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Invasion of human aortic endothelial cells by oral viridans group streptococci and induction of inflammatory cytokine production

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Keywords: cytokine production; endothelial cells; invasion; oral viridans group streptococci
Accepted 20 October 2010
DOI: 10.1111/j.2041-1014.2010.00597.x

SUMMARY

Oral viridans group streptococci are the major commensal bacteria of the supragingival oral biofilm and have been detected in human atheromatous plaque. Atherosclerosis involves an ongoing inflammatory response, reportedly involving chronic infection caused by multiple pathogens. The aim of this study was to examine the invasion of human aortic endothelial cells (HAECs) by oral viridans group streptococci and the subsequent cytokine production by viable invaded HAECs. The invasion of HAECs by bacteria was examined using antibiotic protection assays and was visualized by confocal scanning laser microscopy. The inhibitory effects of catalase and cytochalasin D on the invasion of HAECs were also examined. The production of cytokines by invaded or infected HAECs was determined using enzyme-linked immunosorbent assays, and a real-time polymerase chain reaction method was used to evaluate the expression of cytokine messenger RNA. The oral streptococci tested were capable of invading HAECs. The number of invasive bacteria increased with the length of the co-culture period. After a certain co-culture period, some organisms were cytotoxic to the HAECs. Catalase and cytochalasin D inhibited the invasion of HAECs by the organism. HAECs invaded by *Streptococcus mutans* Xc, *Streptococcus gordonii* DL1 (Challis), *Streptococcus gordonii* ATCC 10558 and *Streptococcus salivarius* ATCC 13419 produced more cytokine(s) (interleukin-6, interleukin-8, monocyte chemoattractant protein-1) than non-invaded HAECs. The HAECs invaded by *S. mutans* Xc produced the largest amounts of cytokines, and the messenger RNA expression of cytokines by invaded HAECs increased markedly compared with that by non-invaded HAECs. These results suggest that oral streptococci may participate in the pathogenesis of atherosclerosis.

INTRODUCTION

Oral viridans group streptococci are the major commensal bacteria of the supragingival oral biofilm, and many studies have found these organisms not only in the oral cavity but also in inflammatory tissues remote from the oral cavity. For example, *Streptococcus sanguinis* and *Streptococcus oralis* are detected frequently in infective endocarditis (Douglas *et al.*, 1993), and the *Streptococcus anginosus* group has been isolated from liver and brain abscesses (Wagner *et al.*, 2006; Ulivieri *et al.*, 2007). Also, *Streptococcus mitis*/*pneumoniae*, *S. oralis*, *Streptococcus gordonii*, *Streptococcus cristatus* and the *S. anginosus* group have been reported in pneumo-

nia patients (Bahrani-Mougeot *et al.*, 2007). These bacteria are thought to be the major causative organisms of these diseases.

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. It is one of the most important contributors to the development of cardiovascular disease and is the leading cause of death and illness in developed countries (Libby, 2002). Although atherosclerosis has been considered a bland lipid storage disease, many cases are not explained by traditional risk factors. Recent research has revealed that atherosclerosis actually involves an ongoing inflammatory response, and associations between human atherosclerosis and chronic infections with multiple pathogens have been reported (Epstein *et al.*, 2009).

Among the oral bacteria, viridans group streptococci have been detected in human atheromatous plaque. *Streptococcus sanguinis* and the periodontopathic bacterium *Porphyromonas gingivalis* were the first oral bacteria detected in a human carotid atheroma (Chiu, 1999). Subsequently, ribosomal DNA of the *S. mitis* and *Streptococcus salivarius* groups (Lehtiniemi *et al.*, 2005) and *Streptococcus mutans* (Kozarov *et al.*, 2006; Nakano *et al.*, 2006) were detected in atheromatous tissue. Furthermore, *S. sanguinis* has been shown experimentally to induce human platelet aggregation *in vitro* (Herzberg *et al.*, 1983), and *S. gordonii* and *S. mutans* have been demonstrated to be capable of inducing foam cell formation, an important characteristic of cardiovascular disease (Kuramitsu *et al.*, 2001). From these findings, it has been suggested that oral viridans group streptococci possess the potential to cause inflammation involving aortic endothelial cells, hence contributing to the process of atherogenesis. Some studies have investigated the interaction between oral viridans group streptococci and endothelial cells (Vernier *et al.*, 1996; Stinson *et al.*, 2003; Abranches *et al.*, 2009), but the invasion and subsequent activation of invaded endothelial cells by bacteria have not been clarified. The aim of this study was to investigate the ability of oral viridans group streptococci to invade human aortic endothelial cells (HAECs) and to evaluate the production of atherosclerosis-related pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-8 and monocyte chemoattractant protein-1 (MCP-1), by invaded HAECs.

METHODS

Bacterial strains

The following viridans streptococcal strains from our laboratory collection were examined: *S. gordonii* DL1 (Challis), *S. gordonii* ATCC 10558, *S. parasanguinis* ATCC 15911, *S. salivarius* ATCC 13419, *S. sanguinis* ATCC 10556, *S. mitis* ATCC 6249, *S. intermedius* ATCC 31412, *S. oralis* ATCC 35037, *S. anginosus* ATCC 33397 and *S. mutans* Xc (Koga *et al.*, 1989). The bacteria were grown in brain–heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD) broth under anaerobic conditions and were harvested in late-logarithmic growth.

Cell culture

Normal HAECs were purchased from Invitrogen Corporation (Carlsbad, CA) and maintained in Humedia-EG2 medium supplemented with low-serum growth supplement (Kurabo Industries Ltd, Osaka, Japan) at 37°C in 5% CO_2. Confluent fourth- to sixth-passage HAECs were used in all experiments.

Invasion of HAECs by oral viridans group streptococci

Antibiotic protection assays were used to examine the invasion of HAECs by oral viridans group streptococci based on the method of Stinson *et al.* (2003), with some modifications. The HAECs were plated in 48-well plates at a density of 2×10^4 cells per well 24 h before adding bacterial cells. The multiplicity of infection (MOI) for each bacterial species was calculated from the number of HAECs per well when seeded. Bacterial cells were collected by centrifugation at 6000 ***g*** 15 min, and then washed and suspended in Humedia-EG2 medium. The HAECs were mixed with 200 μl bacterial suspension at an MOI of 1, then incubated at 37°C in 5% CO_2 for 4, 8 or 24 h. The monolayers were washed, and externally adherent bacteria were killed by incubating the infected HAECs with 400 μl medium containing 200 μg ml^{-1} gentamicin and 20 μg ml^{-1} penicillin G for 1 h. Preliminary experiments showed that these antibiotic concentrations were sufficient to kill 2×10^8 streptococci ml^{-1} in 1 h. All experiments included controls (no HAECs) in which antibiotics were used

to verify the killing of each bacterial species. After exposure to antibiotics, the monolayers were treated with 100 μl trypsin–EDTA (Kurabo Industries) for 5 min at room temperature. Then, 400 μl of 0.1% Tween-20 was added, and the well contents were incubated at 37°C for 20 min. The lysates were pipetted vigorously and plated on BHI agar plates. After the plates had been incubated anaerobically at 37°C for 2 days, the colony-forming units (CFUs) of invasive bacteria were counted. The morphology and viability of HAECs were monitored by microscopic observation and Trypan blue exclusion assay (Higashi *et al.*, 2007) throughout the experiments.

Next, the ability of streptococcal cells to survive after invading HAECs was examined. Invaded HAECs were prepared as described above (time-point 0 h). Then, HAECs invaded by bacteria were incubated in 200 μl medium containing 50 μg gentamicin ml^{-1} for 24 h (time-point 24 h). At both time-points, the monolayers were treated with trypsin–EDTA and Tween-20, and invasive bacteria were enumerated as CFUs on BHI agar plates. The survival rate of invasive bacteria at the 24-h point was expressed as a percentage of the initial bacterial number recovered at the outset (0 h).

Determination of bacterial invasion by confocal scanning laser microscopy

To confirm the invasion of bacteria, a double-fluorescence technique was used according to the method of Inagaki *et al.* (2006) with modifications. Briefly, HAECs were grown in 35-mm collagen type I-coated glass-bottomed culture dishes (Asahi Glass Co. Ltd, Tokyo, Japan) and infected with *S. mutans* Xc or *S. gordonii* ATCC 10558 under the conditions described above. Cells were fixed with 3% paraformaldehyde (Sigma-Aldrich, St Louis, MO) for 10 min, washed with phosphate-buffered saline (PBS), and then incubated with a rabbit anti-*S. mutans* PAc serum (Oho *et al.*, 1999) diluted 1 : 500 with PBS–0.5% bovine serum albumin for 60 min at room temperature. In preliminary experiments, we confirmed that the rabbit anti-*S. mutans* PAc serum detected both *S. mutans* Xc and *S. gordonii* ATCC 10558 cells in a dot-blot assay. Following incubation, culture dishes were washed three times with PBS and incubated with Alexa Fluor 633-conjugated goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR) diluted 1 : 500 with PBS–0.5% bovine serum albumin for 30 min at room temperature to visualize attached bacteria. Invasive bacteria were then stained by first permeabilizing HAECs by dipping culture dishes in 0.4% Triton X-100 solution for 5 min, and then staining with the rabbit anti-*S. mutans* PAc serum followed by Alexa Fluor 555-conjugated goat anti-rabbit immunoglobulin G (Molecular Probes) diluted 1 : 500 as described above. Actin filaments were stained with Alexa Fluor 488 conjugated to phalloidin (Molecular Probes) for 30 min at room temperature to visualize the cellular cytoskeleton. Culture dishes were examined by confocal scanning laser microscopy (CSLM) using a TCS-SP5 microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a DMI6000 B fluorescence microscope (Leica) and a 63× oil immersion objective.

Invasion inhibition studies

The effect of hydrogen peroxide, which is produced by viridans group streptococci, on the ability of bacteria to invade HAECs was examined. To inhibit the activity of hydrogen peroxide produced by bacteria, bovine liver catalase (Sigma-Aldrich) was added to the medium (10,000 U ml^{-1}) in the antibiotic protection assay. Next, the effect of cytochalasin D on the actin polymerization of HAECs was examined. Various amounts of cytochalasin D (Sigma-Aldrich) were added to the medium (0–0.25 μg ml^{-1}) 30 min before co-culture with bacteria and pre-incubated with the HAEC monolayers. In preliminary experiments, we examined the adverse effects of cytochalasin D and catalase on HAECs and bacteria. The viability of HAECs and bacteria was monitored by the Trypan blue exclusion assay and by CFU enumeration on BHI agar plates, respectively. We confirmed that bovine liver catalase and cytochalasin D had no adverse effect on HAECs or bacteria at the concentrations used.

Cytokine production by HAECs

Cytokine production was analysed both for invaded HAECs and for HAECs infected by bacteria. Invaded HAECs possessed only intracellular bacteria; infected HAECs coexisted with extracellular bacteria in the co-culture process. To examine invaded HAECs, cells were co-cultured with each bacterial

strain other than *S. gordonii* DL1 (Challis) at an MOI of 1 for 8 or 24 h. For *S. gordonii* DL1 (Challis), HAECs were co-cultured at an MOI of 1000 for 1 h. After exposure to antibiotics for 1 h, invaded HAECs were incubated in 200 μl fresh Humedia-EG2 medium containing 50 μg gentamicin ml^{-1} for 2, 8 or 24 h. The supernatants were collected and filtered through 0.2-μm pore size filters. To examine the infected HAECs, cells were co-cultured with each bacterial strain at an MOI of 1 for 8 or 24 h. The supernatants were collected and processed similarly. They were stored at −80°C until the cytokine assay was performed. Simultaneously, the cells were harvested and total RNA was extracted from the collected HAECs for real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis using the RNeasy mini kit (Qiagen K.K., Tokyo, Japan).

Cytokine assay

Concentrations of IL-6, IL-8 and MCP-1 in the supernatants from invaded or infected HAEC cultures were analysed using enzyme-linked immunosorbent assay (ELISA) kits (Peprotec, London, UK for IL-8 and MCP-1; Bender MedSystems, Vienna, Austria for IL-6) following the manufacturer's instructions. Unstimulated HAECs were used as controls.

Real-time RT-PCR analysis

For quantitative analysis of messenger RNA (mRNA) expression by the invaded HAECs, total RNA (0.5 μg) from each sample was used to generate complementary DNA (cDNA; High-Capacity cDNA Reverse Transcription kits; Applied Biosystems, Foster City, CA) in a 50-μl volume. Then, 1 μl of the cDNA obtained was used for each subsequent reaction. The samples were amplified using the StepOne™ Real-time PCR System (Applied Biosystems) with 2× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) for IL-6, IL-8 and MCP-1. The amplification consisted of an initial denaturation at 95°C for 20 s, followed by 40 cycles at 95°C for 1 s, and 60°C for 20 s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified in parallel with the gene of interest. Relative changes in gene expression were analysed using the comparative threshold cycle method (Livak & Schmittgen, 2001) and reported as the difference (*n*-fold) relative to the value for a calibrator cDNA (control, non-invaded HAECs) prepared in parallel with the experimental cDNAs.

Statistical analysis

Differences between the control and test samples in the number of invasive bacteria or cytokine production were determined using Student's *t*-test or one-way analysis of variance followed by Dunnett's test.

RESULTS

Invasion of HAECs by oral viridans group streptococci

At an MOI of 1 and a co-culture period of 4 h, *S. salivarius* ATCC 13419 showed the highest invasion efficiency for HAECs among the strains tested (Table 1). *Streptococcus gordonii* DL1 (Challis) and *S. mutans* Xc both showed relatively high invasion efficiencies. The other streptococcal strains, except *S. intermedius* ATCC 31412, showed low invasion efficiencies. The number of invasive bacteria increased with the length of the co-culture period. However, *S. gordonii* DL1 (Challis) induced morphological changes (irregular or round shape different from normal appearance) on HAECs, indicative of cellular damage induced by bacterial virulence, when the co-culture period reached 8 h, and *S. gordonii* ATCC 10558, *S. salivarius* ATCC 13419, *S. intermedius* ATCC 31412 and *S. anginosus* ATCC 33397 induced morphological changes on HAECs when the co-culture period reached 24 h. *Streptococcus mutans* Xc, *S. parasanguinis* ATCC 15911, *S. sanguinis* ATCC 10556, *S. mitis* ATCC 6249 and *S. oralis* ATCC 35037 showed very high invasion efficiencies for HAECs when the co-culture period was 24 h.

Following dual labeling of extracellular and intracellular bacteria, CSLM was performed to access the attachment and invasion of bacteria in HAECs. Figure 1 shows HAEC invasion by *S. mutans* Xc or *S. gordonii* ATCC 10558 after co-culture with bacteria at an MOI of 1. Intracellular bacteria are stained red, and the HAEC skeleton is stained green; because extracellular bacteria are stained blue and red, they appear purple. Bacterial invasion into HAECs was observed at every time-point of the co-culture period.

Table 1 Invasion of human aortic endothelial cells by oral viridans group streptococci[1]

Bacterial strain	Invasion after indicated co-culture period[2]		
	4 h	8 h	24 h
S. gordonii DL1 (Challis)	134 ± 30	nd	nd
S. gordonii ATCC 10558	2 ± 2	126 ± 26	nd
S. mutans Xc	22 ± 6	2776 ± 164	12,084 ± 4184
S. parasanguinis ATCC 15911	6 ± 2	194 ± 48	40,916 ± 4784
S. salivarius ATCC 13419	1004 ± 142	8266 ± 270	nd
S. sanguinis ATCC 10556	4 ± 4	50 ± 14	17,416 ± 2362
S. mitis ATCC 6249	6 ± 2	144 ± 38	24,416 ± 2754
S. intermedius ATCC 31412	0	30 ± 8	nd
S. oralis ATCC 35037	8 ± 4	154 ± 18	27,166 ± 4720
S. anginosus ATCC 33397	8 ± 6	274 ± 18	nd

[1]Human aortic endothelial cells were allowed to interact with bacteria at a multiplicity of infection of 1.
[2]Invasion is expressed as number of invasive bacteria (colony-forming units per well). The values are the means ± standard deviations of triplicate assays.
nd, not determined.

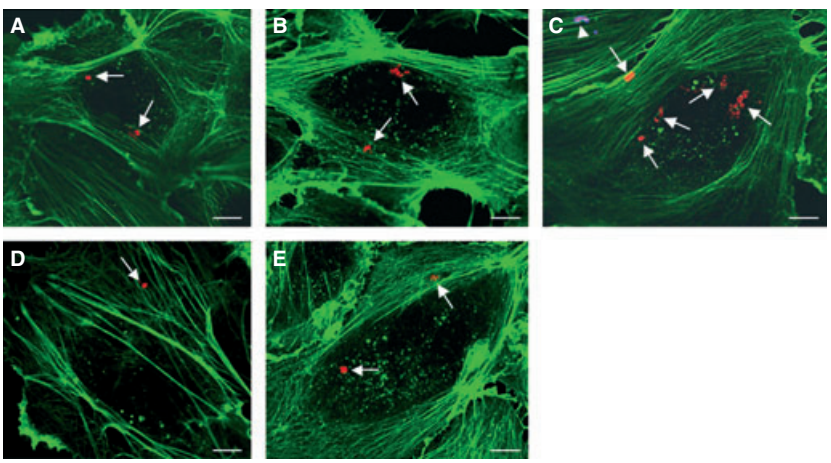

Figure 1 Invasion of bacterial cells into human aortic endothelial cells (HAECs) visualized by confocal scanning laser microscopy following dual labelling. The HAECs were co-cultured with bacteria at a multiplicity of infection of 1 for 4, 8 or 24 h. Bacterial cells invading HAECs are stained red (indicated by arrows), whereas extracellular bacteria are stained purple (indicated by an arrow head). (A) *Streptococcus mutans* Xc, 4 h; (B) *S. mutans* Xc, 8 h; (C) *S. mutans* Xc, 24 h; (D) *Streptococcus gordonii* ATCC 10558, 4 h; (E) *S. gordonii* ATCC 10558, 8 h. Bar represents 10 μm.

Next, the survival of invasive bacteria in HAECs was examined. For the preparation of invaded HAECs, we chose the conditions in which the highest number of invasive bacteria was recovered in an invasion assay for each strain. The numbers of *S. salivarius* ATCC 13419 and *S. mutans* Xc at the 24-h point decreased significantly compared with the numbers at the outset, and their survival rates were 9.5 ± 4.3 and 4.2 ± 0.7%, respectively ($n = 3$). No significant change in the numbers of other strains was observed (data not shown).

Inhibition of bacterial invasion of HAECs

Streptococcus gordonii DL1 (Challis) was used as a representative strain because it produced the strongest cytotoxicity on HAECs among the strains used in the invasion assay. We examined the effects of bovine liver catalase and cytochalasin D on the invasion of HAECs by *S. gordonii* DL1 (Challis) at an MOI of 1000 and a co-culture period of 1 h. In this condition, HAECs were viable and the number of invasive bacteria was the highest (947 ± 129 CFU well^{-1}) in

preliminary experiments. Addition of bovine liver catalase (10,000 U ml^{-1}) inhibited the bacterial invasion of HAECs by 73.4 ± 5.7% compared with the no catalase control (n = 3). Cytochalasin D inhibited bacterial invasion of HAECs in a dose-dependent manner (Fig. 2). The invasive bacteria were inhibited by 98.2 ± 0.5% at a cytochalasin D concentration of 0.125 μg ml^{-1} compared with the no cytochalasin D control.

Cytokine production by invaded or infected HAECs

Pro-inflammatory cytokine production by the invaded or infected HAECs was examined using ELISA. When non-invasive bacteria were killed after an 8-h co-culture period and invaded HAECs were further cultured for 24 h, we observed increases in production of IL-6, IL-8 and MCP-1 from HAECs invaded by *S. mutans* Xc, *S. gordonii* ATCC 10558 and *S. salivarius* ATCC 13419 compared with non-invaded HAECs (Fig. 3). Furthermore, when non-invasive bacteria were killed after a 24-h co-culture period and invaded HAECs were further cultured for 24 h, large increases in the production of IL-6, IL-8 and MCP-1 were observed in HAECs invaded by *S. mutans* Xc. The HAECs invaded by other strains, such as *S. parasanguinis* ATCC 15911, *S. sanguinis* ATCC 10556, *S. mitis* ATCC 6249 and *S. oralis* ATCC 35037, showed low production of cytokines, similar to that in non-invaded HAECs. Because *S. gordonii* DL1 (Challis) induced morphological changes of HAECs at an MOI of 1 and a co-culture period of 8 h, the

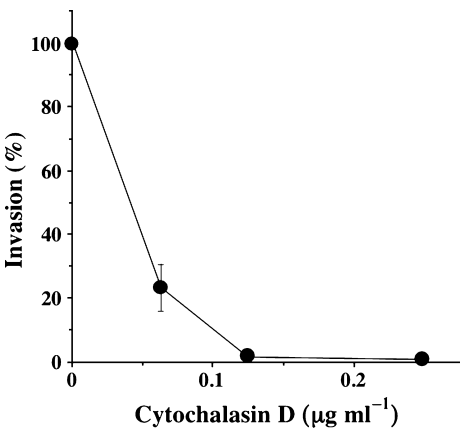

Figure 2 Effect of cytochalasin D on *Streptococcus gordonii* DL1 (Callis) invasion of human aortic endothelial cells (HAECs). Monolayers were co-cultured with *S. gordonii* DL1 at a multiplicity of infection of 1000 for 1 h with cytochalasin D. Percentage invasion is relative to the invasion efficiency with dimethyl sulfoxide (control; 100%). Values represent the means ± standard deviations of triplicate assays.

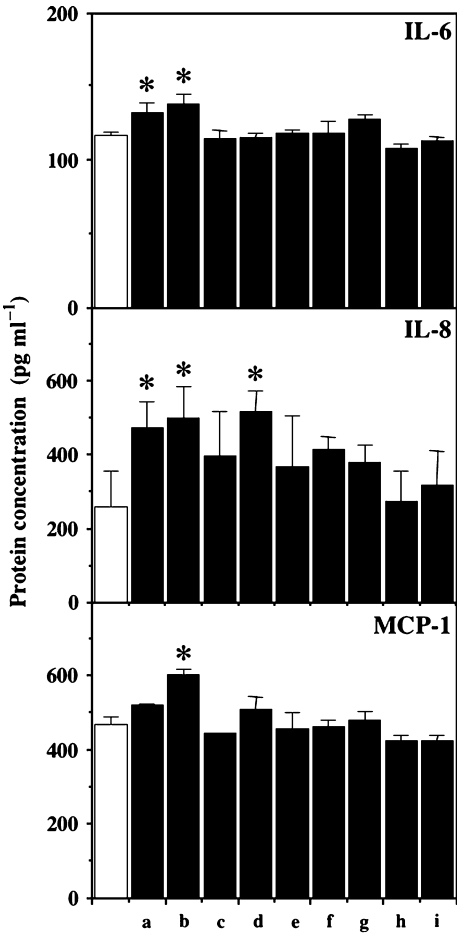

Figure 3 Cytokine production from human aortic endothelial cells (HAECs) invaded by oral viridans group streptococcal strains. Monolayers were co-cultured with each strain at a multiplicity of infection of 1 for 8 h. After killing the non-invasive bacteria using antibiotics, HAECs invaded by bacteria were incubated in medium for 24 h, and the concentrations of cytokines in culture supernatants were determined by enzyme-linked immunosorbent assay (IL, interleukin; MCP-1, monocyte chemoattractant protein 1). Open bar, non-invaded HAECs (control); solid bars, HAECs invaded by *Streptococcus gordonii* ATCC 10558 (a), *Streptococcus mutans* Xc (b), *Streptococcus parasanguinis* ATCC 15911 (c), *Streptococcus salivarius* ATCC 13419 (d), *Streptococcus sanguinis* ATCC 10556 (e), *Streptococcus mitis* ATCC 6249 (f), *Streptococcus intermedius* ATCC 31412 (g), *Streptococcus oralis* ATCC 35037 (h), and *Streptococcus anginosus* ATCC 33397 (i). Values represent the means ± standard deviations of triplicate assays. *$P < 0.05$ compared with control.

cytokine production experiment was performed under the other conditions, at an MOI of 1000 and with a co-culture period of 1 h. We observed an increase in production of IL-6, IL-8 and MCP-1 from HAECs invaded by *S. gordonii* DL1 (Challis) compared with non-invaded HAECs (Fig. 4). Microscopic observation and the Trypan blue exclusion assay revealed that more than 90% of the HAECs invaded by oral streptococci showed no morphological changes and were alive up to 24 h after bacterial invasion in the conditions used for cytokine production. These results were similar to those for non-invaded HAECs. On the other hand, none of the oral viridans group streptococci used in this study could induce detectable levels of IL-6, IL-8 or MCP-1 production from infected HAECs at an MOI of 1 and a co-culture period of 8 or 24 h (data not shown).

Streptococcus mutans Xc was used to examine the kinetics of cytokine production from invaded HAECs. As shown in Fig. 5, the amounts of IL-6, IL-8 and MCP-1 increased in a time-dependent manner. Invaded HAECs produced more IL-6, IL-8 and MCP-1 at every time-point compared with non-invaded HAECs. Expression of all cytokine mRNA in invaded HAECs increased in a time-dependent manner (Fig. 6). Invaded HAECs expressed more IL-6, IL-8 and MCP-1 mRNA at every time-point than non-invaded HAECs.

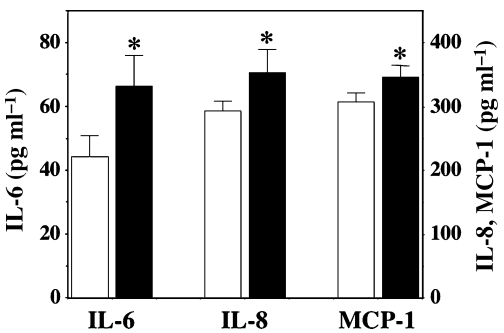

Figure 4 Cytokine production from human aortic endothelial cells (HAECs) invaded by *Streptococcus gordonii* DL1 (Challis). Monolayers were co-cultured with *S. gordonii* DL1 at a multiplicity of infection of 1000 for 1 h. After killing the non-invasive bacteria using antibiotics, HAECs invaded by bacteria were incubated in medium for 24 h, and the concentrations of cytokines in culture supernatants were determined by enzyme-linked immunosorbent assay (IL, interleukin; MCP-1, monocyte chemoattractant protein 1). Open bars, non-invaded HAECs (control); solid bars, invaded HAECs. Values represent the means ± standard deviations of triplicate assays. *$P < 0.05$ compared with control.

DISCUSSION

In this study, we examined the invasion of HAECs by oral streptococci to clarify their possible participation in the pathogenesis of atherosclerosis. All of the strains of oral viridans group streptococci tested were capable of invading HAECs at an MOI of 1 (Table 1). Our purpose was to examine the effects of oral strep-

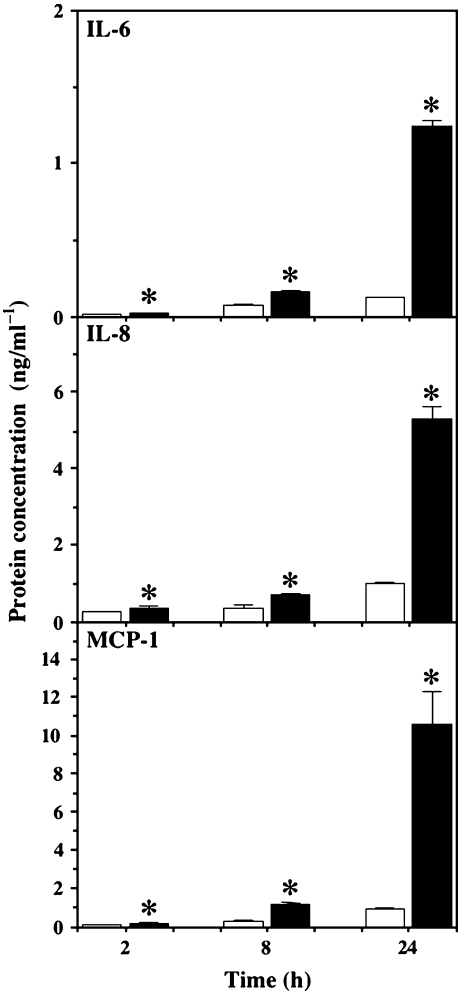

Figure 5 Kinetics of cytokine production from human aortic endothelial cells (HAECs) invaded by *Streptococcus mutans* Xc. Monolayers were co-cultured with *S. mutans* Xc at a multiplicity of infection of 1 for 24 h. After killing the non-invasive bacteria using antibiotics, HAECs invaded by bacteria were incubated in medium for 2, 8 or 24 h, and the concentrations of cytokines in culture supernatants were determined by enzyme-linked immunosorbent assay (IL, interleukin; MCP-1, monocyte chemoattractant protein 1). Open bars, non-invaded HAECs (control); solid bars, invaded HAECs. Values represent the means ± standard deviations of triplicate assays. *$P < 0.05$ compared with control at each time-point.

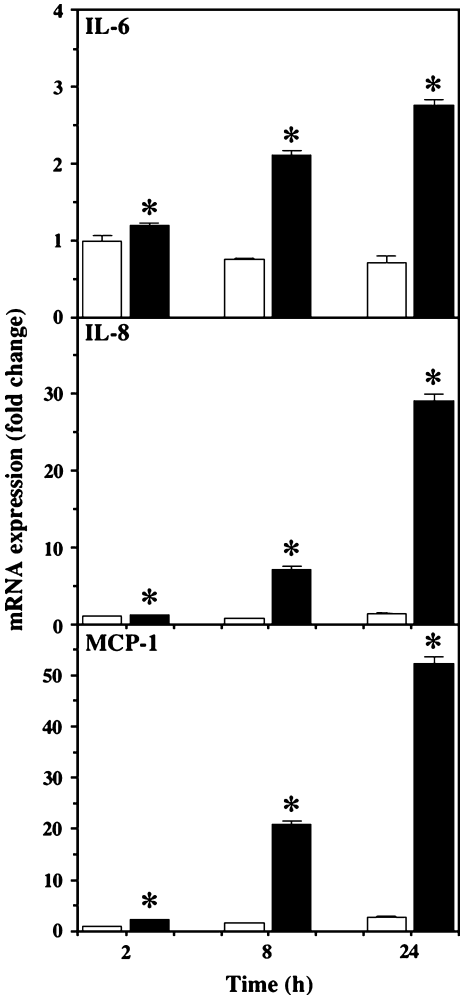

Figure 6 Kinetics of cytokine gene expression in human aortic endothelial cells (HAECs) invaded by *Streptococcus mutans* Xc. Monolayers were co-cultured with *S. mutans* Xc at a multiplicity of infection of 1 for 24 h. After killing the non-invasive bacteria using antibiotics, HAECs invaded by bacteria were incubated in medium for 2, 8 or 24 h, and quantitative analysis of cytokine mRNA expression was performed with real-time polymerase chain reaction (IL, interleukin; MCP-1, monocyte chemoattractant protein 1). Open bars, non-invaded HAECs (control); solid bars, invaded HAECs. Values represent the means ± standard deviations of triplicate assays. $^*P < 0.05$ compared with control at each time-point.

tococci on HAECs at low MOIs during a possibly long period, in which we assumed circumstances where aortic endothelial cells could interact with bacterial cells entering the bloodstream. In preliminary experiments, we found that an MOI of 1 was suitable for this purpose after testing different MOIs using several strains. We observed bacterial growth in Humedia-EG2 medium, indicating that the MOI would change at specific time-points. When 2×10^4 bacterial cells were cultured in the medium, the bacterial number reached 4×10^6 to 1×10^7 after 24 h of incubation. This indicates that the co-culture process started with an MOI of 1, but it ended with a higher MOI, of 200–500. In several bacterial strains tested, the CFU of invasive bacteria recovered after 4 and 8 h of the co-culture period were low (Table 1), and the possibility remained that the recovered bacterial numbers were not derived from intracellular bacteria. Antibiotics were used at sufficient concentrations to kill extracellular bacteria after removal of planktonic bacteria, and we also verified the killing of each bacterial species using controls (no HAECs). The CSLM revealed HAEC invasion by *S. gordonii* ATCC 10558 with low CFU recovered at 4 and 8 h of co-culture. These results suggest that the numbers recovered at these time-points did represent intracellular bacteria.

The invasion efficiency of bacteria increased with the co-culture period of bacteria and HAECs, and it varied depending on the strains. Of the strains tested, *S. salivarius* ATCC 13419 had the highest invasion efficiency at an MOI of 1 and co-culture periods of 4 and 8 h, whereas Stinson *et al.* (2003) reported that the same strain invaded human umbilical vein endothelial cells (HUVECs) weakly. This discrepancy may be attributable to the differences in the endothelial cells used or the experimental conditions.

Some organisms caused morphological changes to the HAECs after a certain co-culture period. Of the strains evaluated, *S. gordonii* DL1 (Challis) produced the strongest cytotoxic effects on HAECs. Stinson *et al.* (2003) reported that *S. gordonii* CH1 (Challis) killed HUVECs via hydrogen peroxide production, and bovine liver catalase reduced the killing of HUVECs by this organism. Bovine liver catalase inactivated the α-hemolysin of *S. gordonii* CH1, which is the same as hydrogen peroxide in terms of chemical and biological properties (Barnard & Stinson, 1996). Bovine liver catalase reduced the number of *S. gordonii* DL1 (Challis) invading HAECs in our study. Hydrogen peroxide produced by *S. gordonii* may be involved in the invasion as well as the killing of endothelial cells by this organism. The invasion of HAECs by *S. gordonii* DL1 (Challis) was almost completely inhibited by cytochalasin D (Fig. 2), indicating that the invasion of HAECs by bacteria occurred via an endocytic mechanism, dependent on the polymerization of actin microfilaments. This invasion

mechanism resembled that by which *P. gingivalis* invades HUVECs (Deshpande *et al.*, 1998).

In previous studies, HUVECs were used primarily in experiments that investigated the roles of bacteria in the induction of vascular disease (Vernier *et al.*, 1996; Stinson *et al.*, 2003). However, Morris *et al.* (1993) demonstrated that the pattern of cytokine mRNA expression in HAECs was different from that of HUVECs. Wang *et al.* (1997) reported that HAECs and HUVECs showed different chemokinetic and mitogenic responses to α-thrombin. Based on their origin, HAECs are a relevant cell type for research on atherosclerosis.

We showed that *S. gordonii* DL1 (Challis), *S. gordonii* ATCC 10558, *S. mutans* Xc and *S. salivarius* ATCC 13419 induced the production of cytokine(s) (IL-6, IL-8, MCP-1) from invaded HAECs (Figs 3 and 4). The recruitment of circulating leukocytes to endothelial cells is a key event in atherosclerosis. Interleukin-8 and MCP-1, which are produced by aortic endothelial cells at sites of infection, are chemoattractant proteins for neutrophils and monocytes, respectively (Gu *et al.*, 1998; Gerard & Rollins, 2001) whereas IL-6 stimulates acute inflammatory-phase proteins and induces endothelial dysfunction (Yudkin *et al.*, 2000). Cytokines produced by invaded HAECs may contribute to the atherogenic process. It should be emphasized that HAECs not infected but invaded by oral viridans group streptococci produced more cytokines than unstimulated HAECs in our study. Additionally, *S. mutans* Xc induced cytokine production in an invasive bacteria-dependent manner. These findings suggest that bacterial invasion could be a necessary process to induce cytokine production from HAECs. Vernier *et al.* (1996) demonstrated that oral viridans group streptococci induced the production of IL-6 and IL-8 from human saphenous endothelial cells when co-cultured with bacterial cells, and they suggested that cell surface adhesins were associated with this response. However, they did not mention whether cytokine production was induced when bacteria invaded cells or when they simply attached to the cell surfaces. In the present study, more than 90% of the HAECs invaded by oral streptococci survived for up to 24 h after bacterial invasion in the experimental conditions used for cytokine production. This indicates that the invasive bacteria could persistently stimulate living HAECs for cytokine production. The numbers of *S. salivarius* ATCC 13419 and *S. mutans* Xc internalized in HAECs decreased during the 24-h incubation. Poor bacterial growth in host cells has been reported for *S. gordonii* CH1 (Stinson *et al.*, 2003) and group B streptococci (Nizet *et al.*, 1997). The reason(s) for these results are complex because various factors, such as bacterial growth capacity, intracellular environment and assay conditions, are probably involved. Interestingly, HAECs invaded by *S. salivarius* ATCC 13419 and *S. mutans* Xc showed an increase in cytokine production in the present study. These strains may have high virulence in stimulating HAECs resulting in the induction of strong responses, such as killing of intracellular bacteria and cytokine production. Further studies are necessary to clarify the mechanism of bacterial survival in host cells and the relationship to cytokine induction.

Streptococcus parasanguinis ATCC 15911, *S. sanguinis* ATCC 10556, *S. mitis* ATCC 6249 and *S. oralis* ATCC 35037 could not induce cytokine production from invaded cells, although each of them showed a high level of invasion efficiency, as did *S. mutans* Xc (Table 1). The mechanism of the large increase in cytokine production by HAECs invaded by *S. mutans* Xc remains unclear. *Streptococcus mutans* possesses various cell-surface substances, including serotype-specific polysaccharide antigens, lipoteichoic acid, glucosyltransferases, glucan-binding proteins and a 190-kDa protein (Ag I/II) (Koga *et al.*, 2002). Among them, Ag I/II and serotype-specific rhamnose glucose polysaccharide have been shown to be potent stimulators of IL-6 and IL-8 production from human saphenous vein endothelial cells (Vernier *et al.*, 1996) and monocytic cells (Engels-Deutsch *et al.*, 2003). It is possible that certain cell-surface components of *S. mutans* Xc functioned to induce a large increase in cytokine production from HAECs. Further studies are necessary to investigate the characteristics of *S. mutans* Xc compared with other strains, and to identify the bacterial components responsible for cytokine induction.

In a previous report, a real-time PCR method revealed bacterial ribosomal DNA in blood specimens from healthy individuals that had previously been considered sterile most of the time (Nikkari *et al.*, 2001). Periodontal disease causes ulceration of the junctional epithelium of the gingiva and increases the permeability of blood vessels. Forner *et al.* (2006) showed that the incidence and magnitude of bactere-

mia after scaling was significantly higher in periodontitis patients than in gingivitis patients and healthy control individuals. In addition to dental procedures, daily oral practices, such as tooth brushing, could cause transient bacteremia, and most of the prevalent bacteria detected in blood specimens belong to the genus *Streptococcus* (Roberts *et al.*, 1997; Lockhart *et al.*, 2008). We demonstrated that some oral viridans group streptococci have the capacity to invade HAECs at a low MOI of 1, and induce cytokine production by the invaded HAECs. Oral care practices are routine daily activities throughout life, and it is possible that aortic endothelial cells are exposed to frequent challenges by oral viridans group streptococci over the long term, even if the impact of each bacterial challenge is small. Furthermore, because oral bacteria are present as biofilm constituents in the oral cavity, synergistic effects among multiple bacterial species, such as oral viridans group streptococci and periodontal bacteria, may facilitate the invasion and activation of cytokine production by aortic endothelial cells. Our results indicate the importance of considering oral viridans group streptococci as possible causative organisms in the pathogenesis of atherosclerosis.

ACKNOWLEDGEMENTS

We thank Dr. Ayumi Nakayama, Department of Oral Physiology, Kagoshima University Graduate School of Medical and Dental Sciences for technical help with the CSLM. This study was supported in part by Grants-in-Aid for Scientific Research (B)19390543 (to T.O.) and (C)20592459 (to E.N.) from the Japan Society for the Promotion of Science.

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