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Infection and Immunity

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E-Selectin Mediates *Porphyromonas gingivalis* Adherence to Human Endothelial Cells

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***Porphyromonas gingivalis*, a major periodontal pathogen, may contribute to atherogenesis and other inflammatory cardiovascular diseases. However, little is known about interactions between *P. gingivalis* and endothelial cells. E-selectin is a membrane protein on endothelial cells that initiates recruitment of leukocytes to inflamed tissue, and it may also play a role in pathogen attachment. In the present study, we examined the role of E-selectin in *P. gingivalis* adherence to endothelial cells. Human umbilical vein endothelial cells (HUVECs) were stimulated with tumor necrosis factor alpha (TNF-α) to induce E-selectin expression. Adherence of *P. gingivalis* to HUVECs was measured by fluorescence microscopy. TNF-α increased adherence of wild-type *P. gingivalis* to HUVECs. Antibodies to E-selectin and sialyl Lewis X suppressed *P. gingivalis* adherence to stimulated HUVECs. *P. gingivalis* mutants lacking OmpA-like proteins Pgm6 and -7 had reduced adherence to stimulated HUVECs, but fimbria-deficient mutants were not affected. E-selectin-mediated *P. gingivalis* adherence activated endothelial exocytosis. These results suggest that the interaction between host E-selectin and pathogen Pgm6/7 mediates *P. gingivalis* adherence to endothelial cells and may trigger vascular inflammation.**

Periodontitis is a disease of the supporting structures of the teeth, causing loss of attachment to the alveolar bone and eventual exfoliation of teeth (5). Severe periodontitis affects up to 20% of the population, and mild to moderate periodontitis is observed in the majority of adults (6). Gram-negative bacteria play an important role in the pathogenesis of human periodontal diseases (15, 42), and *Porphyromonas gingivalis* is one of the species most strongly implicated in periodontal diseases (14, 43). Several recent studies have demonstrated that *P. gingivalis* is able to invade and activate different cell types in the tissue surrounding teeth (endothelial and gingival epithelial cells as well as periodontal ligament cells) (12, 26, 40). Moreover, recent studies have demonstrated a transient bacteremia with potential systemic infection after a variety of dental treatment procedures (2, 19, 20, 41). Therefore, endothelial cells can act as primary target cells during infection with *P. gingivalis*. However, little is known about mechanisms of infection and activation of endothelial cells by *P. gingivalis*.

The endothelium has several important functions, which include providing a nonadhesive, nonthrombotic barrier between the blood and the underlying tissues. In atherosclerosis or in response to injury or inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), the endothelium becomes activated, and selectins and cell adhesion molecules (CAMs) are rapidly induced (36, 39). In particular, members of the immunoglobulin superfamily of CAMs, such as intercellular cell adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), as well as the selectin family members E-selectin and P-selectin, are expressed and play crucial roles in the adhesion and migration of monocyte/macrophage infiltration into atherosclerotic lesions during the early and subsequent stages of atherosclerosis in a variety of animal models (21, 47, 49). Increased expression of E-selectin and production of proinflammatory cytokines in the endothelium play a pivotal role in the generation of leukocyte infiltrates and subsequent atherosclerotic plaque formation (16, 28). *P. gingivalis* infection significantly increases endothelial expression of VCAM-1, ICAM-1, and E-selectin, enhances production of interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1), and increases adhesion of THP-1 monocytes to endothelial cells (18, 46). Therefore, *P. gingivalis* elicits a proatherogenic response in endothelial cells. Although E-selectin is involved in vascular inflammation and is induced with *P. gingivalis*, the interaction between *P. gingivalis* and endothelial cells is not understood. In the present study, we explored the ability of E-selectin to facilitate *P. gingivalis* adherence to human umbilical vein endothelial cells (HUVECs). We found that activated endothelial cells interact with *P. gingivalis* via E-selectin on endothelial cells and via OmpA-like proteins Pgm6 and -7 of the bacterium.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* ATCC 33277 was used as a wild-type strain in this study. *P. gingivalis* defective mutants lacking *fimA* were constructed as described previously (17). A *P. gingivalis* Pgm6/7-deficient mutant was constructed as described previously (32). This mutant did not show any sign of a polar effect on the downstream gene (data not shown). All *P. gingivalis* strains were grown at 37°C under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) on brucella HK agar (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) supple-

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mented with 5% laked rabbit blood, hemin (2.5 μg/ml), menadione (5 μg/ml), and dithiothreitol (0.1 mg/ml) and in Trypticase soy broth (BD, Franklin Lakes, NJ) supplemented with yeast extract (2.5 mg/ml), hemin (2.5 μg/ml), menadione (5 μg/ml), and dithiothreitol (0.1 mg/ml). Bacterial growth was monitored by measuring the optical density at 660 nm (OD_{660}). For infection assays, an inoculum with an infection ratio (multiplicity of infection [MOI]) of 100 bacteria per cell was added to the cell culture medium.

Cell culture conditions. HUVECs were cultured in endothelial cell growth medium 2 (EGM-2) (Lonza, Basel, Switzerland) supplemented with fetal bovine serum, hydrocortisone, human recombinant fibroblast growth factor, vascular endothelial growth factor, recombinant insulin growth factor 1, ascorbic acid, human recombinant epidermal growth factor, gentamicin, and amphotericin B at 37°C in a humidified atmosphere of 5% CO_2.

E-selectin expression. E-selectin cDNA was constructed as described previously (53). The E-selectin cDNA was amplified by PCR with specific primers (5′-GAC AGC TAG CAT GAT TGC TTC ACA G-3′ [includes an additional NheI site] and 5′-CGG CCT CGA GTT AAA GGA TGT AAG AAG GC-3′ [includes an additional XhoI site]) and then cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). For preparation of a soluble E-selectin vector, a stop codon and a unique EcoRV site were introduced by site-directed mutagenesis (Promega, Madison, WI) into the boundary between the sixth consensus repeat and the transmembrane domain, using the following oligonucleotide, which starts at nucleotide 1776: 5′-CC AAC ATT CCC TAG ATA TCT AGA CTT TCT GCT G-3′.

Measurement of E-selectin production. An enzyme-linked immunosorbent assay (ELISA)-based method was used for quantification of E-selectin protein expression in endothelial cells. HUVECs (3.5×10^5 cells/ml) were seeded into 6-well plates and grown overnight. The cells were then stimulated with 10 ng/ml of TNF-α (PeproTec Inc., Rocky Hill, NJ) for 1, 2, 3, 4, 8, and 24 h. After removing the medium, the cell layers were washed twice with phosphate-buffered saline (PBS). Cells were lysed in a cell lysis reagent (CelLytic P; Sigma-Aldrich, St. Louis, MO) with a protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan). Concentrations of E-selectin in the cell lysates were determined using a commercial ELISA kit for E-selectin (eBioscience, San Diego, CA). The cell lysates were also mixed with 4× Laemmli sample buffer without reducing agents and were fractionated by 7.5% SDS-PAGE and immunoblotted with a monoclonal antibody to E-selectin (BBIG-E4 [5D11]; R&D Systems, Abingdon, United Kingdom).

Analysis of *P. gingivalis* adhesion to endothelial cells. HUVECs (2×10^6 cells) were seeded in a Lab-Tek II chamber slide system (Nalge Nunc International, Rochester, NY) that had been coated with 50 μg/ml of rat tail collagen (BD), and the cells were incubated for 24 h before administration of *P. gingivalis*. HUVECs grown to near confluence in each well were stimulated with TNF-α for 3 h, and then *P. gingivalis* cells which had been washed with EGM-2 and resuspended in EGM-2 without antibiotic at a concentration of 10^8 cells/ml were added to the monolayer cells at an MOI of 1:100 under 5% CO_2 at 37°C for 0.5 to 3 h. Cells were then washed three times with PBS, followed each time by gentle rinsing for 5 min at room temperature, and fixed with 4% (wt/vol) paraformaldehyde at 4°C overnight. After washing three times with PBS, the cells were permeabilized with PBS containing 0.05% Triton X-100 at room temperature for 30 min. They were washed again and then blocked with PBS containing 5% (wt/vol) bovine serum albumin (BSA) at room temperature for 30 min. Bacterial cells on chamber slides were labeled with an antiserum for *P. gingivalis* whole cells (1:1,000 dilution) for 60 min at room temperature and then washed five times with PBS. The bacterial cells were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000 dilution; Invitrogen Co., Carlsbad, CA). Actin filaments in HUVECs or 293 cells were stained simultaneously with Alexa Fluor 568-conjugated phalloidin (1 μg/ml; Invitrogen Co.) for 60 min at room temperature in the dark. After washing 10 times with PBS, chamber slides were mounted onto a slide containing ProLong Gold antifade reagent (Invitrogen). Adherent bacteria on the cell surface were examined by fluorescence microscopy (Keyence, Osaka, Japan). We measured the area stained with Alexa 488 (corresponding to *P. gingivalis*) in a visual field (corresponding to 0.06 mm^2) by using the Image J program. We then calculated bacterial number by dividing the area by the size (in pixels) of a *P. gingivalis* cell. To determine whether E-selectin is involved in *P. gingivalis* adherence to endothelial cells, TNF-α-pretreated HUVECs were incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 30 min to 3 h in the presence of various concentrations of an antibody for E-selectin (R&D Systems, Inc., Minneapolis, MN), recombinant E-selectin, or sialyl Lewis X (Calbiochem, San Diego, CA). *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) was also incubated with HEK 293 cells transfected with a human E-selectin-inserted vector for 30 min. To explore ligands for E-selectin on *P. gingivalis*, *P. gingivalis* ATCC 33277 (wild type), a FimA-deficient mutant (Δ*fimA*), and a Pgm6/7-deficient mutant (Δ*pgm6/7*) (10^8 cells/ml) were incubated with TNF-α-pretreated HUVECs for 3 h. TNF-α-pretreated HUVECs were incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml) for 30 min in the presence or absence of envelopes isolated from wild-type or mutant *P. gingivalis*. TNF-α-pretreated HUVECs were incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml) for 30 min in the presence or absence of purified FimA fimbriae and Pgm6/7.

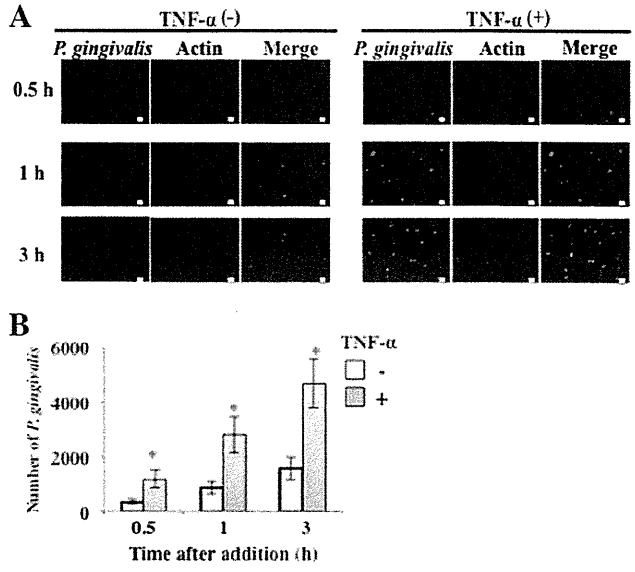

FIG 1 Adherence of *P. gingivalis* to HUVECs is enhanced by stimulation with TNF-α. (A) HUVECs were incubated with TNF-α (10 ng/ml) for 0.5 to 3 h. *P. gingivalis* ATCC 33277 cells (10^8 cells/ml in each well) were then added to the culture medium for 0.5 to 3 h. Cells were then washed, and attachment of *P. gingivalis* to the cells was observed by fluorescence microscopy. *P. gingivalis* was stained with Alexa Fluor 488 (green), and actin of endothelial cells was visualized with Alexa Fluor 568 (red). Bars, 10 μm. (B) HUVECs were incubated with TNF-α (10 ng/ml) for 0.5 to 3 h. *P. gingivalis* ATCC 33277 cells (10^8 cells/ml in each well) were then added to the culture medium for 0.5 to 3 h. Cells were then washed, and attachment of *P. gingivalis* to the cells was observed by fluorescence microscopy. The attachment levels are expressed as numbers of *P. gingivalis* cells per 60,430 mm^2 (means ± standard deviations [SD] [$n = 3$]). *, $P < 0.01$ versus no TNF-α.

Measurement of VWF and nitric oxide. HUVECs (3.5×10^5 cells/ml) were seeded into 12-well plates and grown overnight. The cells were then stimulated with 10 ng/ml of TNF-α for 3 h. *P. gingivalis* cells were inoculated into cultures at an MOI of 100, and the cultures were incubated for 30 min and 1 h. The culture media were then collected and centrifuged at 13,000 rpm for removal of bacterial cells. Concentrations of von Willebrand factor (VWF) in the supernatants were measured by use of an ELISA kit according to the manufacturer's instructions (VWF ELISA kit;

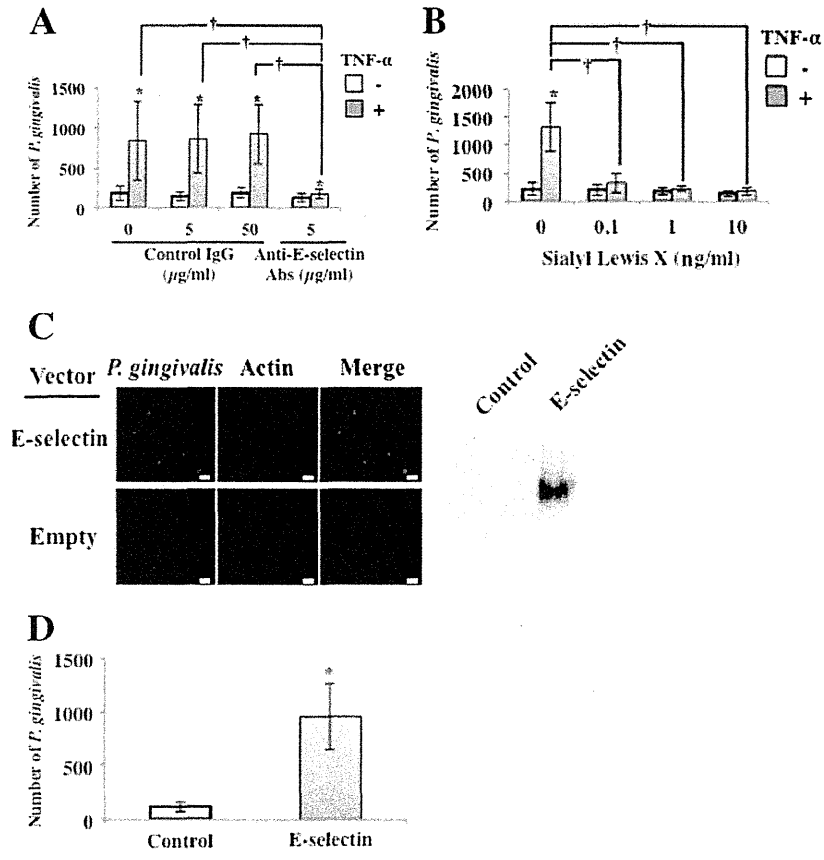

FIG 2 Adherence of *P. gingivalis* to TNF-α-activated endothelial cells was mediated by E-selectin. (A) Inhibitory effect of anti-E-selectin antibodies. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 30 min in the presence of antibodies for E-selectin or control IgG. Other procedures are described in the legend to Fig. 1B. Data are means ± SD ($n = 3$). *, $P < 0.01$ versus no TNF-α; †, $P < 0.01$ versus no anti-E-selectin antibodies. (B) Inhibitory effect of sialyl Lewis X. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 30 min in the presence of purified sialyl Lewis X (0 to 10 ng/ml). Other procedures are described in the legend to Fig. 1B. Data are means ± SD ($n = 3$). *, $P < 0.01$ versus no TNF-α; †, $P < 0.01$ versus no sialyl Lewis X. (C) Adherence of *P. gingivalis* was augmented in HEK293 cells transfected with an expression vector for E-selectin. *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) was incubated with 293 cells transfected with a human E-selectin-inserted vector for 0 min. Other procedures are described in the legend to Fig. 1A. Bars, 10 μm. (D) Adherence of *P. gingivalis* was augmented in 293 cells transfected with an expression vector for E-selectin. *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) was incubated with 293 cells transfected with a human E-selectin-inserted vector for 30 min. Other procedures are described in the legend to Fig. 1B. Data are means ± SD ($n = 3$). *, $P < 0.01$ versus control.

American Diagnostic Inc., Stanford, CT). The concentration of NO_2^-/NO_3^- was also measured by 2,3-diaminonaphthalene (DAN) assay (24).

Preparation of *P. gingivalis* envelope. Separation of whole envelopes and the outer membrane from *P. gingivalis* strains was performed essentially as described previously (30). Briefly, bacterial cells were washed with PBS (pH 7.5) and then resuspended in PBS (pH 7.5) containing 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin. The cells were disrupted by sonication, and remaining, undisrupted bacterial cells were removed by centrifugation at 1,000 × *g* for 10 min. The envelope was collected as a pellet by centrifugation at 100,000 × *g* for 60 min at 4°C. The pellet was washed once by resuspension in PBS and recentrifuged. The final pellet was suspended in PBS.

Purification of FimA. The major fimbriae from *P. gingivalis* ATCC 33277 were purified as described previously (52). The purity was ascertained by scanning of the stained SDS-polyacrylamide gel.

Purification of Pgm6/7 complex. The functional Pgm6/7 complex was purified by two methods. First, we purified it electrophoretically from bacterial envelopes as previously reported (32). Briefly, an envelope fraction of *P. gingivalis* was subjected to SDS-PAGE under nonreducing conditions. A 120-kDa protein band, corresponding to Pgm6/7 heterotrimers, was excised, and then the complex was extracted electrically from a piece of gel. We used these samples for the experiments in Fig. 3E and File S3B in the supplemental material. Second, we constructed C-terminally hexahistidine-tagged Pgm6 and purified the Pgm6/7 complex from a *P. gingivalis* mutant by using a nickel affinity column. Briefly, we inserted a DNA fragment consisting of the *pgm7* open reading frame (ORF) associated with the DNA sequence encoding Gly-Ser-Ser-hexahistidine into the vector pT-COW (13), bearing a powerful promoter of the 350-bp upper region of *ragA* (31). The constructed plasmid was introduced into a *pgm7* deletion mutant of *P. gingivalis* (32). The cell lysate was applied to a nickel affinity column, and the bound proteins were eluted. Although a hexahistidine tag was associated with Pgm7 alone, the Pgm6/7 complex was obtained. We used these samples for the experiments in Fig. 3F and G and File S3C in the supplemental material.

RESULTS

TNF-α augments adherence of *P. gingivalis* to endothelial cells by inducing expression of E-selectin. We first examined induc-

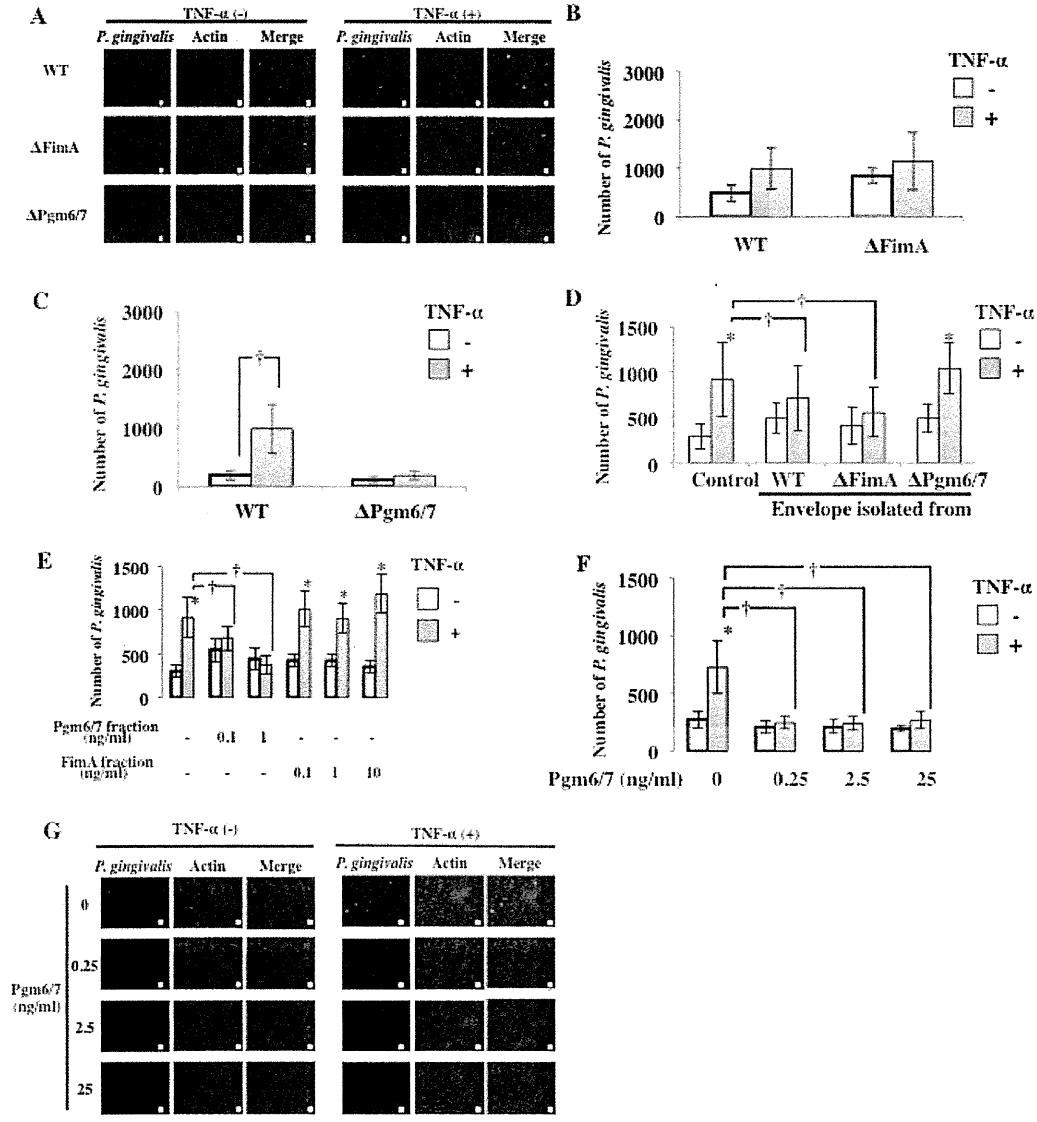

FIG 3 Pgm6/7 in *P. gingivalis* mediates the interaction with activated endothelial cells. (A) *P. gingivalis* ATCC 33277 (wild type), a FimA-deficient mutant (ΔFimA), and a Pgm6/7-deficient mutant (ΔPgm6/7) (10^8 cells/ml in each well) were incubated with TNF-α-pretreated HUVECs for 3 h. Other procedures are described in the legend to Fig. 1A. Bars, 10 μm. (B) *P. gingivalis* ATCC 33277 (wild type) and a FimA-deficient mutant (ΔFimA) (10^8 cells/ml in each well) were incubated with TNF-α-pretreated HUVECs for 30 min. Other procedures are described in the legend to Fig. 1A. (C) *P. gingivalis* ATCC 33277 (wild type) and a Pgm6/7-deficient mutant (Pgm6/7) (10^8 cells/ml in each well) were incubated with TNF-α-pretreated HUVECs for 30 min. Other procedures are described in the legend to Fig. 1B. Data are means ± SD ($n = 3$). *, $P < 0.01$ versus no TNF-α. (D) Inhibitory effects of *P. gingivalis* envelopes on TNF-α-induced adhesion of *P. gingivalis* to HUVECs. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 30 min in the presence or absence of envelopes isolated from wild-type or mutant *P. gingivalis*. Other procedures are described in the legend to Fig. 1B. Data are means ± SD ($n = 3$). *, $P < 0.01$ versus no TNF-α; †, $P < 0.01$ versus control. (E) Effects of extracted Pgm6/7 and FimA on TNF-α-induced adhesion of *P. gingivalis* to HUVECs. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 30 min in the presence or absence of purified Pgm6/7 and FimA. Other procedures are described in the legend to Fig. 1B. Data are means ± SD ($n = 3$). *, $P < 0.01$ versus no TNF-α; †, $P < 0.01$ versus Pgm6/7 fraction. (F) Inhibitory effect of *P. gingivalis* Pgm6/7 on TNF-α (10 ng/ml)-induced adhesion of *P. gingivalis* to HUVECs. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 30 min in the presence or absence of purified Pgm6/7. Other procedures are described in the legend to Fig. 1B. Data are means ± SD ($n = 3$). *, $P < 0.01$ versus no TNF-α; †, $P < 0.01$ versus Pgm6/7 (0 ng/ml). (G) Inhibitory effect of *P. gingivalis* Pgm6/7 on TNF-α-induced adhesion of *P. gingivalis* to HUVECs. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 30 min in the presence or absence of purified Pgm6/7. Other procedures are described in the legend to Fig. 1A. Bars, 10 μm.

tion of E-selectin expression by TNF-α by using ELISA and Western blotting of HUVEC cultures. TNF-α induced a time-dependent expression of E-selectin in HUVECs (see Files S1 and S2 in the supplemental material). E-selectin expression was maximal 3 h after TNF-α addition. No basal expression of E-selectin was found. To determine whether E-selectin expression in endothelial cells is involved in adhesion of *P. gingivalis* to the cells, we incubated HUVECs with TNF-α (10 ng/ml) for 0.5 to 3 h, and then *P.*

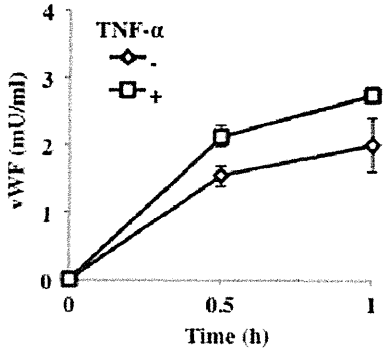

FIG 4 Endothelial VWF exocytosis in response to *P. gingivalis* is augmented by pretreatment with TNF-α. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 0 to 1 h. The release of VWF into the medium was measured by ELISA. Data are means ± SD ($n = 3$).

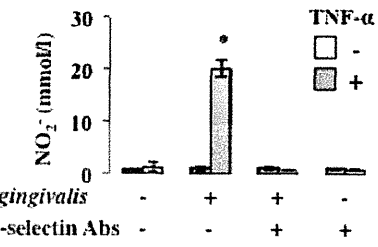

FIG 5 *P. gingivalis*-induced nitric oxide release from activated endothelial cells is mediated by E-selectin. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml in each well) for 30 min in the presence or absence of an antibody for E-selectin. The release of nitric oxide into the medium was measured by DAN assay. Data are means ± SD ($n = 3$). *, $P < 0.01$ versus no TNF-α.

gingivalis ATCC 33277 cells (10^8 cells/ml in each well) were added to the culture medium for 0.5 to 3 h. Cells were then washed, and attachment of *P. gingivalis* to the cells was observed by fluorescence microscopy. Attachment of *P. gingivalis* to HUVECs increased time dependently without pretreatment of TNF-α (Fig. 1A and B). Pretreatment with 10 ng/ml of TNF-α significantly enhanced the level of attachment in HUVEC cultures.

To clarify the role of E-selectin in *P. gingivalis* adherence to HUVECs, we examined the effect of anti-E-selectin antibodies on *P. gingivalis* adherence to HUVECs. HUVECs were pretreated with TNF-α and then incubated with *P. gingivalis* for 30 min in the presence of antibodies for E-selectin or control IgG. Antibodies to E-selectin inhibited *P. gingivalis* adherence to TNF-α-pretreated HUVECs (Fig. 2A).

E-selectin mediates the rolling of leukocytes on activated endothelial cells through binding of the carbohydrate antigen sialyl Lewis X (37). Therefore, we examined the effect of sialyl Lewis X on interactions between *P. gingivalis* and endothelial cells. Sialyl Lewis X inhibited TNF-α-induced *P. gingivalis* adherence to HUVECs at a concentration of 0.1 μg/ml (Fig. 2B). To assess the effect of E-selectin overexpression on the upregulation of *P. gingivalis* adherence to endothelial cells, we transfected an E-selectin-inserted plasmid into HUVECs. Expression of E-selectin was confirmed by Western blotting 24 h after transfection (Fig. 2C). Adherence of *P. gingivalis* significantly increased in E-selectin-transfected HEK 293 cells (Fig. 2D). These results suggest that TNF-α augments *P. gingivalis* adherence to HUVECs by inducing expression of E-selectin.

***P. gingivalis* interacts with TNF-α-stimulated endothelial cells via Pgm6/7.** The initial adherence of *P. gingivalis* to host cells is mediated by multiple adhesins, including FimA and HagB (44, 45). To determine whether an interaction occurs between the major fimbriae and E-selectin, we examined adherence to endothelial cells of *P. gingivalis* defective in FimA alone (ΔFimA). TNF-α increased the adherence to endothelial cells of FimA-deficient *P. gingivalis* as well as wild-type *P. gingivalis*, and the degrees of adherence were similar (Fig. 3A and B). We next examined whether a major outer membrane protein of *P. gingivalis* that is homologous to the OmpA protein in *Escherichia coli*, namely, the Pgm6/7 complex, mediates *P. gingivalis* adherence to HUVECs. The Pgm6/7-deficient mutant (ΔPgm6/7) was incubated with TNF-α-pretreated HUVECs, and attachment of *P. gingivalis* to the cells was observed. TNF-α increased adherence of wild-type *P. gingivalis* to endothelial cells but failed to increase adherence of ΔPgm6/7 *P. gingivalis* to endothelial cells (Fig. 3C). To clarify whether Pgm6/7 mediates *P. gingivalis* adherence to HUVECs, we prepared envelopes from wild-type, ΔFimA, and ΔPgm6/7 *P. gingivalis* cells and examined the effects on the interaction between wild-type *P. gingivalis* and HUVECs. Envelope peptides from wild-type *P. gingivalis* or ΔFimA *P. gingivalis* suppressed adherence of *P. gingivalis* to TNF-α-pretreated HUVECs (Fig. 3D). However, envelope peptides from ΔPgm6/7 *P. gingivalis* did not affect *P. gingivalis* adherence. In addition, the Pgm6/7 fraction from *P. gingivalis* ATCC 33277 suppressed TNF-α-augmented *P. gingivalis* adherence, but the FimA fraction from the same strain did not (Fig. 3E). Furthermore, purified Pgm6/7 inhibited TNF-α activation of *P. gingivalis* adherence to HUVECs at concentrations as low as 0.25 ng/ml (Fig. 3F and G). These results suggest that the *P. gingivalis* peptide Pgm6/7 plays a role in the adherence of *P. gingivalis* to endothelial cells.

***P. gingivalis* interaction with endothelial cells via E-selectin induces endothelial exocytosis and NO production.** Finally, to determine whether E-selectin-mediated adherence of *P. gingivalis* activates endothelial cells and increases vascular inflammation, we investigated induction of VWF and nitric oxide in TNF-α-pretreated endothelial cells by stimulation with *P. gingivalis*. HUVECs were incubated with TNF-α (10 ng/ml) for 3 h, and then the cells were washed and incubated with *P. gingivalis* for 0 to 1 h. The release of VWF into the medium was measured by ELISA. *P. gingivalis* triggers endothelial exocytosis, as measured by endothelial release of VWF. Release of VWF by stimulation with *P. gingivalis* was also enhanced by pretreatment of HUVECs with TNF-α (Fig. 4). TNF-α pretreatment of HUVECs before *P. gingivalis* stimulation for 30 min significantly increased NO_2^- release into the medium (Fig. 5). Anti-E-selectin antibodies inhibited activation of NO release by *P. gingivalis* in TNF-α-pretreated HUVECs. These results suggest that interaction of *P. gingivalis* with endothelial cells via E-selectin activates the endothelial cells and enhances proinflammatory responses of the cells to this bacterium.

DISCUSSION

P. gingivalis adherence to and invasion of endothelial cells have been reported by several investigators (9, 46). However, this is the first report on the adhesion of activated endothelial cells by *P.*

gingivalis. HUVECs activated by TNF-α increased the adherence of *P. gingivalis* through E-selectin expression, interacting with the OmpA-like proteins Pgm6 and -7 in *P. gingivalis*.

One of the initial events in atherogenesis is the activation of endothelial cells, which then express cell surface adhesion molecules such as endothelial leukocyte adhesion molecule (E-selectin), VCAM-1, and ICAM-1 (8, 10, 22). These endothelial adhesion molecules in turn facilitate the attachment of blood leukocytes to endothelial surfaces (34). In the present study, we demonstrated that one of the periodontopathogens adheres to endothelial cells via E-selectin.

P. gingivalis can invade many cell types, including human oral epithelial cells (33, 51), human gingival fibroblasts or epithelial cells (3, 26), human coronary artery smooth muscle cells, and HCAECs (11). Adhesion of *P. gingivalis* to host cells is multimodal (27) and involves a variety of cell surface and extracellular components, including fimbriae, proteases, hemagglutinins, and lipopolysaccharide (LPS) (8). Among the large array of virulence factors produced by *P. gingivalis*, the major fimbriae (FimA) as well as cysteine proteinases (gingipains) contribute to the attachment to and invasion of many types of mammalian cells, including oral epithelial cells (4) and endothelial cells. *P. gingivalis* strains deficient in FimA fimbriae had an attenuated capacity to adhere to and invade epithelial cells and endothelial cells (33, 46, 51). Invasive *P. gingivalis* strains and their purified fimbriae activate expression of cytokines and cell adhesion molecules in endothelial cells (46). However, our data showed that Pgm6/7 rather than FimA is associated with *P. gingivalis* adherence to TNF-α-treated endothelial cells. Although we do not know exact mechanisms, *P. gingivalis* cells adhere to activated endothelial cells through their Pgm6/7 complex, in a manner different from the fimbria-integrin interaction. TNF-α activates endothelial cells to express adhesion molecules as well as proinflammatory cytokine and chemokine receptors and promotes synthesis and release of a variety of inflammatory cytokines and chemokines to support recruitment of activated leukocytes to an inflammatory lesion (38). TNF-α promotes the inflammatory cascade within the arterial wall during development of atherosclerosis (1). In addition, *P. gingivalis* has been detected within atherosclerotic plaques from vascular tissues (25, 54). Therefore, TNF-α may also augment adherence of *P. gingivalis*, as well as that of leukocytes, in part through inducing E-selectin expression. Weibel-Palade bodies (WPBs) are endothelial granules that store VWF and other vascular modulators (48, 50). Endothelial cells secrete WPBs in response to vascular injury, releasing VWF, which triggers platelet rolling. Endothelial exocytosis is one of the earliest responses to vascular damage and plays a pivotal role in thrombosis and inflammation (29). In this study, we demonstrated that *P. gingivalis* interaction with endothelial cells via E-selectin activates endothelial cells, enhances endothelial exocytosis (Fig. 4), and may enhance atherogenesis and thrombosis (e.g., Buerger disease) (7, 23).

Pgm6/7 in *P. gingivalis*, which shares a low level of homology with *E. coli* OmpA, exists as a heterotrimer comprising Pgm6 and Pgm7 and plays a role in the outer membrane integrity of this organism. OmpA in *E. coli* K1 has been reported to interact with a glycoprotein (Ecgp) of human brain microvascular endothelial cells for invasion (35). Therefore, *P. gingivalis* invasion into endothelial cells should be investigated in the near future, especially regarding whether Pgm6/7 is involved in the invasion. How does Pgm6/7 bind to E-selectin? The adhesion activity of E-selectin is mediated primarily by the binding of sialyl Lewis X on the leukocyte to the carbohydrate-binding domain of the protein. E-selectin recognizes the carbohydrate structure of sialyl Lewis X. Pgm6/7 is also a glycoprotein, and therefore it may bind to E-selectin through its carbohydrate side chain. However, we need additional experiments to reveal the mechanism.

Collectively, in the present study, we clarified a new host-pathogen interaction, i.e., the interaction between Pgm6/7, a major outer membrane protein of *P. gingivalis*, and E-selectin of activated endothelial cells. This finding raises the possibility that chronic infection of the vasculature by pathogens such as *P. gingivalis* could exacerbate systemic vascular diseases such as coronary heart disease, stroke, and diabetes mellitus.

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5. *Porphyromonas gingivalis* ジンジパインによるヒト歯肉上皮細胞における IL-33 発現誘導

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はじめに

口腔，肺，皮膚や腸管など粘膜組織において，上皮細胞は細菌，アレルゲンや寄生虫の感染に対して物理的障壁となるのみならず，種々の炎症性サイトカインを生産することで炎症の惹起に係わることが知られている。上皮細胞はさまざまな刺激に応答して interleukin（IL）-33，IL-25 ならびに thymic stromal lymphopoietin（TSLP）などを生産し，これら上皮性サイトカインが肥満細胞や好塩基球を直接刺激することにより Th2 型アレルギー性炎症が誘導される[1]。

歯周炎は細菌感染症であり直接の原因は歯周病関連細菌とデンタルプラークであるが，歯周組織の破壊は主として免疫応答による炎症反応により引き起こされる。歯周炎患者の歯周組織では B 細胞とともに多数の $CD4^+$ T 細胞が検出され，多年にわたり歯周炎の病態における Th1/Th2 バランスについて議論がなされており Th2 細胞が多く検出される報告が優勢であるが，歯周炎の病態形成における Th2 細胞の役割は十分に解明されていない[2]。本稿では，IL-33 の特徴および免疫学役割と歯周炎関連細菌 *Porphyromonas gingivalis* による歯肉上皮細胞からの IL-33 誘導機構についてわれわれの知見を概説したい。

1. IL-33

IL-33 は 2005 年に IL-1 受容体ファミリーに属するコンポーネントである ST2 のリガンドとして発見された[3]。ST2 は Th2 細胞，肥満細胞，好塩基球や好酸球に発現することから，IL-33 は Th2 応答に係わるサイトカインであると考えられた。その点で，IL-33 は IL-17 ファミリーサイトカインである IL-25（IL-17E）と機能が類似している。IL-1 受容体ファミリーの IL-1 や IL-18 はプロドメインを有しており，caspase-1 による蛋白分解を受けて生物活性を有する成熟型に変換される。当初 Schmitz らは，IL-33 と caspase-1 を *in vitro* にて反応させると 30 kDa の前駆体型から 18 kDa の成熟型に変換されることで生理活性が示されると報告したが，その後の実験において caspase-1 による活性型 IL-33 への変換は確認されず，caspase-1 ならびにアポトーシスで活性化される caspase-3 と caspase-7 は IL-33 の C 末端側にある生理活性を有する IL-1 様ドメインを切断し，不活性型 IL-33 へ変換させることが明らかにされた。また，IL-33 は通常のサイトカインの分泌経路を経て生産されるのではなく，感染や傷害により細胞がネクローシスに陥ると生理活性を有する 30 kDa の全長体型 IL-33 が細胞外に放出されることが示された[4]。これらの事実は，IL-33 が HMGB1（high mobility group box 1）や IL-1α のように細胞傷害により細胞外に放出される DAMPs（damage-associated molecular patterns）の機能を有していると考えられる。他方，興味深いことに IL-33 は構成的に核内のヘテロクロマチン領域に局在しており，さまざまな遺伝子の転写制御に係わる可能性が示唆されている[5]。しかしながら，核内 IL-33 がどのように免疫応答および炎症反応に係わるかは不明である。

IL-33 mRNA は組織では脳や脊髄などの中枢神経系，リンパ節，肺，皮膚やパイエル板に発現

しており，細胞では上皮細胞，血管内皮細胞，線維芽細胞，樹状細胞やマクロファージに発現する[3]。IL-33 受容体は膜結合型 ST2（IL-33Rα 鎖）と IL-1RAcP（IL-1 receptor accessory protein）（IL-33Rβ 鎖）のヘテロ二量体から構成される。IL-33 の ST2 への結合に伴い IL-1RAcP からシグナルが伝達され，細胞質内領域の TIR（Toll/IL-1R）ドメインに MyD88 および IRAK が会合し，TRAF6 を介したシグナルにより NF-κB および MAP kinase 経路が活性化される。

2. IL-33 の免疫学的役割

ST2 はヘルパー T 細胞のうち Th2 サブセットに選択的に発現するほか，肥満細胞，好塩基球，好酸球，$CD8^+$T 細胞および調節性 T 細胞に発現が認められ，IL-33 に応答して Th2 サイトカインが生産されることが示されている[6]。IL-33 は組織損傷の初期段階に検出されることがいくつかの実験モデルにおいて示されている。鞭虫 *Trichuris muris* を野生型マウスに感染させると，感染 3 日後をピークとして腸管組織における IL-33 mRNA 発現が亢進される[7]。感染防御に Th2 応答が必要とされる *T. muris* 感染モデルにおいて IL-33 は感染防御に働く。同感染における IL-33 生産は一過性であり，感染初期に生産される IL-33 の刺激により TSLP を始めとする他の Th2 サイトカイン誘導因子が生産されることから，IL-33 は Th2 応答の増幅および持続に作用すると捉えられる。また，IL-33 は炎症反応の初期段階において肥満細胞や好酸球など自然免疫系の誘導に係わる多様な細胞を活性化させる。炎症性疾患において IL-33 はアレルギー性疾患を増悪させる因子と考えられており，気管支喘息，炎症性腸疾患や慢性関節リウマチなどさまざまな疾患の発症に係わることが報告されている。近年，腸間膜の脂肪組織に存在する新たなリンパ球集積に IL-33 や IL-25 に反応し高い Th2 サイトカイン生産能を持つ innate lymphoid cell［ナチュラルヘルパー細胞，nuocyte，multipotent progenitor type 2（MPP^{type2}）細胞，innate helper type 2（Ih2）細胞など］が局在することが相次いで発見され，IL-33 が炎症反応の増幅および慢性化に係わる可能性が注目されている[8]。他方，アルツハイマー病の発症は脳に蓄積されるアミロイドβ（Aβ）による神経細胞の機能傷害が一因とされるが，IL-33 は Aβ の発現を減少させることが示されており IL-33 がアルツハイマー病の進行を抑制させる可能性が注目されている。

3. *P. gingivalis* ジンジパインによる歯肉上皮細胞の IL-33 発現誘導

成人性歯周炎は歯周炎関連細菌 *Porphyromonas gingivalis* による細菌感染症であり，同菌により引き起こされる炎症反応による歯周組織の破壊は歯を喪失する最大の要因である。最近われわれは，*P. gingivalis* により歯肉上皮細胞から IL-33 が誘導される可能性について検討を行った。ヒト歯肉上皮細胞株 Ca9-22 細胞を凍結乾燥 *P. gingivalis* 全菌体で刺激すると，48 時間後に IL-33 mRNA 発現が約 30 倍以上亢進されることを見出した（図 1）。同様に，*P. gingivalis* 全菌体の刺激により IL-25 ならびに TSLP mRNA 発現も著明に亢進された。そこで *P. gingivalis* により誘導された IL-33 蛋白の局在を免疫染色法ならびにウェスタンブロット法にて検討した結果，IL-33 蛋白は刺激 4 日後をピークに細胞質に局在していた。それでは，歯肉上皮細胞における IL-33 誘導活性を担う *P. gingivalis* の菌体成分はどのようなものであろうか。われわれは *P. gingivalis* 由来の fimbriae（線毛），リポペプチドないしリポポリサッカライドをそれぞれ歯肉上皮細胞に刺激した結果，いずれの菌体成分も IL-33 誘導活性を示さなかった。これらの実験結果から，歯肉上皮細胞における IL-33 誘導活性は Toll-like receptor アゴニストではなく他の成分により担われる可能性が推測された。これまでの報告から，肺上皮細胞を真菌 *Alternaria alternata* 由来抽出液で刺激すると TSLP が生産され，同誘導作用はシステインプロテアーゼ阻害剤により抑制されることが示されている[9]。*P. gingivalis* はジンジパインというシステインプロテアーゼを分泌する。ジンジパインは同菌が生産する総プロテアーゼ活性の約 85% を占めており，多彩な病原性を発揮することで歯周炎の増悪に深く関与することが明らかにされて

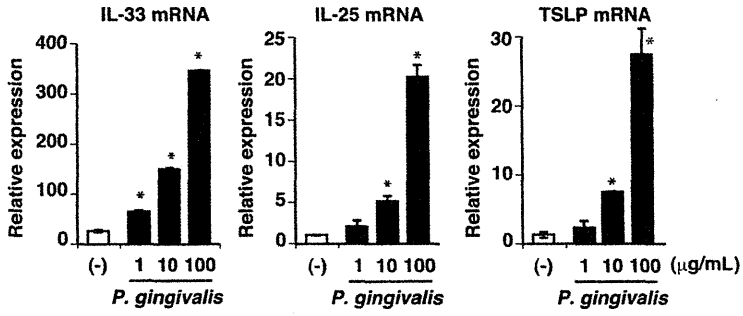

図 1　*P. gingivalis* によるヒト歯肉上皮細胞からの IL-33, IL-25 ならびに TSLP mRNA 発現誘導

ヒト歯肉上皮細胞株 Ca9-22 を *P. gingivalis* W83 株全菌体で 48 時間刺激した際の IL-33, IL-25 ならびに TSLP mRNA 発現をリアルタイム PCR 法により測定した。

*：ANOVA 検定にて無刺激群と刺激群間に有意差（$p<0.01$）が認められた。

いる[10]。ジンジパインはアルギニン残基を切断するジンジパイン-R（Rgp）とリジン残基を切断するジンジパイン-K（Kgp）にそれぞれ分類される。そこで *P. gingivalis* 全菌体を Rgp ないし Kgp の特異的阻害剤でそれぞれ前処理した後に Ca9-22 細胞に刺激した結果，IL-33 発現誘導は完全に抑制された。また，すべてのジンジパインを変異する KDP136 株（長崎大学口腔病原微生物，中山浩次博士より分与）では IL-33 誘導活性はみられなかった。これらの事実から，歯肉上皮細胞における IL-33 誘導活性は *P. gingivalis* が発現するジンジパインにより担われることが示された。

それでは，ジンジパインによる IL-33 誘導活性はどのようなシグナル伝達機構により担われるのであろうか。Rgp はヒト好中球に発現する G 蛋白共役 7 回膜貫通型受容体 protease-activated receptor-2（PAR-2）を活性化させることが明らかにされている[11]。Ca9-22 細胞は mRNA レベルおよび蛋白レベルにおいて構成的に PAR-2 を発現していることから，RNA 干渉法により PAR-2 mRNA 発現を抑制させた同細胞に *P. gingivalis* 全菌体を刺激した結果，コントロール siRNA 導入細胞に比べて PAR-2 siRNA 導入細胞では IL-33 mRNA 発現誘導は有意に減少した。PAR-2 を介したシグナルにより MAP キナーゼ経路ならびに NF-κB 経路が活性化されることから，*P. gingivalis* による MAP キナーゼ ERK，JNK ならびに p38 の活性化について検討した。全菌体を刺激すると 18 時間後に p38 の著明なリン酸化が検出され，IL-33 誘導活性は p38 阻害剤の処理により著明に抑制された。次に *P. gingivalis* による NF-κB 経路の活性化について，NF-κB プロモーターをトランスフェクションした Ca9-22 細胞を *P. gingivalis* 全菌体で刺激した際のルシフェラーゼ活性を測定した結果，刺激 3 時間後から NF-κB の活性化がみられ IL-33 誘導活性は NF-κB 阻害剤により抑制された。以上の結果から，ジンジパインによる歯肉上皮細胞からの IL-33 発現は，PAR-2，p38 および NF-κB を介した活性化により誘導されることが明らかになった（図 2）。今後，*P. gingivalis* により歯肉上皮細胞に誘導された IL-33 の放出メカニズムならびに IL-33 が歯肉炎へおよぼす影響について検討する必要がある。

おわりに

IL-33 は IL-25 や TSLP とともに皮膚や粘膜組織を構成する上皮細胞を中心に発現しており，Th2 応答の誘導を調節する。IL-33 はアレルギー性炎症の誘導を促進させることにより，気管支喘

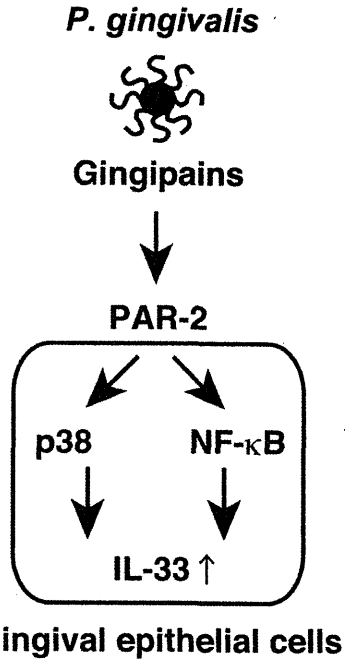

図 2 *P. gingivalis* によるヒト歯肉上皮細胞からの IL-33 誘導機構

息，炎症性腸疾患や慢性関節リウマチなどさまざまな疾患を増悪させる知見が報告されているが，IL-33 の発現および生産機構は十分に解明されていない。われわれは *P. gingivalis* 由来プロテアーゼであるジンジパインにより歯肉上皮細胞において IL-33 発現が亢進されることを示した。今後，歯周炎の病態形成において IL-33 が炎症の増悪因子となる可能性が想定され，今後の検討課題といえる。

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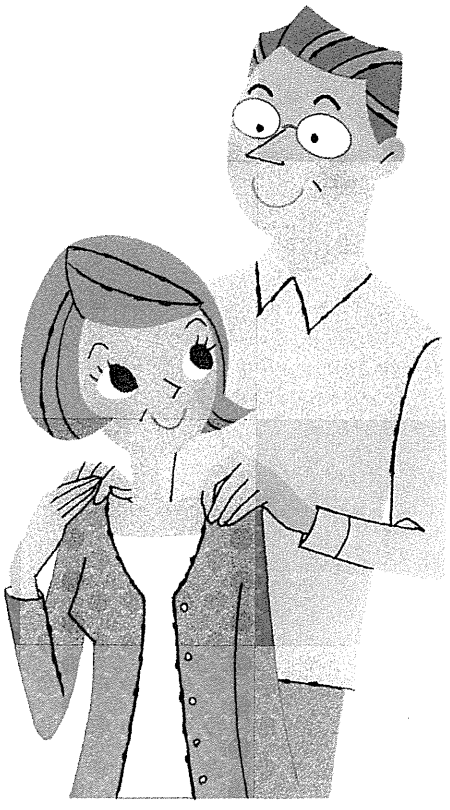
世界口腔保健学術大会記念

第18回口腔保健シンポジウム



健康寿命の鍵は、口の健康！

歯周病と全身の密接な関係

最近の研究によって、お口の病気である歯周病が、
糖尿病などのさまざまな全身疾患と関係していることが判明しつつあります。
また21世紀における国民の健康運動の取り組み「第二次健康日本21」が
本年から始まる予定で、歯と口腔の健康もクローズアップされています。
すべての人たちが健康寿命を延ばし、毎日を楽しく過ごせますよう、
歯周病の正しい知識と予防はもちろん、正しいお口のケアを身につけましょう。

監修●松下 健二 先生（独立行政法人 国立長寿医療研究センター 口腔疾患研究部 部長）／髙柴 正悟 先生（岡山大学大学院 医歯薬学総合研究科 教授）

健康寿命と口腔の密接な関係

口腔は、全身の健康を保つためにとても重要です。特に健康寿命と口腔には密接な関係があります。

健康寿命とは、日常で介護を必要とせずに自立した生活のできる期間を指します。厚生労働省は今年、初めてその数値を発表しました。2010年の平均は男性70・42歳、女性は73・62歳でした。一方、同じ2010年の平均寿命は男性79・55歳、女性86・30歳。両者の間に男性約9年、女性約13年のギャップがあります。これは、介護などを必要とする期間にあたります。

厚労省は、運動や食習慣などを改善することで、健康寿命を1・6年以上延ばすことを提案していますが、高齢になるとどうしても認知症や寝たきりといった問題が生まれます。

この問題に口腔、つまり口や歯の健康が深く関わっているのです。また脳卒中や心臓病、糖尿病などの発症と悪化にも口腔が深く関わっていることも分かっています。自分の歯がたくさん残っていると、全身疾患のリスクが低く、長生きになるというデータが、さまざまな国の研究から報告されています。

具体的には、歯がある人と無い人を比べると、残りの寿命が全然違います。歯を失った人のその後を観察すると、急に身体にいろいろな症状が出たり、生活習慣病の発症率が高くなったりしているのです。この他にも歯が無くなることは、がんの発症や死亡に関連があるというのです。歯が無いということは、特に消化器系のがんの発症と関連が高いと言われています。

第18回口腔保健シンポジウムが開催されました。

1994年に東京でおこなわれた世界口腔保健学術大会を記念し、口腔保健に関するさまざまな話題を取り上げて、理解を深める、第18回口腔保健シンポジウム(主催:社団法人日本歯科医師会、協賛:サンスター株式会社)が「健康寿命の鍵は、口の健康！～歯周病と全身の密接な関係～」をテーマに、2012年7月7日・津田ホール(東京)にて開催されました。松下健二先生からは「口腔と全身の密接な関係」と題して、健康長寿と口腔ケアについてお話いただきました。続いて高柴正悟先生からは同じテーマに関し、健康長寿と歯周病についてご説明いただきました。
また大林素子さんのミニトーク「マイドリーム～あきらめないで頑張れる、大林流・健康管理～」では、元バレーボール全日本代表の経験を活かした健康管理術やお口の健康について、楽しくお話しいただきました。

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国立長寿医療研究センター
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健康寿命を延ばすために

今後、日本でますます課題になると思われるのは認知症です。この認知症についても、歯との関係についていろいろな報告があります。健康な歯が多いこと、むし歯があってもちゃんと治療していること、もしくは歯が残っていることは、認知機能と関係があるというのです。

また高齢の方にとって肺炎は深刻な疾患です。日本人全体の死因に占める肺炎の割合は約10%ですが、そのうち96%までが65歳以上の高齢者です。高齢になると飲み込む機能が低下するため、誤嚥性肺炎が原因で亡くなる人が多いからです。

そうしたことから口のなかをきれいにする口腔ケアをおこなうと、肺炎の予防になると言われています。うがいや歯みがきはいつもしていると思いますが、顔の表情をうまく作ったり、口のまわりの筋肉を鍛えたり、舌の運動をしたり、唾液腺のマッサージで唾液の分泌をスムーズにすることも大切です。

歯の健康とともに口腔の機能を維持すると、おいしく食べられます。これは健康寿命を延ばすことにつながるのです。

OnePoint 知っていますか。「唾液の大切な役割」

唾液には重要な役割がたくさんあります。
何らかの原因で唾液が減ってしまうと歯周病やむし歯になりやすくなります。
もし下記の症状に心当たりがあるならば、歯医者さんにご相談ください。

唾液の主な役割

抗菌作用	再石灰化作用	浄化作用
むし歯、歯周病から肺炎まで感染症から身体を守る	脱灰で失われたミネラルの回復、歯質の保護	唾液の水分と嚥下により口のなかがきれいになる
緩衝作用	粘膜修復作用	円滑作用・作用
口のなかの酸性化を和らげる	傷を治し、老化を防止する神経成長因子を全身に届ける	食物や粘膜を湿らせて咀嚼・嚥下・発音などの運動を円滑にする

こんな症状があれば要注意！

口が乾く	のどが渇く	味がおかしい
口のなかがネバネバする	目が乾く	パンなど乾いたものが食べにくい

歯科医院での定期的なケアを

まず口のなかのばい菌が感染して炎症が起こることから始まります。その結果、歯ぐきが腫れたり、骨がやせたりして、歯のまわりの組織が壊れます。やがて歯が抜けて、機能を失います。これが歯周病で、いま話題になっている健康寿命と密接に関わっています。

歯周病になると、歯肉の上の皮が欠落して壊れ、ばい菌がどんどん身体のなかに入ります。その結果、動脈硬化などの病気が進行することもあります。

歯周病は生活習慣病や呼吸器系などの病気にも関わっています。早産や低体重児の出産、糖尿病にも関係があります。末期の腎疾患に悪い影響を与えるとも言われています。

歯は、特に治療を必要とする場合でなくても、定期的に専門的なクリーニングを受けていると、非常によい状態を保てます。歯周病対策の基本は「プラーク（歯垢）を除去する」「炎症を抑える」「形態を改善する」「リハビリ（歯を削ってかぶせたり、つないだりする補てつ治療）をする」というものです。

歯周病の治療としては歯みがきのほか、外科治療もあります。再生治療などを用い、歯周組織を再び感染が起きにくい形態へと改善する治療です。ただ、歯周病治療のいちばんの基本は「プラークの除去」。それがうまくできれば、高価な治療を受けなくても済むようになります。

GUEST SPEAKER

マイドリーム
～あきらめないで頑張れる、大林流健康管理～

イタリアのプロリーグでプレーしていたとき、私は1年の1/3が海外遠征でした。1年に10カ国ほど回っていたのです。その遠征中、むし歯になったこともありました。

日本だと、どんな地方でも歯科医院はありますよね。でも海外だと、無い地域も多くて、結構困りました。

あるときのこと、むし歯治療中なのに遠征で1ヶ月、家に戻ることができず、とても痛むので抜くことにしました。アジアのある国で病院に行ったら麻酔を打てる医師がおらず、結局断られてしまいました。そのときは海外に身を置いてしまったことで、当たり前のことをしっかりとやっている日本のすごさを感じました。それをしっかりやり続けることが、とても大切なのですね。

歯が痛むとプレーに集中できません。試合中であっても痛いものは痛いですから。そうしたことを避けるために、日々の歯みがきに気を付けるようになりました。

スポーツキャスター
大林 素子 氏

中学1年からバレーボールを始め、中学3年の時に東京都中学選抜に選出される。その後、高校バレーボール界の名門八王子実践高校に進む。86年日立入社、88年ソウル五輪、92年バルセロナ五輪に出場する。95年にイタリアセリエA・アンコーナに所属、日本人初のプロ選手となる。帰国後、東洋紡オーキスに所属、96年アトランタ五輪出場後、97年に引退する。現在、日本スポーツマスターズ委員会シンボルメンバー、JOC環境アンバサダー、福島県・しゃくなげ大使、環境省チャレンジ25キャンペーン応援団、JVA（日本バレーボール協会）広報委員、2012オリンピックデーラン・アンバサダー、観光庁2012「スポーツ観光マイスター」。

Effects of Exercise and Amino Acid Supplementation on Body Composition and Physical Function in Community-Dwelling Elderly Japanese Sarcopenic Women: A Randomized Controlled Trial

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OBJECTIVES: To evaluate the effectiveness of exercise and amino acid supplementation in enhancing muscle mass and strength in community-dwelling elderly sarcopenic women.

DESIGN: Randomized controlled trial.

SETTING: Urban community in Tokyo, Japan.

PARTICIPANTS: One hundred fifty-five women aged 75 and older were defined as sarcopenic and randomly assigned to one of four groups: exercise and amino acid supplementation (exercise + AAS; n = 38), exercise (n = 39), amino acid supplementation (AAS; n = 39), or health education (HE; n = 39).

INTERVENTION: The exercise group attended a 60-minute comprehensive training program twice a week, and the AAS group ingested 3 g of a leucine-rich essential amino acid mixture twice a day for 3 months.

MEASUREMENTS: Body composition was determined using bioelectrical impedance analysis. Data from interviews and functional fitness parameters such as muscle strength and walking ability were collected at baseline and after the 3-month intervention.

RESULTS: A significant group × time interaction was seen in leg muscle mass ($P = .007$), usual walking speed ($P = .007$), and knee extension strength ($P = .017$). The within-group analysis showed that walking speed significantly increased in all three intervention groups, leg muscle mass in the exercise + AAS and exercise groups, and knee extension strength only in the exercise + AAS group (9.3% increase, $P = .01$). The odds ratio for leg muscle mass and knee extension strength improvement was more than four times as great in the exercise + AAS group (odds ratio = 4.89, 95% confidence interval = 1.89–11.27) as in the HE group.

CONCLUSION: The data suggest that exercise and AAS together may be effective in enhancing not only muscle strength, but also combined variables of muscle mass and walking speed and of muscle mass and strength in sarcopenic women. **J Am Geriatr Soc 60:16–23, 2012.**

Key words: sarcopenic women; exercise; amino acid supplementation; muscle mass; muscle strength

Sarcopenia, defined as age-related involuntary loss of skeletal muscle mass and strength,[1,2] has been associated with physical disability, functional decline, falls, impaired mobility, and mortality in elderly people.[3,4] Therefore, treating or reversing sarcopenia is important in the maintenance of health and life expectancy in the elderly population. Although many factors, such as chronic disease, physical inactivity, and decreased muscle protein synthesis, may contribute to loss of muscle mass,[5–7] it has been suggested that only skeletal muscle disuse and undernutrition are potentially preventable or reversible with targeted interventions.[8]

Many studies have shown a strong relationship between resistance exercise and strength improvement, through which the efficacy of resistance exercise for the prevention and treatment of sarcopenia has been confirmed.[9] The previous studies have also shown that ingestion of essential amino acids can induce muscle protein anabolism in elderly adults.[10,11] One study showed that the combination of resistance exercise and essential amino acid supplementation (AAS) augmented muscle protein

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synthesis, suggesting it as a strategy to reverse sarcopenia[12] but in a small sample size. There are few randomized controlled trials (RCTs) on the effects of exercise and AAS on body composition and functional capacity.

The purpose of this study was to investigate the effects of exercise and AAS on muscle mass, strength, and walking ability in sarcopenic women.

METHODS

Subjects

A letter outlining the comprehensive geriatric health examination survey, describing its objective and the way that the personal data would be used, was mailed to the women randomly selected from the Basic Resident Register of 5,932 people aged 75 and older residing in the Itabashi ward of metropolitan Tokyo inviting them to participate in the study. Two thousand eighteen people responded to the mailed letters of invitation to participate in the study, with 1,670 people agreeing and 348 people declining to participate. The baseline assessment was conducted at the Tokyo Metropolitan Institute of Gerontology (TMIG) from October 12 to November 3, 2008. One thousand three hundred eighty-three women aged 75 and older were screened; 287 who originally agreed to participation were absent. Written informed consent was obtained for baseline screening; six people did not sign the informed consent form and were not included in this study.

Three hundred four of 1,377 women (22.1%) were operationally defined as sarcopenic (Figure 1), with selection based on categorization into one or more of the following inclusion criteria groups: appendicular skeletal muscle mass/height^2 less than 6.42 kg/m^2 and knee extension strength less than 1.01 Nm/kg[13,14] (n = 68), appendicular skeletal muscle mass/height^2 less than 6.42 kg/m^2 and usual walking speed less than 1.22 m/s (n = 65),[14] body mass index (BMI) less than 22.0 kg/m^2[14] and knee

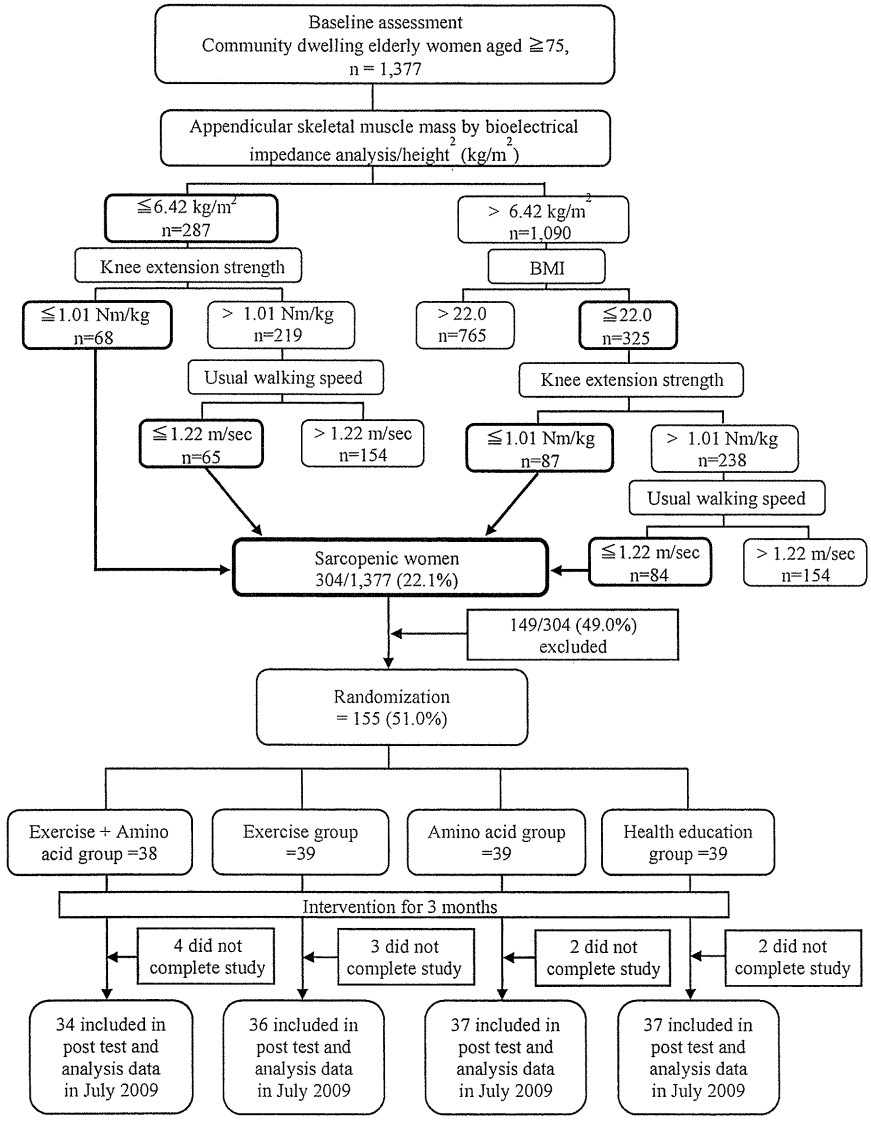

Figure 1. Algorithm for the selection of women who were operationally defined as sarcopenic and flowchart of participants in the randomized controlled trial of exercise and amino acid supplementation.

extension strength less than 1.01 Nm/kg (n = 87), and BMI less than 22.0 kg/m^2 and usual walking speed less than 1.22 m/s (n = 84). Exclusion criteria were severe knee or back pain; severely impaired mobility; impaired cognition (Mini-Mental State Examination (MMSE) score < 24);[16] missing baseline data; and unstable cardiac conditions such as ventricular dysrhythmias, pulmonary edema, or other musculoskeletal conditions. One hundred forty-nine (49.0%) of the potential sarcopenic participants were excluded because they were classified into one or more of the exclusion criteria or declined participation. The Clinical Research Ethics Committee of TMIG approved the study protocol. The intervention procedures were fully explained to all participants, and written informed consent was obtained (Figure 1).

Randomization

Randomization was performed after the baseline assessment; any variable that identified personal information was not included in the randomization process. Computer-generated random numbers were assigned to 155 participants who were then sorted and divided into four equal groups. The groups were randomly assigned to one of the four interventions groups: exercise + AAS (n = 38), exercise (n = 39), AAS (n = 39), or health education (HE; n = 39). All participants agreed to the group allocations that were mailed to them. There was no attempt to equalize the size of the groups based on their characteristics or to recruit subjects with specific characteristics. The co-investigators were blind to the randomization procedure and group allocations, separate physical therapy staff members who were also blind to the allocation of treatments collected data.

Outcome Measures

Outcome measures were evaluated according to data collected from interviews, body composition assessments using bioelectrical impedance analysis (BIA), and physical fitness tests at baseline and after the 3-month intervention.

Interview Survey

Face-to-face interviews were conducted to assess the individual's history of fractures and falls over the previous year, number of falls, cause of falls, urinary incontinence, exercise habits, smoking status, and MMSE score.

Body Composition Assessment

Measurements of height and weight were used to calculate BMI (kg/m^2). Body composition was measured using a segmental multifrequency BIA instrument that operated at frequencies of 5, 50, 250, and 550 kHz (Well-Scan 500, Elk Corp., Tokyo, Japan). Participants removed their socks, stood on two metallic electrodes on the floor scale barefoot, and held metallic grip electrodes placed in the palm of the hand with the fingers wrapped around the handrails. Using segmental body composition and muscle mass values of both legs, both arms, and the trunk, appendicular skeletal muscle mass and leg muscle mass values were obtained and used for analysis by summing the appropriate segmental muscle mass values.[13,17,18] Reliability of body composition measurements in all 155 participants in this study was not analyzed, although for the AAS group (n = 39), measurements were taken for a second time 1 week after baseline testing, and reliability was examined; the intraclass correlation coefficients (ICC) were: 0.98 for the right arm, 0.97 for the left arm, 0.97 for the right leg, 0.96 for the left leg, and 0.93 for the trunk.

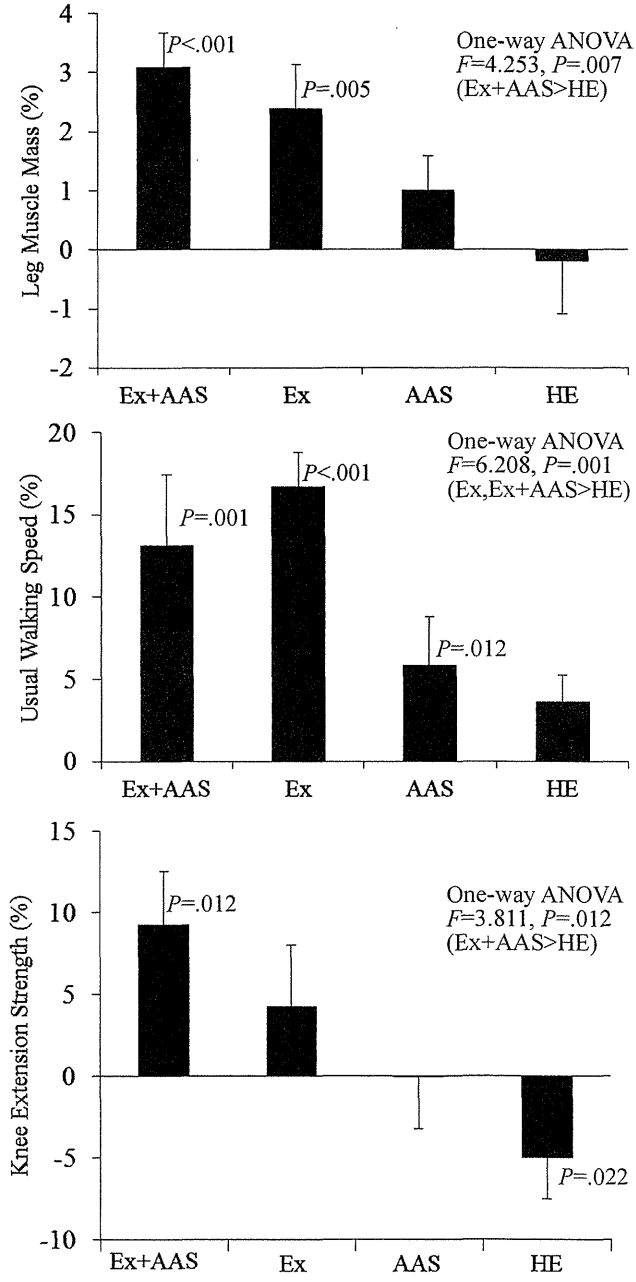

Figure 2. Mean percentage changes (standard errors) in leg muscle mass, usual walking speed, and knee extension strength after exercise (Ex), amino acid supplementation (AAS), both (Ex + AAS), or health education (HE). Bars indicate average changes from baseline to after the 3-month intervention. ANOVA = analysis of variance.

Functional Fitness Test

Calf girth and functional fitness variables including usual and maximum walking speeds and knee extension strength were measured. In measures of walking speed, participants were allowed to use assistive walking devices only if they expressed strong concerns about walking without a device or if there was any danger of falling. The knee extension strength measurement was taken twice, and the higher value divided by body weight (Nm/kg) were analyzed. The procedures for the functional fitness tests have been described in detail in previous reports.[19,20]

Intervention

Exercise

A comprehensive physical fitness and muscle mass enhancement training program of moderate intensity was provided for the participants in the exercise groups. The exercise intervention consisted of 60-minute exercise sessions held at the TMIG twice per week for 3 months. Each exercise intervention group was divided into two subgroups, with participants exercising together within their assigned group in one of four exercise sessions offered per day.

Each exercise session consisted of a 5-minute warm-up, 30 minutes of strengthening exercise, 20 minutes of balance and gait training, and 5 minutes of cool down. The strengthening exercises were performed in a progressive sequence from seated to standing positions. For each type of exercise, participants were instructed to complete up to eight repetitions of the movements. When the exercises were properly executed without significant fatigue or loss of proper execution, the resistance was increased. The progressive resistance was provided through the use of resistance bands or ankle weights. Intensity was maintained at approximately 12 to 14 on the Borg Rate of Perceived Exertion scale.[21] The principal investigator, along with the exercise instructor and assistant trainers, assessed each individual's ability to increase intensity.

Chair exercise: The chair-seated exercises were used in the early stages of the program because the participants were frail older adults and it provided a secure and stable position. Repetitions of toe raises, heel raises, knee lifts, knee extensions, and others were performed while seated on a chair. Hip flexions, lateral leg raises, and repetitions of other exercises were performed standing upright behind the chair and holding the back of the chair for stability.

Ankle-weight exercise: To strengthen lower extremities, a fixed weight was placed on the ankle while participants performed strengthening exercises. Weights of 0.50, 0.75, 1.00, and 1.50 kg were prepared and used in accordance with each participant's strength level as the resistance progressively increased. The exercises performed using these ankle weights included seated knee flexion and extension and standing knee flexion and extensions.

Exercises using a resistance band: Resistance bands were used to strengthen the upper and lower body. Lower body exercises included leg extension and hip flexion. Upper body exercises included double-arm pull downs and biceps curls.

Balance and gait training: The balance training was focused on improvement of static, dynamic, and lateral balancing ability. Exercises included standing on one leg, multidirectional weight shifts, tandem stand, and tandem walk. Participants practiced proper gait mechanics that focused on the maintenance of stability during walking and increasing stride length, toe elevation of the forward limb, heel elevation of the rear limb, frequency of stepping, and heel–floor angle. Exercises included raising the toes (dorsiflexion) during the forward swing of the leg, kicking off the floor with the ball of the foot, walking with directional changes, and gait pattern variations.

Amino Acid Supplementation

Essential AAS was provided for the participants in the AAS groups every 2 weeks. Packets of powdered amino acid supplements (42.0% leucine, 14.0% lysine, 10.5% valine, 10.5% isoleucine, 10.5% threonine, 7.0% phenylalanine, and 5.5% other) were provided for the participants to be taken with water or milk, and they were instructed to take the 3-g supplement two times a day (6 g daily) every day for 3 months.[22] To monitor their amino acid intake accurately, participants were given record sheets that were collected every 2 weeks on which they recorded what time of day they took the supplement and the amount of amino acid taken every day.

Health Education

Participants in the HE group took a class once a month for 3 months, a total of three times. The classes focused on cognitive function, osteoporosis, and oral hygiene. Participants were asked to continue their regular lifestyle habits, and no specific instructions on diet or physical activity were given.

Data Analysis

Sample size calculations using univariate one-factor repeated-measures analysis of variance (ANOVA) to examine significant differences in means at baseline and after the 3-month intervention ($\alpha = 0.05$, power = 0.80) with an effect size of 0.15 required a sample size of 28 participants. Esimating a potential attrition rate of 25%, 38 subjects per group were required.[23] One-way ANOVA was used to test any differences in baseline measures and percentage changes between groups, and chi-square tests were performed on categorical variables. Percentage changes in muscle mass and functional fitness after the intervention were calculated using the following formula: % change = ((postintervention value–baseline value)/(baseline value) × 100). Two-way repeated-measures ANOVA was used to evaluate the differences in the effect of the intervention on the outcome measures between groups, and a post hoc test was done on variables showing significant differences to determine which groups were different. Multiple logistic regressions were performed to compare the effects of the four intervention groups on each outcome variable after 3 months of intervention. All analyses were performed using SPSS version 15.0 of Windows (SPSS, Inc., Tokyo, Japan).