by using a microplate reader set at 450 nm.

Caspase-9 and Caspase-8 Detection

Caspase-9 and caspase-8 were measured by using Human Caspase-9 ELISA and Human Caspase-8 ELISA, respectively (Bender MedSystems). Caspase-9 is the most upstream member of the apoptotic proteases and is activated by cytochrome C. Caspase-8 binds the death effector domain of Fas-associated death domain protein and is activated by death, including the signal complex. After incubation for 48 hours, with treatment with H_2S for the test group, the cells were trypsinized, centrifuged, and resuspended in lysis buffer at a concentration of 1.2×10^6 cells/mL. The amounts of caspase-9 and caspase-8 were determined by using a microplate reader set to 450 nm.

Statistical Analysis

The results are presented as the means ± standard deviation or the median (lower quartile – upper quartile). Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison or the Mann-Whitney *U* test. SPSS version 12.0 (SPSS Japan Inc, Tokyo, Japan) was used for analyses. Statistical significance was accepted at a P value of $<.05$.

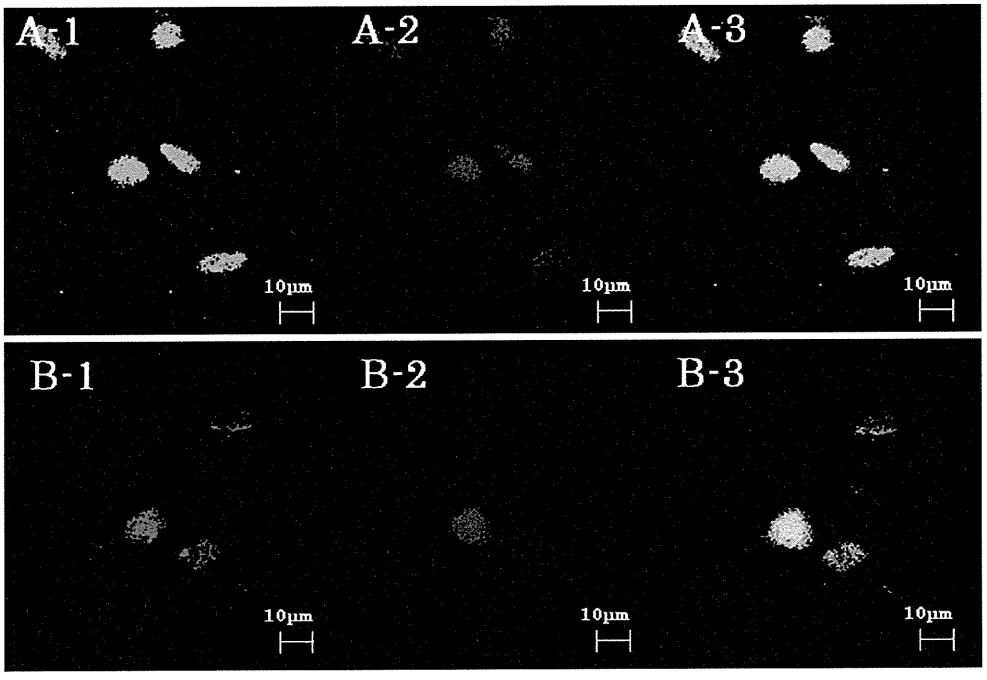

Figure 2. (*A-1*) Immunocytochemical staining of HPSCs for NANOG (green). (*A-2*) Nuclei were stained by DAPI (blue). (*A-3*) Overlay shows NANOG exists in nuclei. (*B-1*) Immunocytochemical staining of HPSCs for Oct (red). (*B-2*) Nuclei were stained by DAPI (blue). (*B-3*) Overlay shows Oct exists in nuclei.

Results

Stem Cell Markers

CD117(+) HPSC cultures retained spindle-shaped morphology (Fig. 1*A*). In immunocytochemical staining, CD117, CD44H, ALP, nestin, osteonectin, CK19, and p63 were stained red (Fig. 1*B–H*), whereas CK18, a marker for differentiated cells, was negative (Fig. 1*I*). NANOG was stained green (Fig. 2*A-1*). Oct was stained red (Fig. 2*B-1*). Overlays show that NANOG and Oct both exist in the nuclei (Fig. 2*A-3, B-3*). All HPSCs at passage 17 CD117(+) retained CD117 and other stem cell markers mentioned above (data not shown).

Cell Viability

The number of living cells was significantly lower in the H_2S group than in the control (61.3% ± 12.4% vs 90.0% ± 6.6%, n = 5; $P < .01$) and was also significantly lower in the positive control group than in the control group (43.2% ± 13.5% vs 90.0% ± 6.6%, n = 5; $P < .01$) (Fig. 3*A*).

Early Apoptosis, Late Apoptosis, and Necrosis

A significant difference in early apoptosis was found between the H_2S group and the control group (16.3% ± 1.9% vs 1.6% ± 0.3%, n = 5; $P < .01$) and also between the positive control group and the control group (36.1% ± 10.2% vs 1.6% ± 0.3%, n = 5; $P < .01$) (Fig. 3*B*).

In addition, late apoptosis and necrosis were significantly increased in the H_2S group compared with the control group (6.1% ± 2.1% vs 1.6% ± 1.0%, n = 5; $P < .01$) and also significantly increased in the positive control group compared with the control group (5.7% ± 1.7% vs 1.6% ± 1.0%, n = 5; $P < .01$) (Fig. 3*C*).

Mitochondrial Membrane Depolarization

The number of depolarized cells was significantly increased in the H_2S group compared with the control group (29.2% ± 8.0% vs 7.2% ± 1.5%, n = 5; $P < .05$) and was also significantly increased in the positive control group compared with the control group (67.2% ± 16.8% vs 7.2% ± 1.5%, n = 5; $P < .01$) (Fig. 3*D*).

Cytochrome C Release

Cytocrome C release was significantly increased in the H_2S group compared with the control group (0.8 [0.8–0.8] vs 0.1 [0.1–0.1] ng/mL, n = 5; $P < .01$; Fig. 4*A*).

Caspase-9, Caspase-3, and Caspase-8 Activity

The amount of caspase-9 was significantly higher in the H_2S group than in the control group (10.3 [10.2–10.3] vs 3.7 [3.6–3.7] ng/mL, n = 5; $P < .01$; Fig. 4*B*). Activated caspase-3 was also significantly higher in the H_2S group than in the control group (0.7 [0.6–0.7] vs 0.0 [0.0–0.0] ng/mL, n = 5; $P < .01$; Fig. 4*C*). However, the H_2S-exposed group showed the same amount of caspase-8 as the control group, and the level stayed low (0.0 [0.0–0.1] vs 0.0 [0.0–0.0] ng/mL, n = 5; Fig. 4*D*).

Discussion

Volatile sulfur compounds (VSCs) are produced by oral gram-negative anaerobic and microaerophilic bacteria (23, 24). H_2S is one of the components of VSCs. *Treponema denticola*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Capnocytophaga ochracea*, and *Peptostreptococcus anaerobius* are able to produce a high density of H_2S. *S. mutans* and *Lactobacillus minutus* are also able to produce H_2S (24). It is believed that VSCs are produced in periodontal

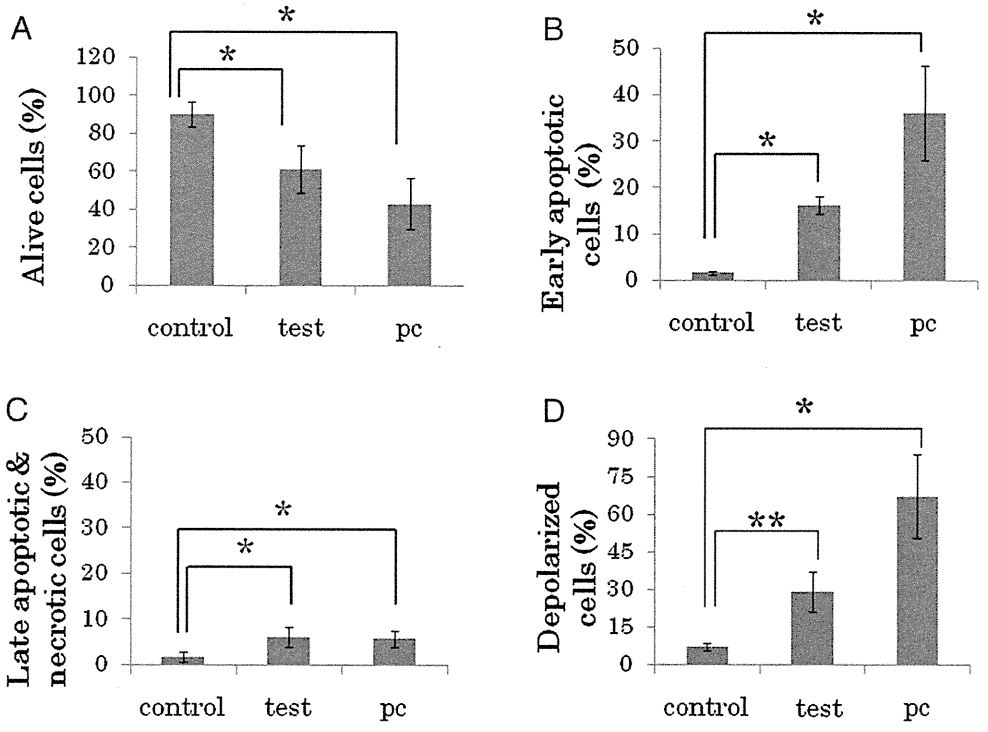

Figure 3. (*A*) Cell viability. After 48-hour exposure to 50 ng/mL H_2S, the percentage of live cells was significantly decreased in both the H_2S group and the positive control (pc) by using 30 μmol/L camptothecin (*$P < .01$, n = 5, Bonferroni multiple comparison). (*B*) Early apoptotic cells. After exposure to H_2S, the number of early apoptotic cells was significantly increased in both the H_2S group and the positive control (*$P < .01$, n = 5, Bonferroni multiple comparison). (*C*) Late apoptotic and necrotic cells. After exposure to H_2S, the number of late apoptotic and necrotic cells was significantly increased in both the H_2S group and the positive control (*$P < .01$, n = 5, Bonferroni multiple comparison). (*D*) Mitochondrial membrane potential. After exposure to H_2S, the number of depolarized cells was significantly increased in both the H_2S group and the positive control (*$P < .01$, **$P < .05$, n = 5, Bonferroni multiple comparison).

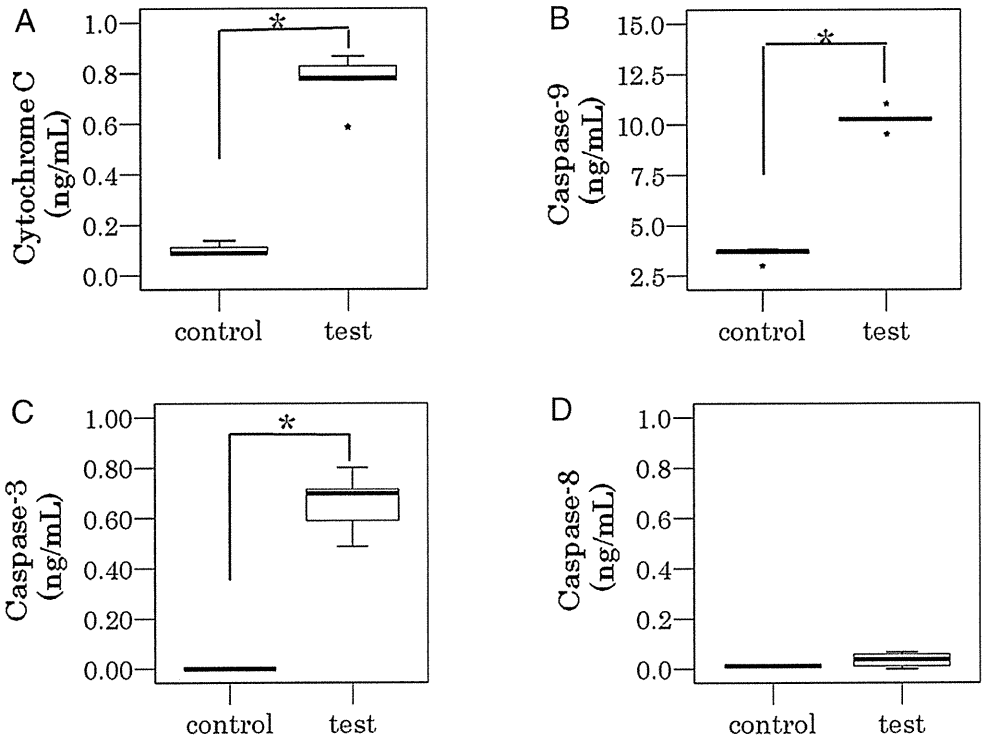

Figure 4. (*A*) Release of cytochrome C from mitochondria into cytosol was significantly increased by exposure to 50 ng/mL H_2S for 48 hours (*$P < .01$, n = 5, Mann-Whitney *U* test). (*B–D*) Activation of caspase-9, caspase-3, and caspase-8. Caspase-9 and caspase-3 levels were significantly increased by exposure to H_2S (*$P < .01$, respectively; n = 5, Mann-Whitney *U* test) (*B*, *C*). Caspase-8 activity remained low (*D*). The line in the box represents the median, the top of the box represents the lower quartile, and the bottom of the box represents the upper quartile.

pockets. However, Yaegaki and Sanada (25, 26) and Yaegaki et al (27) found that bacteria in the surface of the tongue produced 60% of the VSCs in mouth air. These same bacteria are detected in deep caries lesions and in the infected pulp, and it has been reported that bacteria will produce H_2S in the infected pulp (14, 24, 28).

In the present study, we demonstrated that H_2S induces apoptosis in HPSCs through a mitochondrial pathway. The immunocytochemical studies show that the magnetic separation worked satisfactorily. After H_2S incubation, trypan blue staining showed that the numbers of living cells were significantly lower in the H_2S group than in the control. The results indicate that H_2S induces cell necrosis in the HPSC population. However, 16% of the cells were found to be in the early apoptotic stage, whereas late apoptosis and necrosis levels were lower than 7%. These data indicate that H_2S mainly induced early apoptosis rather than necrosis during the 48-hour incubation.

Apoptosis involves 2 main signal pathways, a receptor ligand–mediated pathway and a mitochondrial pathway (29). Once later pathway is triggered, the mitochondrial inner transmembrane potential collapses, followed by the release of cytochrome C from the intermembrane space of mitochondria into the cytosol (30–32). We found that H_2S caused a significant increase in the percentage of depolarized cells and in the release of cytochrome C. Cytochrome C activates caspase-9 and initiates the apoptotic caspase cascade. We also found that H_2S caused a significant increase in caspase-9 and activated caspase-3. Caspase-3 is an effector caspase (33), which in turn is activated by each pathway, regulating the initial apoptotic process. Caspase-8, an initiator caspase activated by receptor ligand–mediated pathways (34), stayed at a low level in the H_2S group, with no significant difference when compared with the control. These results clearly indicate H_2S-induced apoptosis of HPSCs through a mitochondrial pathway. Thus, H_2S produced by bacteria might affect the pathogenesis of pulpitis.

Hyperplastic pulpitis is associated with a vital tooth, usually asymptomatic (35), and exhibits significant crown destruction. In theory, H_2S could also suppress the formation of hyperplastic pulpitis, because H_2S reduces the viability of HPSCs and induces apoptosis in HPSCs. On the other hand, in teeth with wide destruction of the crown, H_2S is likely to be washed away by saliva or diffuse into mouth air and could not have the same negative impact on HPSCs as in a closed situation.

Acknowledgments

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The authors deny any conflicts of interest related to this study.

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Combined Effects of Hydrogen Sulfide and Lipopolysaccharide on Osteoclast Differentiation in Rats

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Background: Lipopolysaccharide (LPS) stimulates osteoclast differentiation through toll-like receptors (TLRs) 2 and 4, and hydrogen sulfide (H_2S) induces osteoclast differentiation. If H_2S activates TLRs, H_2S may enhance the effects of LPS on osteoclast differentiation. The purpose of the present study is to examine the combined effects of sodium hydrogen sulfide (NaHS, an H_2S donor drug) and LPS on osteoclast differentiation and TLR expression in rat periodontal tissue.

Methods: Twenty-eight male Wistar rats (8 weeks old) were divided into four groups (n = 7 per group): a control (no treatment) group and three experimental groups (NaHS group, LPS group, and a combination [NaHS + LPS] group). At 1 day after topical application of NaHS and/or *Porphyromonas gingivalis* LPS into the gingival sulcus of first molars, the number of tartrate-resistant acid phosphate (TRAP)-positive osteoclasts in the periodontal tissue was counted. Expression of TLR2 and TLR4 mRNAs and proteins in the gingival was also assessed.

Results: The number of TRAP-positive osteoclasts was significantly higher in the combination group than in any other group ($P<0.01$). The combination group had 11.0-fold higher TLR4 mRNA levels than the control group. TLR4 protein levels were also higher in the combination group than in the NaHS or LPS group. However, the TLR2 mRNA and protein levels were not significantly different in the combination group and the LPS group.

Conclusion: In rat periodontal tissue, NaHS and LPS had an additive effect on osteoclast differentiation through activation of the TLR4 pathway but not the TLR2 pathway. *J Periodontol 2012;83:522-527.*

KEY WORDS

Hydrogen sulfide; lipopolysaccharides; osteoclasts; toll-like receptor 2; toll-like receptor 4; rats.

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Oral malodor is mainly attributed to volatile sulfur compounds (VSCs), such as hydrogen sulfide (H_2S), methyl mercaptan, and dimethyl sulfide.[1] The relationship between periodontitis and oral malodor is supported by a number of studies.[2,3] There are positive correlations between sulcular VSC levels and the degree of gingival inflammation and probing depth (PD).[4] VSCs are toxic to oral tissue even at low concentrations.[2,3] Together, these observations indicate that VSCs are pathogenic compounds that mediate periodontitis and are a causative factor of oral malodor.[4] In fact, we have found that topical application of H_2S increases the production of receptor activator of nuclear factor-κB ligand (RANKL) and induces osteoclast differentiation in a rat periodontitis model.[5]

Lipopolysaccharide (LPS) stimulates RANKL expression and osteoclast differentiation through toll-like receptors (TLRs) 2 and 4.[6] Periodontitis progression induced by LPS is augmented by protease.[7] Therefore, it is possible that molecules other than protease augment the detrimental effect of LPS through TLR expression. If H_2S activates TLRs, H_2S may enhance the LPS-induced osteoclast differentiation. However, how H_2S affects TLRs in the periodontal tissue is unknown.

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The purpose of the present study is to examine the combined effects of sodium hydrogen sulfide (NaHS, an H_2S donor drug) and LPS on osteoclast differentiation and TLR expression in rat periodontal tissue. Gingival levels of tumor necrosis factor-α (TNF-α) and RANKL were assessed as markers of inflammation and osteoclastic activity, respectively. Furthermore, TLRs are activated by high-mobility group box-1 (HMGB-1) signaling.[8] HMGB-1 is a nuclear protein involved in nucleosome stabilization and gene transcription, released from injured cells, and identified as a novel inflammatory cytokine.[8] Therefore, expression of HMGB-1 was also assessed to investigate a possible mechanism of NaHS-mediated effects on TLR expression and activity.

MATERIALS AND METHODS

Animals

Twenty-eight male Wistar rats (8 weeks old) were housed under standard conditions, and the room was maintained on a 12 hours light/12 hours dark cycle at temperatures of 23°C to 25°C. The experimental protocol was approved by the Animal Research Control Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences. Animals had access ad libitum to a powdered diet‡ and tap water.

Experimental Design

Rats were divided into four groups (n = 7 per group): a control group without any treatment and three experimental groups. The NaHS, LPS, and combination (NaHS + LPS) groups received topical application of 1.0 mM NaHS§ (0.5 μL, six times daily), 25 mg/mL LPS of *Porphyromonas gingivalis*‖ (0.5 μL, three times daily), or both NaHS and LPS (0.5 μL, six times daily; 0.5 μL, three times daily), respectively, into the gingival sulcus of both first maxillary molars. A micropipette was used to deliver the topical treatments.[5] The animals were sacrificed under general anesthesia 1 day after application of NaHS and/or LPS.

Histologic Analysis

Samples of the left maxillary were resected from each rat and were immediately fixed in 4% paraformaldehyde in 0.1 mol/L buffered-phosphate (pH 7.4). The fixed samples were decalcified with 10% tetrasodium–EDTA aqueous solution (pH 7.4) for 2 weeks at 4°C. The decalcified samples were embedded in paraffin after dehydration with ethanol (70%, 80%, 90%, and 100%) and immersion in xylene. The paraffin-embedded bucco-lingual 4-μm sections were stained with hematoxylin and eosin and subjected to other procedures as described below.

Immunohistochemistry

Immunohistochemical labeling of TNF-α, RANKL, and HMGB-1 were performed with a commercial kit.¶[9] The sections were treated consecutively with polyclonal antibodies for TNF-α (diluted 1:200),# RANKL (1:100),** or HMGB-1 (1:400)†† overnight at 4°C.[5,10,11] The sections were counterstained with Mayer's hematoxylin. Control sections included buffer alone or non-specific purified goat, rabbit, or mouse immunoglobulin G. After immunostaining for RANKL, tartrate-resistant acid phosphatase (TRAP) activity was measured using the azo dye method to identify osteoclastic cells.[12]

A single examiner (KI), who was masked to the treatment assignment, performed all histometric analyses using a light microscope. The number of TNF-α– and HMGB-1-positive cells in standard areas (0.1 × 0.1 mm each) adjacent to the alveolar bone surface within the periodontal ligament (five serial areas from the top of the alveolar bone crest) was determined.[13] TRAP-positive osteoclasts and RANKL-positive osteoblasts occurring along the whole edge of the bone surface were counted and reported as numbers per millimeters.[14]

Gene Expression Analyses

Total RNA was isolated from the gingival biopsy samples using a commercial reagent.‡‡ The isolated RNA was quantified by measurement of the absorbance at 260 nm, and its purity was determined by the 260/280 nm absorbance ratio. Only samples with a 260/280 ratio >1.8 were used in the procedure. Total RNA (2 μg) was reverse transcribed using reverse transcription§§ at 42°C for 30 minutes.[15] The resulting cDNA was diluted 10-fold with yeast RNA (10 μg/mL). Real-time polymerase chain reaction (PCR) was performed using a kit‖‖ and the PCR system,¶¶ for 50 cycles of 95°C for 15 seconds, the appropriate annealing temperature for 20 seconds, and 72°C for 20 seconds. Primers were designed as follows: TNF-α, 5′-AAATGGGCTCCCTCTCATCAGTTC-3′ and 3′-CAGCATCGTTTGGTGGTTCGTCT-5′;[16] RANKL, 5′-GCTCACCTCACCATCAATGCT-3′ and 3′-ATTTCAGTCAGACAGGAGAACCATGG-5′[5]; TLR2, 5′-TCTGAGTTCCGTGACATAGG-3′ and 3′-AGATGTAACGCAACAGATTC-5′; TLR 4, 5′-GTGAGCATTGATGATGAGTTCAG-3′ and 3′-CATCTAATGATTGATAAGGATT-5′;[17] and β-actin, 5′-TGTTGCCCTAGACTTCGAGCA -3′ and 5′-GGACCCAGGAAGGAAGGCT-

‡ Oriental Yeast, Tokyo, Japan.
§ Sigma-Aldrich, St. Louis, MO.
‖ Invitrogen, Carlsbad, CA.
¶ Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan.
# R & D Systems, Minneapolis, MN.
** Santa Cruz Biotechnology, Santa Cruz, CA.
†† LifeSpan Bioscience, Minneapolis, MN.
‡‡ Invitrogen.
§§ TaKaRa Bio, Shiga, Japan.
‖‖ Toyobo, Osaka, Japan.
¶¶ Roche Applied Science, Mannheim, Germany.

3′.[18] The mRNA levels were expressed in terms of the relative copy number ratio of TNF-α, RANKL, TLR2, or TLR4 to β-actin for each sample.

Western Blot Analysis of TLR2 and TLR4

Samples of total protein were extracted from gingival tissue samples using a detergent-based extraction buffer[##] containing a protease inhibitor cocktail.*** The tissue samples were macerated in buffer (20 μL/mg tissue) and centrifuged for 10 minutes at 11,700 × *g* at 4°C. Aliquots (20 μg protein) were mixed with sample buffer (125 mM Tris-HCl [pH 6.8] with 20% glycerol, 4% sulfate polyacrylamide gel, 0.04% bromophenol blue, and 10% 2-mercaptoethanol) and heated at 95°C for 3 minutes. The samples and controls along with molecular weight marker were loaded onto 10% polyacrylamide gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were separated over 30 minutes at 200 V. After gel electrophoresis, the separated proteins were electroblotted onto a 100% non-ionic surfactant-free nitrocellulose membrane (pore size: 0.2 μm)[†††] in a transfer buffer (0.3% Tris-HCl, 1.44% glycine) using 24 V for 60 minutes. The membrane was blocked with 5% (weight/volume) non-fat milk in 0.1% Tween-20 in Tris-buffered saline for 1 hour at room temperature. The membranes were probed with rabbit polyclonal antibody to TLR2 (1:20,000),[‡‡‡] goat polyclonal antibody to TLR4 (1:20,000),[§§§] and mouse monoclonal antibody to α-tubulin (1:30,000).[‖‖11,19] Membranes were washed in a washing buffer three times for 5 minutes each, incubated with secondary antibodies conjugated to horseradish peroxidase (1:50,000) for 1 hour at room temperature, and given a final wash in buffer. Subsequently, signals were visualized with a Western blotting analysis system.[¶¶¶] The α-tubulin was used as internal control.

Sample Size Calculation

The sample size was calculated using statistical software[###] based on the data of the number of RANKL-positive osteoblasts among control and NaHS groups in our preliminary study. A sample size of six per group was required for detection of a significant difference (80% power; two-sided 5% significance level).

Statistical Analyses

All data analysis was done using a statistical software package.**** Comparisons among the groups were made using the Mann-Whitney *U* test with the Bonferroni correction. The level of significance was set at $P<0.01$.

RESULTS

The NaHS, LPS, and combination groups showed 1.5, 2.1, and 3.9 times higher TNF-α mRNA levels, 8.8, 8.9, and 11.3 times higher expression of RANKL, and 6.8, 7.8, and 11.0 times higher expression of TLR4 compared to the control group, respectively (Fig. 1). However, the TLR2 mRNA levels in the NaHS group were similar (1.1 times higher) to those in the control group, and TLR2 mRNA levels were modestly increased in the LPS (1.8 times) and combination (1.7 times) groups relative to the control group (Fig. 1).

The numbers of TNF-α-positive and HMGB-1-positive cells and TRAP-positive osteoclasts in the combination group were significantly higher than those in the other groups ($P<0.01$) (Table 1). The number of TNF-α-positive cells was significantly higher in the LPS group than in the control group (Table 1). The numbers of RANKL-positive osteoblasts in the NaHS, LPS, and combination groups were significantly higher than the control group ($P<0.01$) (Table 1).

The signals from the TLR2 protein in the control and NaHS groups were very weak relative to those in the LPS and combination groups (Fig. 2). The signal from TLR4 in the combination group was the strongest among the four groups.

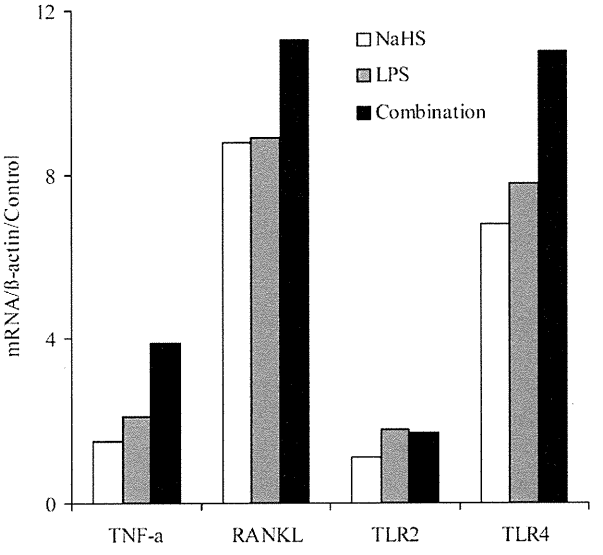

Figure 1.
Fold changes of gene expression in rat gingival tissue. Gene expression was expressed in terms of the copy number of TNF-α, RANKL, TLR2, or TLR 4 relative to β-actin in each sample.

Thermo Fisher Scientific, Rockford, IL.
*** Bio-Rad Laboratories, Hercules, CA.
††† Bio-Rad Laboratories.
‡‡‡ LifeSpan Bioscience.
§§§ Cell Signaling Technology, Beverly, MA.
‖‖ Sigma-Aldrich.
¶¶¶ Amersham ECL Plus Western Blotting Analysis System, GE Healthcare, Little Chalfont, UK.
nQuery Advisor, Statistical Solutions, Saugus, MA.
**** SPSS 15.0J for Windows, IBM Japan, Tokyo, Japan.

Table 1.

Numbers of TNF-α-Positive Cells, HMGB-1-Positive Cells, RANKL-Positive Osteoblasts, and TRAP-Positive Osteoclasts (mean ± SD)

Parameter	Control (n = 7)	NaHS (n = 7)	LPS (n = 7)	Combination (n = 7)
TNF-α-positive cells (number/0.1 × 0.1 mm)	2.23 ± 1.1	3.54 ± 0.6	4.43 ± 0.9*	6.61 ± 1.1*†‡
HMGB-1-positive cells (number/0.1 × 0.1 mm)	5.11 ± 0.9	7.99 ± 0.8*	8.40 ± 0.8*	10.9 ± 1.2 *†‡
RANKL-positive osteoblasts (number/mm)	8.99 ± 2.8	15.0 ± 2.6*	15.2 ± 2.4*	18.9 ± 2.2*
TRAP-positive osteoclasts (number/mm)	1.88 ± 1.0	3.33 ± 1.3	3.18 ± 1.3	5.76 ± 2.5*†‡

* $P<0.01$ compared to the control group (Bonferroni correction of the Mann-Whitney U test).
† $P<0.01$ compared to the NaHS group (Bonferroni correction of the Mann-Whitney U test).
‡ $P<0.01$ compared to the LPS group (Bonferroni correction of the Mann-Whitney U test).

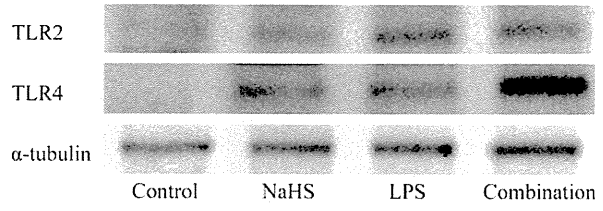

Figure 2.
Western blot analysis of TLR2 and TLR4 in rat gingival tissue. The signals from the TLR2 protein in the control and NaHS groups were weaker than the signals from the LPS and combination groups.

DISCUSSION

In the present study, the combination group (NaHS + LPS) shows the highest numbers of TRAP-positive osteoclasts, TNF-α-positive cells, and HMGB-1-positive cells among the four groups. Furthermore, mRNA and protein expression of TLR4 in the combination group seemed to be highest among the four groups. These results suggest that NaHS and LPS had additive effects on osteoclast differentiation in the rat periodontal tissue through a TLR4-dependent pathway. Previous studies have demonstrated that both the NaHS and LPS can individually stimulate osteoclast differentiation.[5,7,20] In the present study, the combination group (NaHS+LPS) shows the highest number of TRAP-positive osteoclasts among the four groups. These findings indicate that NaHS and LPS had additive effects on osteoclast differentiation in the periodontal tissue. Alveolar bone loss plays an important role in progression of periodontitis.[21] It is feasible that NaHS could enhance LPS-induced periodontitis.

Our results also reveal that the LPS group had more TNF-α-positive cells than the control group. There was no significant difference in the number of TNF-α-positive cells between the NaHS and control groups. Proinflammatory cytokines, such as TNF-α, can stimulate osteoclast differentiation.[22] LPS and H_2S lead to significant increase in production of TNF-α.[5,11,23,24] It is also possible that NaHS strengthened the effects of LPS on osteoclast differentiation by increasing TNF-α production in the periodontal tissue.

Activation of TLRs induces osteoclast differentiation.[25,26] Based on our findings, NaHS induced expression of TLR4 more than the control group. Furthermore, expression of the TLR4 gene was more upregulated relative to the control group in the combination group than in the NaHS or LPS group. In addition, protein expression of TLR4 seemed to be higher in the combination group than in either the NaHS or the LPS group. These results indicate that NaHS augmented the effect of LPS on osteoclast differentiation by activating the TLR4 pathway. In contrast, mRNA and protein expression of TLR2 in the combination group did not differ from that of the LPS group. NaHS may not affect activation of TLR2 in our model.

In this study, the levels of HMGB-1 protein are higher in the combination group than in the NaHS or the LPS group. HMGB-1 is a DNA-binding protein that is released from injured cells, and it has been shown to activate TLR4.[8] Therefore, the increased level of HMGB-1 may be involved in NaHS-mediated TLR4 activation.

The numbers of RANKL-positive osteoblasts were significantly higher in the NaHS, LPS, and combination groups than in the control group. Topical application of NaHS increases the production of RANKL in a rat periodontitis model.[5] LPS stimulates RANKL expression.[6] Our results are consistent with the previous studies.

The concentrations of H_2S in human feces and gingival fluid were 0.3 to 3.4 mM and ≤1.9 mM, respectively.[27,28] In our preliminary study, using

rats as an animal model, we confirmed that, among treatments ranging from 0.1 to 10 mM NaHS, application of 1 mM NaHS resulted in the highest inflammatory response in periodontal tissue (unpublished data). Therefore, we used a dose of 1 mM NaHS in this study.

P. gingivalis LPS was also applied at a dose of 25 μg/μL. This concentration was based on the result of our previous study using LPS from *Escherichia coli*.[9] However, assessing the dose–response relationship between *P. gingivalis* LPS and inflammation in periodontal tissue might be required for understanding the detailed effects of *P. gingivalis* LPS on the periodontal tissue. This is a limitation of our study.

Another limitation of this study is that the number of TNF-α-positive cells was evaluated within the periodontal ligament area, whereas the expression of TNF-α mRNA was investigated using gingival biopsy samples. Although the gingival samples for real-time PCR were not completely extracted from the same area in which the immunohistochemical analyses were performed, gingival samples included a part of periodontal ligament area. Thus, upregulation of TNF-α mRNA may reflect expression of a part of periodontal ligament area and support the data of increased TNF-α expression in the periodontal ligament area.

Clinical studies[28,29] demonstrated the elevated VSC levels in periodontally involved pockets. For instance, positive correlations are noted between the amounts of H_2S in the gingival crevice and the depth of periodontal pockets[28] and between gingival index, gingival crevicular fluid volume, and H_2S production.[29] It is also known that VSCs rise significantly with an increase in radiographic bone loss and are correlated with other clinical parameters, such as PD, clinical attachment level, and bleeding on probing.[4,30] These findings are in agreement with the current concept that H_2S could contribute to periodontitis progression.

CONCLUSIONS

NaHS and LPS had additive effects on osteoclast differentiation in the rat periodontal tissue. In addition, NaHS may augment LPS-induced osteoclast differentiation through a TLR4-dependent pathway but not a TLR2-dependent pathway.

ACKNOWLEDGMENT

The authors report no conflicts of interest related to this study.

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High-purity Hepatic Lineage Differentiated from Dental Pulp Stem Cells in Serum-free Medium

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Abstract

Introduction: We have previously differentiated hepatocyte like cells from deciduous tooth pulp stem and extracted third molar pulp stem cells with a protocol that used fetal bovine serum, but it showed high contaminations of nondifferentiated cells. Both the lower purity of hepatically differentiated cells and usage of serum are obstacles for application of cell therapy or regenerative medicine. Objective of this study was to investigate the capacity for and purity of hepatocyte-like differentiation of CD117-positive dental pulp stem cells without serum. **Methods:** Mesenchymal cells from human deciduous and extracted third molar pulp were isolated and expanded *in vitro*. We separated CD117-positive cells by using a magnetic-activated cell sorter. The cells were characterized immunofluorescently by using known stem cell markers. For hepatic differentiation, the media were supplemented with hepatic growth factor, insulin-transferrin-selenium-x, dexamethasone, and oncostatin M. Expression of hepatic markers alpha fetoprotein, albumin, hepatic nuclear factor-4 alpha, insulin-like growth factor-1, and carbamoyl phosphate synthetase was examined immunofluorescently after differentiation. The amount of differentiated cells was assessed by using flow cytometry. Glycogen storage and urea concentration in the medium were defined. **Results:** Both cell cultures demonstrated a number of cells positive for all tested hepatic markers after differentiation, ie, albumin-positive cells were almost 90% of differentiated deciduous pulp cells. The concentration of urea in the media increased significantly after differentiation. Significant amount of cytoplasmic glycogen storage was found in the cells. **Conclusions:** Without serum both cell types differentiated into high-purity hepatocyte-like cells. These cells offer a source for hepatocyte lineage differentiation for transplantation in the future. *(J Endod 2012;38:475–480)*

Key Words

Adult stem cells, dental pulp, hepatic differentiation, serum-free medium, stem cell pluripotency

We have recently differentiated a hepatic lineage of cells from human dental pulp cells (1), because dental pulp stem cells (DPSCs) are an easily accessed and convenient reserve of stem cells compared with bone marrow stem cells (BMSCs). In the study we found that the purity of hepatic differentiated cells was not to be high (1). An increase of the undifferentiated cells involving stem cells might be one of the concerns for future cell therapy or regenerative medicine because of the possibility of malignancy (2). Many previous studies have also characterized hepatic differentiation from stem cells by using hepatic marker expressions, sometimes at the mRNA level (3–6). Most studies with mesenchymal cells do not describe the purity of the cell lineage from stem cells. Jin et al (4) reported that 68% of the cells were differentiated into alpha fetoprotein/cytokeratin 18 (CK18) double-positive cells, but the cells were negative to albumin. The purity of cell lineage from the stromal stem cells in previous research might not be high. Moreover, even in lineage from human embryonic stem cells (hESCs), the purity of differentiated albumin-positive cells was only 67% (7), although higher purity was expected because of hESCs.

CD117 (proto-oncogene c-kit or stem cell growth factor receptor) is a surface marker present in stem cells from human exfoliating teeth (SHED) and adult human DPSCs from extracted third molars. CD117 has been found in many nondifferentiated cell types, including dental pulp cells, but it disappears when the cells undergo differentiation (8). We have previously reported that 50%–60% of SHED or DPSCs expressed CD117 (1). In recent years, dramatic improvement in magnetic cell separation techniques has made it more popular for isolating target cells compared with the use of a flow cytometer. Magnetic separation might not affect the integrity and scrupulousness of DNA in nuclei because fluorescent dye is not used (9, 10). This is a huge advantage to use magnetic separation for future regenerative medicine. Moreover, magnetic separation requires fewer cells than other separation methods and therefore can be applied to the small numbers of original cells residing in the dental pulp. Recently, we developed a protocol that uses CD117 antibody and magnetic separation techniques for separating stem cells from human dental pulp (11). Furthermore, the previous studies used a 10% fetal bovine serum (FBS)–complemented medium for the differentiation. The presence of animal products in the medium for differentiation also presents a huge obstacle for application of *in vitro* cell therapy methods in clinical practice. FBS has been advanced as a cause of delayed hypersensitivity reaction and to be a potential vector for prion transmission (12). Even autologous serum might

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cause unknown latent biological reactions during the differentiation process of stem cells. In regenerative medicine, we must lower the possibility of such an obstruction as much as possible. Therefore, we have recently developed a serum-free medium (SFM) for the differentiation of human DPSCs (13).

In this study we investigated a novel protocol for differentiating SHED and DPSCs into a high-purity hepatic lineage. We assessed the capacity of the CD117-positive cell fraction to proliferate in SFM. Furthermore, we determined the potential of the cultured SHED and DPSCs to differentiate into a hepatogenic lineage by using SFM during the differentiation process.

Materials and Methods

Primary Cultures from Human Tooth Pulp Cells

All protocols for this study were reviewed and approved by the Research Ethics Committee of Nippon Dental University. Deciduous and maxillary extracted third molars were supplied after their extraction in patients who received treatment at several collaborating dental clinics. Root formation was completed in all third molars. Informed consent was obtained from all subjects who voluntarily agreed to participate in this study. The pulp was accessed through the resorbed tooth root canal and extracted by a sterile barbed broach. Extracted third molar surfaces were cleaned and cut around the cementoenamel junction by using sterilized dental fissure burs and then cracked open to reveal the pulp chamber. The pulp tissue was gently separated from the crown and root. The pulp was then digested in a solution of 3 mg/mL collagenase type I (Wako Pure Chemicals, Osaka, Japan) for 1 hour at 37°C. Cell suspensions of deciduous or extracted third molar pulp were seeded into 25-cm^2 flasks (Iwaki, Tokyo, Japan) and cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Eugene, OR) supplemented with 10% FBS (HyClone, Logan, UT), 100 U/mL penicillin (Meiji, Tokyo, Japan), and 100 mg/mL kanamycin (Meiji). They were incubated at 37°C in 5% CO_2.

To obtain sufficient numbers of cells for magnetic separation, cells were grown in SFM for up to 4 passages. However, to obtain cell attachment, DMEM supplemented with 10% FBS was used only for the first day after every passage. On the second day after passage, the medium was replaced with SFM.

Isolation and Magnetic Separation of CD117-positive Cells

CD117 is a membrane receptor for the stem cell factor and is related to mesenchymal, endothelial, and endodermal lineage cells (8). The cells were incubated with mouse monoclonal human CD117 fluorescein isothiocyanate–conjugated antibody (Invitrogen). After excess antibodies were removed, the cells were further reacted with mouse anti-human CD117 immunoglobulin G conjugated with magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany); the cell suspension was then loaded into a column placed in the magnetic field of a mini-MACS Separator (Miltenyi Biotec Inc, Auburn, CA). The unlabeled cells ran through the column, whereas the magnetically labeled cells, representing the CD117-positive cell fraction, were retained in the column. After the column was removed from the magnetic field, the CD117-positive cell fraction was obtained.

Hepatogenic Differentiation

On the day after the separation when the cells attached to the surface, the serum-containing medium was replaced with serum-free DMEM supplemented with 1% insulin-transferrin-selenium-x (ITS-X) (Invitrogen) (13, 14) and 100 μg/mL of embryotrophic factor produced according to the method described by Ishiwata et al (15).

When the cells reached 70% confluence, differentiation agents were added to the SFM. Recombinant human hepatocyte growth factor (HGF) (R&D Systems Inc, Minneapolis, MN) 20 ng/mL was added for 5 days; then a mixture of 10 ng/mL oncostatin M (R&D Systems Inc) and 10 nmol/L dexamethasone (Waco Pure Chemical Industries Ltd, Osaka, Japan) was added for another 15 days. All the media were changed every third day.

Immunofluorescence

Magnetically separated cells were subcultured in 4-chamber slides (5000 cells/slide) (Nalge Nunc International, Naperville, IL) to determine the characteristics of the stem cells. After being fixed with 4% paraformaldehyde, the slides were labeled with the different primary antibodies, followed by Alexa Fluor 568-conjugated secondary antibodies (Invitrogen). The following antibodies produced in mouse were used: anti-CD44H, anti-cytokeratin 19 (R&D Systems); anti-alkaline phosphatase, anti-nestin (AbCam, Cambridge, MA); anti-Nanog (Sigma-Aldrich, St Louis, MO), anti-SPARC (secreted protein acidic and rich in cysteine, osteonectin); anti-p63, anti-STRO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-CD117 (Miltenyi Biotec, Bergich Gladbach, Germany). Anti-Oct3/4 (R&D Systems) produced in rat was also used. Different sample was stained with different antibody; one sample was stained with one antibody. As a secondary antibody, Alexa Fluor 568-conjugated donkey anti-mouse immunoglobulin G (Invitrogen) was used to detect mouse primary antibodies. The samples were washed 3 times with phosphate buffered saline (PBS) after each antibody layer, and the stained cells were observed under a confocal scanning laser fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

To determine whether the cells were differentiated into hepatic cells, the characteristic expression of hepatic phenotype markers was examined at mRNA levels or immunohistochemically (3). In this study, we determined the expression of the markers, and then the number of cells positive to each antibody was determined to identify the concentration of hepatic cells, because a higher concentration of the cells is expected to be required for successful future studies of transplantation. The following specific hepatic markers were used in both stem cells from human exfoliating teeth (SHED) and adult human DPSCs: anti-serum albumin; anti-alpha fetoprotein (αFP) (R&D Systems); anti-insulin-like growth factor I (IGF-I) (Raybiotech, Norcross, GA); anti-hepatic nuclear factor-4 alpha (HNF-4α) (Perseus Proteomics, Tokyo, Japan); and carbamoyl phosphate synthetase (CPS-1) (Santa Cruz Biotechnology) (16). Alexa fluor 568-conjugated antibodies (Invitrogen) were used as secondary antibodies.

Flow Cytometric Analysis

Cells were detached with 0.25% trypsin solution (Invitrogen), followed by incubation for 1 hour at room temperature with anti-albumin, anti-αFP, anti-HNF-4α, anti-IGF-I, and anti-CPS-1 antibodies (all at a concentration of 2 μg/mL). Nonlabeled cells were used as a negative control. The cells were analyzed with Guava EasyCyte flow cytometer (Guava Technologies, Billerica, MA).

Glycogen Storage

To determine glycogen storage capability, we used periodic acid–Schiff (PAS) staining. After 4% formaldehyde fixation, the slides were oxidized in 1% periodic acid for 5 minutes and rinsed in pure water. Samples were then treated with Schiff's reagent (Sigma) for 15 minutes, rinsed in deionized water for 5 minutes, and then assessed under a phase contrast light microscope (Nikon Corp, Tokyo, Japan) for glycogen accumulation.

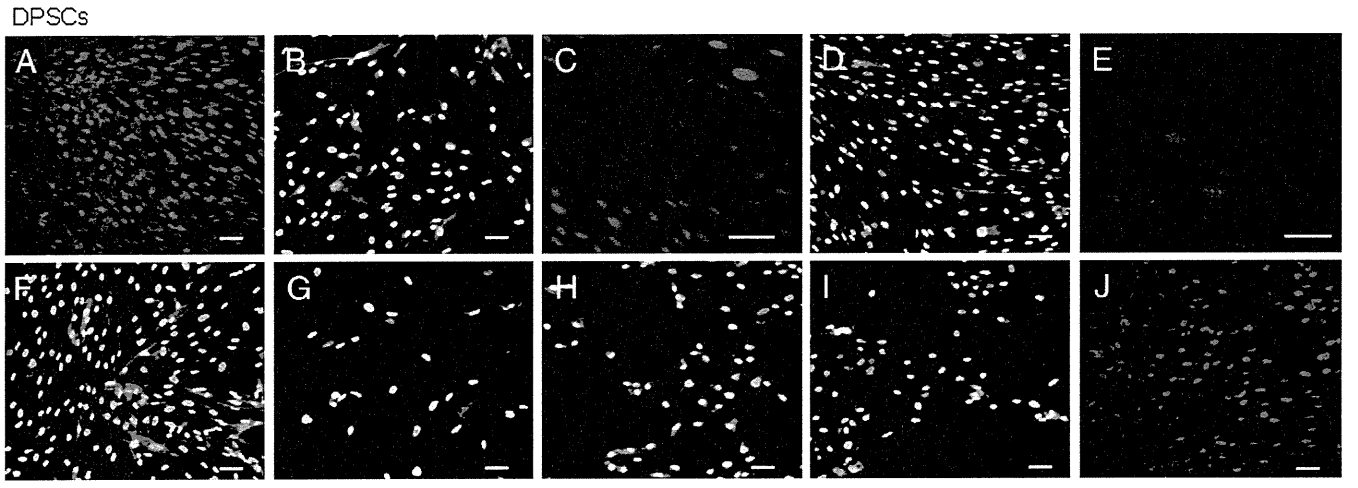

Figure 1. Immunocytochemical staining of DPSCs. (*A*) CD117, (*B*) CD44H, (*C*) Oct3/4, (*D*) nestin, (*E*) nanog, (*F*) osteonectin, (*G*) alkaline phosphatase, (*H*) CK19, (*I*) P63, (*J*) STRO-1, all stained red. Nuclei of the cells are stained with SybrGreen (yellow) except for (*A*) and (*J*) (stained blue with DAPI) and (*C*) and (*E*) (not stained). Scale bar = 100 μm.

Urea Assay

Urea concentrations were measured in the culture medium by using enzyme-linked immunosorbent assay (Quantichrom Urea assay kit; Bioassay Systems, Hayward, CA) according to the manufacturer's instructions (17), because urea cycle exists only in hepatocytes. Three days before the end of the differentiation protocol, the medium was replaced with fresh medium. Culture medium was collected on the last day of the protocol and analyzed colorimetrically. Fresh medium with additives was used as a negative control.

Statistics

Statistical analysis was performed by using Student's *t* test after angle transformations were carried out for the percentage data.

Results

Characteristics of CD117-positive Cells

Flow cytometry revealed that about 45% of SHED and 50% of DPSCs were positive for CD117 in this study (n = 5, respectively). After culturing the isolated SHED and DPSCs in SFM for 3–5 passages in DMEM supplemented with 10% FBS to acquire a sufficient quantity of cells (1×10^7 to 1×10^8), we separated the fraction of CD117-positive cells from the primary cultures by means of magnetic separation.

Immunocytochemistry revealed that all cells in this fraction were CD117-positive. After subculturing the isolated CD117-positive cells in 4-chamber glasses (NUNC), we characterized them for a panel of stem cell markers related to embryonic (nanog, oct3/4), mesenchymal (CD44H), endodermal (nestin, CK19), ectodermal (p63), and mesodermal (SPARC, alkaline phosphatase, STRO-1) cells, and we found both SHED and DPSCs positive for all of them (Figs. 1 and 2). Even after 70 population doublings by using CD117-positive magnetic separation every 4 passages, the CD117-positive fraction showed the same characteristics as found in Figure 1 (data not shown).

Hepatogenic Differentiation

As we have previously reported, clusters of oval-shaped cells appeared after the cultures were exposed to the differentiation media for a total of 22–28 days (1). In this study, the morphology of the cells

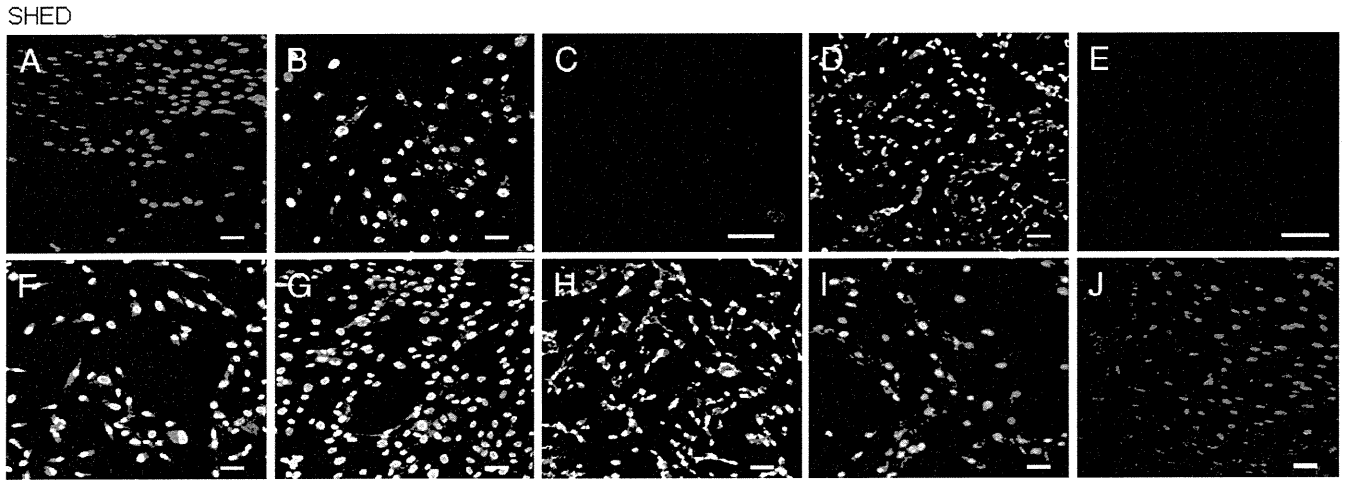

Figure 2. Immunocytochemical staining of SHED. (*A*) CD117, (*B*) CD44H, (*C*) Oct3/4, (*D*) nestin, (*E*) nanog, (*F*) osteonectin, (*G*) alkaline phosphatase, (*H*) CK19, (*I*) P63, (*J*) STRO-1, all stained red. Nuclei of the cells are stained with SybrGreen (yellow) except for (*A*) and (*J*) (stained blue with DAPI) and (*C*) and (*E*) (not stained). Scale bar = 100 μm.

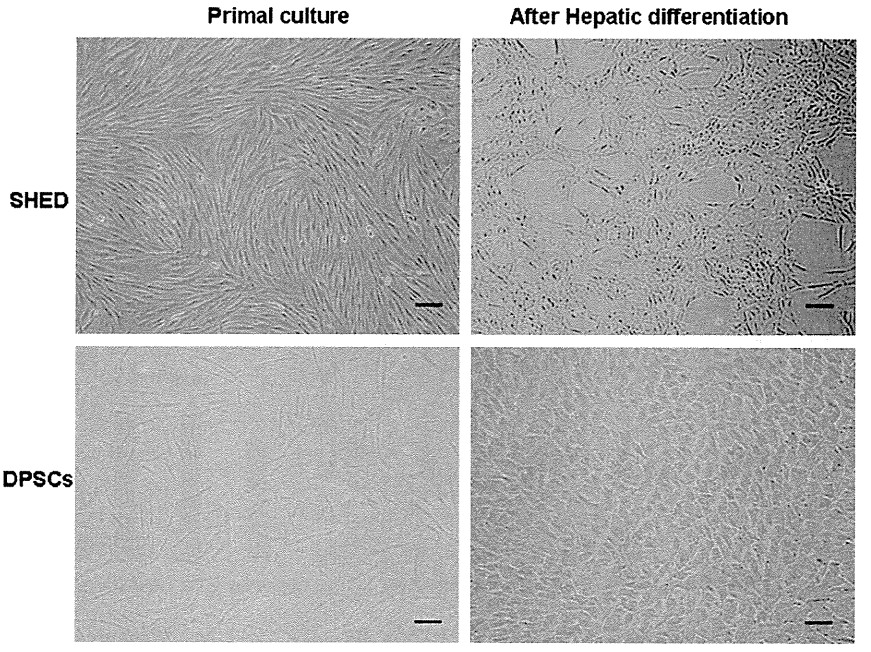

Figure 3. Cell shape of CD117-positive fractions of SHED and DPSC after hepatic induction. Shapes of cells from both cultures after hepatic induction changed from spindle-shaped and fibroblast-like to polygonal and oval-shaped. Polygonal cells were surrounded by small fraction of spindle-shaped cells after 20 days of differentiation. Scale bar = 100 μm.

in the clusters in both SHED and DPSC cultures after magnetic separation of the CD117-positive fraction also changed from spindle-shaped to polygonal; moreover, a significant number of cells changed their shape to polygonal (Fig. 3).

We found that all specific hepatic markers such as albumin, αFP, HNF-4α, IGF-I, and CPS-1 were positive in both SHED and DPSCs. Glycogen storage in the differentiated cells was found by PAS reaction (Fig. 4). The number of positive cells for hepatic markers was determined with flow cytometry, and nondifferentiated cells were used as a negative control. The high purity of the hepatic cells after the differentiation was confirmed in this study, as shown in Figure 5. The urea concentration in the disposed differentiation media was examined colorimetrically (n = 5). We found 3.45 $\pm$ 0.3 mg/L of urea in the medium disposed from DPSCs and 2.46 $\pm$ 0.22 mg/L urea in the

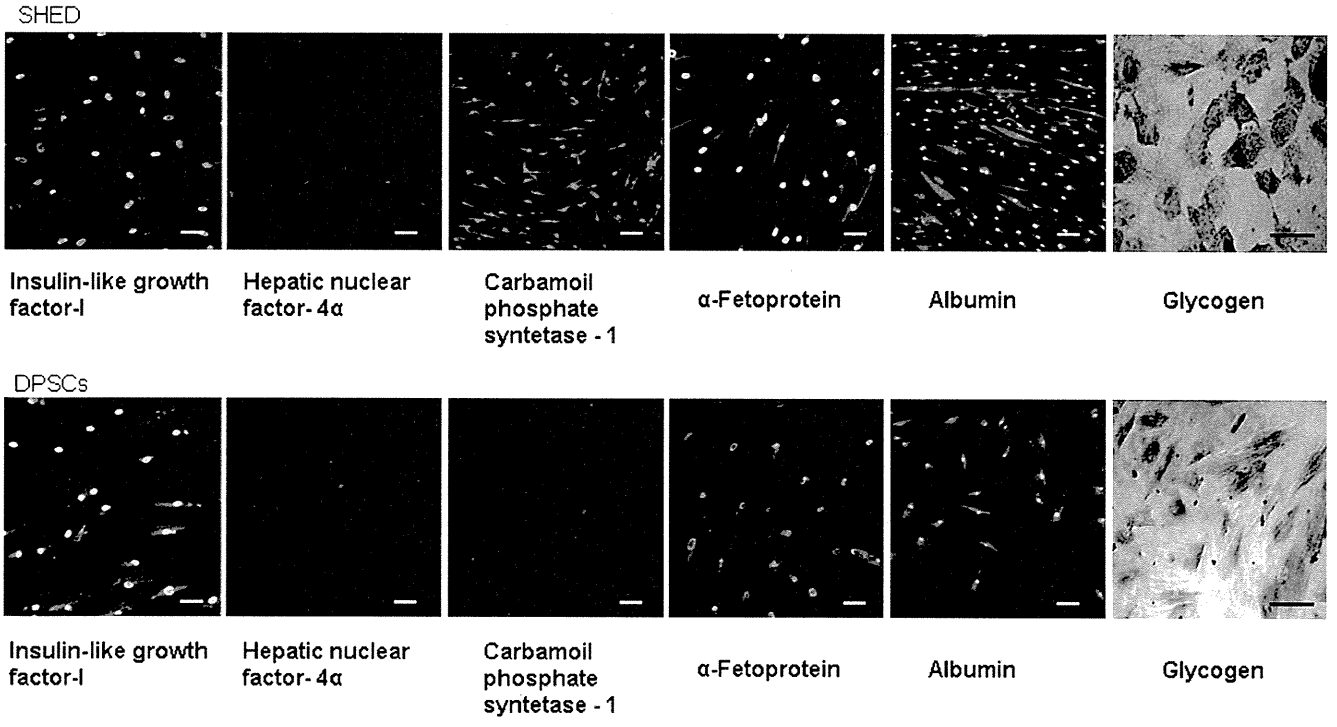

Figure 4. Immunofluorescent staining of hepatically differentiated cells. IGF-1, HNF-4α, CPS-1, α-FP, albumin (all red). Nuclei were counterstained yellow (Sybr-Green). PAS reaction showed cytoplasmic collections of glycogen after the differentiation period (pink). Scale bar = 100 μm.