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Selective Proteolysis of Apolipoprotein B-100 by Arg-Gingipain Mediates Atherosclerosis Progression Accelerated by Bacterial Exposure

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Epidemiological studies suggest the association of periodontal infections with atherosclerosis, however, the mechanism underlying this association remains poorly understood. *Porphyromonas gingivalis* is the primary etiologic agent of adult periodontitis and produces a unique class of cysteine proteinases consisting of Arg-gingipain (Rgp) and Lys-gingipain (Kgp). To elucidate key mechanisms for progression of atherosclerosis by *P. gingivalis* infection, we tested the effects of the disruption of genes encoding Rgp and/or Kgp and inhibitors specific for the respective enzymes on atherosclerosis progression in apolipoprotein E–knockout mice. Repeated intravenous injection of wild-type *P. gingivalis* resulted in an increase in atherosclerotic lesions as well as an increase in the serum LDL cholesterol and a decrease of HDL cholesterol in these animals. LDL particles in *P. gingivalis*–injected animals were modified as a result of selective proteolysis of apoB-100 in LDL particles. This modification of LDL by *P. gingivalis* resulted in an increase in LDL uptake by macrophages and consequent foam cell formation *in vitro*. The atherosclerotic changes induced by *P. gingivalis* infection were attenuated by disruption of Rgp-encoding genes or by an Rgp-specific inhibitor. Our results indicate that degradation of apoB-100 by Rgp plays a crucial role in the promotion of atherosclerosis by *P. gingivalis* infection.

Key words: apolipoproteins, atherosclerosis, bacterial proteinases, gingipains, periodontal disease.

Atherosclerosis is a complex disease with multiple risk factors and has been suggested by epidemiological studies to be associated with periodontal disease, a chronic inflammatory condition that results in destruction of periodontal tissue and alveolar bone (*1*, *2*). Although the mechanism underlying this association remains poorly understood, it is likely related to the virulence properties of the infecting organisms. *Porphyromonas gingivalis* is the primary etiologic agent of adult periodontitis (*3*). This bacterium is able to invade and infect epithelial, endothelial, and vascular smooth muscle cells, and it has been shown to alter endothelial function (*4*). Recently, it has been identified in human carotid and coronary atheromas (*5*–*7*), implying its direct involvement in atheroma lesion development. Accumulating evidence has also demonstrated that *P. gingivalis*, but not other periodontal pathogens, is able to induce platelet aggregation (*8*–*12*), LDL aggregation (*13*), and macrophage foam cell formation (*14*), which are characteristics of atheromas. Given their frequent access to the systemic circulation of individuals with periodontitis, through events such as toothbrushing, scaling and root planing, tooth extraction, and periodontal surgery (*15*), periodontal pathogens including *P. gingivalis* may participate in the development of atherosclerosis. Although many *in vitro* studies, besides epidemiological studies, have suggested a possible association between *P. gingivalis* and atherosclerosis, the real significance of this bacterium in etiology of athrosclerosis is still unidentified. Also, whether and to what extent long-term *P. gingivalis* challenge accelerates atheroma lesion development remain to be fully understood. It goes without saying that little is known of the mechanism by which *P. gingivalis* might contribute to promotion of atherosclerosis.

P. gingivalis produces a wide variety of virulence factors, including proteases, lipopolysaccharide, capsular polysaccharides, hemagglutinins, and fimbriae (*16*). Among these factors, a unique class of cysteine proteinases, termed gingipains which consist of arginine-specific (Rgp) and lysine-specific (Kgp) proteases, are implicated in a wide range of both pathological and physiological processes of *P. gingivalis*, including destruction of periodontal tissue, disruption of host defense mechanisms, processing of bacterial cell surface and secretory proteins, and acquisition of heme and amino acids (*17*, *18*). Based on the finding that Rgp is encoded by two genes, *rgpA* and *rgpB*, and Kgp is encoded by a single gene, *kgp*, we constructed various *P. gingivalis* mutants lacking Rgp (*rgpA* and/or *rgpB*) and/or Kgp and revealed that these two enzymes play a crucial role in bacterial virulence (*19*–*23*). In addition, we recently developed novel proteinase inhibitors specific for Rgp (KYT-1) and Kgp (KYT-36) and substantiated the importance of these enzymes in bacterial virulence both *in vivo* and *in vitro* (*24*, *25*). However, whether or how Rgp or Kgp are associated with the

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progression of atherosclerosis *in vivo* has still remained unclear. This study was therefore undertaken to determine whether and how *P. gingivalis* infection promotes atherosclerosis *in vivo*, especially any possible involvement of Rgp and/or Kgp in this process.

MATERIALS AND METHODS

Materials and Bacteria—KYT-1 and KYT-36 were synthesized as described previously (*25*) and dissolved in 0.01% dimethyl sulfoxide (final)/phosphate-buffered saline (PBS). Human native LDL and HDL cholesterols were obtained from Calbiochem (La Jolla, CA). Polyclonal antibodies to apolipoprotein (apo) B and to apoA-I were from Santa Cruz Biotechnology (Santa Cruz, CA) and Rockland (Gilbertsville, PA), respectively. Bacterial strains used were *P. gingivalis* ATCC 33277 (wild type), the Rgp/Kgp-null (*rgpA rgpB kgp*–deficient) triple-mutant KDP136 (*26*), the Kgp-null (*kgp*) mutant (KDP129) (*20*), and an Rgp-null (*rgpA rgpB*–deficient) double-mutant (KDP133) (*26*). Cell growth for bacterial strains and preparation of their culture supernatants were performed as described previously (*19*).

Mice—Mice homozygous for disruption of the apoE gene were obtained from The Jackson Laboratories (Bar Harbor, ME). Mutant mice were maintained as heterozygotes by crossing male heterozygotes with wild-type C57BL/6 females. The male apoE-null mice used in this study were generated by crossing heterozygotes. All animal experiments were performed in accordance with the ethical guidelines of the Animal Care and Use Committee of Kyushu University. Male apoE-null mice weaned at 4 weeks after birth were fed a high-fat diet (PMI Nutrition International, Richmond, IN) and, after 4 weeks, injected intravenously once a week for up to 12 weeks with wild-type (WT) *P. gingivalis* or KDP136 [1×10^7 colony-forming units (CFU) in 100 μl of PBS] or with vehicle. In some experiments, WT were also intravenously injected into the mice in combination with KYT-1 or KYT-36 (100 μl of 1 μM).

Analysis of Atherosclerotic Lesions—Mice were deeply anesthetized by diethyl ether and the heart was gently perfused through the pulmonary vein with saline. Sixteen serial cross-sections were cut at intervals of 7 μm from the aortic sinus of each animal, fixed with 4% paraformaldehyde, stained with 2% Sudan IV in 70% ethanol, counterstained with hematoxylin, and examined with a light microscope. The area of atherosclerotic lesions was measured with a computer-assisted image analysis system (Image Gauge; Fujifilm, Tokyo, Japan). The maximal lesion area determined from the 16 sections was taken as the lesion size for each animal and expressed as percentages of the total area of the aortic sinus.

Measurement of Serum Cholesterol Levels—Venous blood was collected from the tail of mice, and serum was isolated from the blood by centrifugation at $1,200 \times g$ for 10 min after clotting at room temperature. The serum levels of total cholesterol, LDL cholesterol, and HDL cholesterol were determined with the use of a quantitative kit (Cholestest; Daiichi Pure Chemicals, Tokyo, Japan). In some experiments, serum lipoproteins were also analyzed by fractionation of serum cholesterols using a dual-detection high performance liquid chromatography (HPLC) system (Skylight Biotech, Akita, Japan) fitted with two tandem TSK gel Lipopropak columns (300 by 7.8 mm; Tosoh, Tokyo, Japan) according to the procedure of Usui *et al*. (*27*).

Electrophoretic Analysis of Lipoproteins—For electrophoretic mobility-shift analysis of lipoproteins, serum (2 μl), LDL (20 μg), or HDL (20 μg) was applied to 0.6% (for serum and LDL) or 0.8% (for HDL) agarose gels and subjected to electrophoresis for 90 min (serum or LDL) or 50 min (HDL). The gels were fixed for 15 min in 75% ethanol containing 5% acetic acid, stained with 2% Sudan IV for 60 min, and washed with 30% ethanol. SDS-PAGE under reducing conditions and subsequent immunoblot analysis were performed as described (*28*).

Detection of P. gingivalis in ApoE-Knockout Mice—Whole blood was collected from mice at the indicated times after the first injection with *P. gingivalis* WT or KDP136 (1×10^7 CFU in 100 μl) and then plated on CDC Anaerobe Blood Agar (Nippon Becton Dickinson, Tokyo, Japan). The number of generated colonies was counted and is presented as CFU per 100 μl of blood. For detection of *P. gingivalis* in the heart tissue, DNA was extracted from the heart of male apoE-knockout mice injected weekly with vehicle, *P. gingivalis* WT or KDP136 using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and then subjected to PCR with primers specific for a 197-bp region of the gene for 16S ribosomal RNA of *P. gingivalis* (5′-TGTAGATGACTGATGGTGAAA-3′, 5′-ACGTCATCCCCACCTTCCTC-3′). Polymerase chain reaction (PCR) was performed as described previously (*29*), and the PCR product was detected by agarose gel electrophoresis. Genomic DNA extracted from *P. gingivalis* WT or KDP136 was also subjected to PCR as a positive control.

Treatment of Lipoproteins with P. gingivalis in Vitro and Foam Cell Formation—Human native LDL or HDL (50 μg) was incubated for 30 min at 37°C with *P. gingivalis* WT or KDP136 (1×10^7 CFU, unless indicated otherwise) in 100 μl of PBS. The mixture was then centrifuged at $25,000 \times g$ for 20 min to separate the lipoprotein from the bacteria. The lipoprotein sample was then added to mouse peritoneal macrophages (1×10^6 cells/well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3% bovine serum albumin, penicillin (50 U/ml), and streptomycin (50 μg/ml), and the mixture was incubated for 18 h at 37°C under 5% CO_2. The cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, stained for 15 min with 2% Sudan IV in 70% ethanol, and counterstained with hematoxylin.

Preparation of Oxidized LDL—Native human LDL was dialyzed against Ca^{2+}- and Mg^{2+}-free PBS, and adjusted to a 100 μg/ml concentration with the same buffer. $CuSO_4$ was added to the LDL solution at final concentration of 0.1 mM, and incubated at 37°C for 14 h. The reaction was stopped by addition of 1 mM EDTA, and then extensively dialyzed against Ca^{2+}- and Mg^{2+}-free PBS.

Statistical Analysis—Quantitative data are presented as means ± SD. Differences in the extent of atherosclerosis among groups were evaluated by two-way analysis of variance followed by Student's *t* test. Differences in plasma cholesterol levels were assessed by the paired Student's *t* test. A P value of <0.05 was considered statistically significant.

RESULTS

Effect of Deficiency in Both Rgps- and Kgp-Encoding Genes on Atherosclerosis Progression in ApoE-Knockout Mice Injected with P. gingivalis—To directly examine the connection between *P. gingivalis* infection and the acceleration of atherosclerosis progression *in vivo*, we injected intravenously *P. gingivalis* WT or its mutant deficient in both Rgp and Kgp (*rgpA rgpB kgp* triple-mutant, designated KDP136) into apoE knockout mice, which are animal models known to spontaneously develop pronounced hyperlipidemia and manifest enhanced atheroma formation in response to feeding with a high-fat diet (*30*, *31*). Injections were administered once a week from 8 weeks of age, 4 weeks after weaning and the onset of feeding with the high-fat diet. Six weeks after the initiation of injections, there was no apparent difference in the extent of lipid-rich lesions (stained with Sudan IV) of the aortic sinus among mice injected with *P. gingivalis* WT, the KDP136 mutant, or vehicle (Fig. 1A). The lesions were flat or partially protruding into the vascular lumen of the aortic sinus, with the gross appearance of early atheromas. At 9 weeks after the onset of injections, the lesion area was increased in all three groups of animals but was 50 or 64% greater for mice injected with *P. gingivalis* WT than for those injected with KDP136 or with vehicle, respectively, as determined by morphometric analysis (Fig. 1, B and D). Accumulation of smooth muscle cells and macrophages in the atherosclerotic lesions of mice injected with *P. gingivalis* WT was also evident at this time, resulting in a marked thickening of the intima that is characteristic of the established stage of atherosclerosis. At 12 weeks, the extent of the atherosclerotic lesions in mice injected with *P. gingivalis* WT had not increased further compared with that apparent at 9 weeks, whereas the area of lesions in mice injected with KDP136 or vehicle had increased to the level manifested by the animals injected with the WT (Fig. 1C). These results thus indicated that atherosclerotic lesions progress more rapidly in apoE-knockout mice injected with *P. gingivalis* WT than in those injected with KDP136 or vehicle.

Effect of Infection with P. gingivalis WT and KDP136 on Serum Lipid Levels in ApoE-Knockout Mice—The hypercholesterolemia with elevated low density lipoprotein (LDL) cholesterols and decreased high density lipoproteins (HDL) is well accepted as a major direct etiological factor for atherosclerotic diseases. The lipid profiles in the serum of mice injected with vehicle and *P. gingivalis* WT for 9 weeks were determined by fractionation of serum cholesterols using a dual detection HPLC size exclusion system with two tandem TSK gel columns. The total cholesterol level in *P. gingivalis*–injected mice (2,047.4 ± 103.5 mg/dl) was significantly higher than that in vehicle-injected mice (1,557.8 ± 67.5 mg/dl) (Table 1). The concentrations of LDL, very low density lipoprotein (VLDL), and chylomicron (CM) cholesterols also revealed statistically significant increases in the serum of *P. gingivalis*–injected mice compared to the vehicle-injected mice. In contrast, the HDL cholesterol concentration was significant lower in the serum of *P. gingivalis*–injected mice (38.9 ± 2.6 mg/dl) than that in the serum of vehicle-injected mice (57.4 ± 1.8 mg/dl). The serum concentrations of LDL- and HDL-cholesterols in the mice injected with

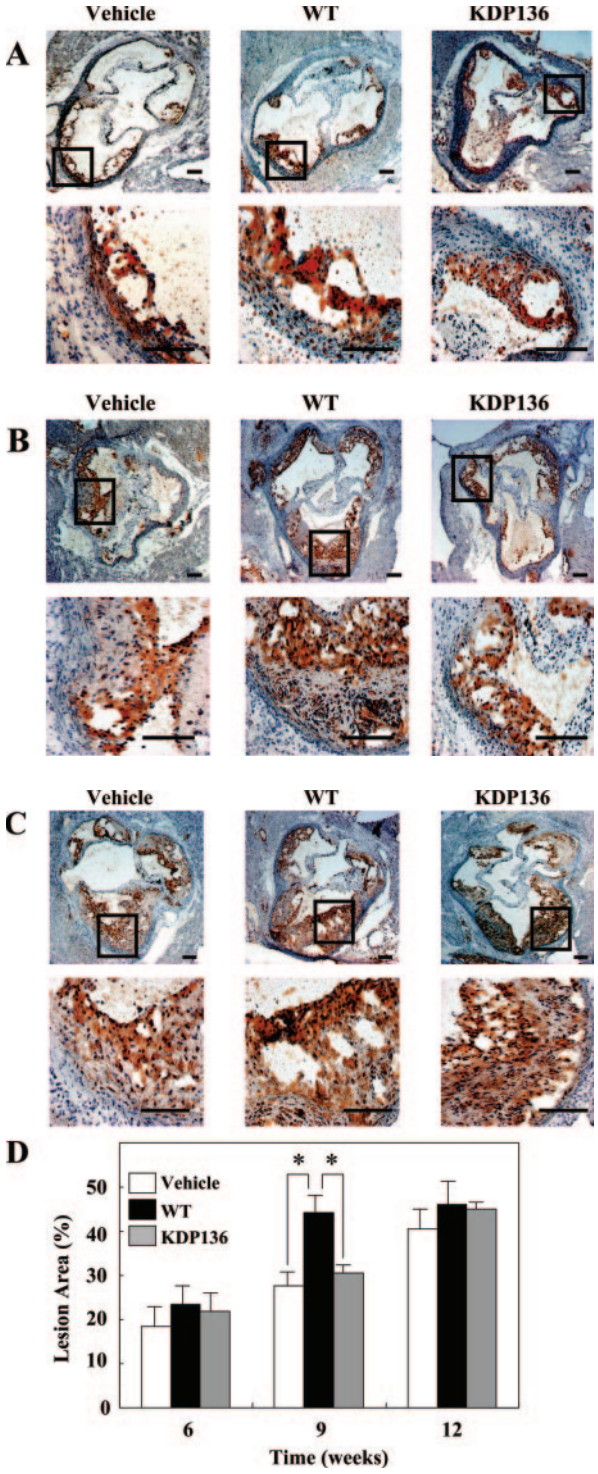

Fig. 1. **Arterial lesions in apoE-knockout mice fed a high-fat diet, injected with *P. gingivalis* WT or KDP136.** Representative Sudan IV–stained sections of the aortic sinus from mice injected weekly with vehicle, *P. gingivalis* WT, or KDP136 for 6 (A), 9 (B), or 12 (C) weeks. The boxed regions in the upper panels for the animals at each stage are shown at higher magnification in the lower panels. Scale bars, 100 μm. D: Morphometric analysis of the total area of atherosclerotic lesions in animals treated with wild-type *P. gingivalis* or KDP136. Data are means ± SD of values from at least six mice per group. **P*< 0.001 *versus* the corresponding value for wild-type *P. gingivalis*–injected mice.

vehicle, *P. gingivalis* WT, and KDP136 were also compared at each time point from initiation of injection up to 12 weeks. The serum concentration of LDL cholesterol in the animals injected with *P. gingivalis* WT is consistently higher than in those injected with KDP136 or vehicle (Fig. 2). Values for the relative LDL concentrations of *P. gingivalis* WT– and vehicle–treated sera at 2, 8 and 12 weeks after initiation of injection were 1.36, 1.33, and 1.67, respectively. There were no significant differences in serum LDL levels between KDP- and vehicle-injected apoE knockout mice. On the contrary, the serum HDL cholesterol levels were significantly lower in mice injected with *P. gingivalis* WT than in vehicle- and KDP136-injected mice. These results were thus indicative of a close relation between changes in serum lipoprotein profile and acceleration of atherosclerosis by *P. gingivalis* in apoE knockout mice.

Detection of P. gingivalis in Blood Circulation and Atherosclerotic Lesions of ApoE-Knockout Mice—It has recently been shown that human atherosclerotic plaques contain periodontal pathogens, including *P. gingivalis* and *Actinobacillus actinomycetemcomitance* (*5–7*, *32*). We thus determined the clearance rate of *P. gingivalis* WT and KDP136 in blood and their presence in atherosclerotic lesions of apoE knockout mice. The persistence of the WT bacterium in blood was significantly greater than that of KDP136 (Fig. 3A). PCR analysis revealed that DNA isolated from heart of apoE-knockout mice injected with *P. gingivalis* WT at 9 and 12 weeks, likewise genomic DNA extracted from the WT bacterium and KDP136, reacted with primers specific for 16S ribosomal RNA of this bacterium, while no reaction was detectable for that from animals injected vehicle and KDP136 (Fig. 3B), suggesting the presence of *P. gingivalis* WT, but not that of KDP136, in atherosclerotic lesions at 9 and 12 weeks after initiation of weekly injections.

Effects of Inhibitors Specific for Rgp and Kgp on Promotion of Atherosclerosis by P. gingivalis Infection—In contrast to *P. gingivalis* WT, KDP136 is structurally devoid of cell surface adhesion molecules such as fimbriae and functionally lacks abilities to coaggregate, bind hemoglobin, and induce hemagglutination (*27*, *33*). Therefore, to exclude the possibility that the inability of KDP136 to promote atherosclerosis *in vivo* was due to such structural or functional defects for this mutant, we examined the effects of proteinase inhibitors specific for Rgp (KYT-1) or for Kgp (KYT-36) on the development of atherosclerosis in the *P. gingivalis*–injected animals. These inhibitors have proved informative in assessment of whether and to what extent the proteolytic activities of Rgp and Kgp contribute to biological activities of *P. gingivalis in vivo* and *in vitro* (*25*). The results indicated that the extent of atherosclerotic lesions in mice injected for 9 weeks with *P. gingivalis* WT was markedly reduced by treatment with KYT-1 but not by that with KYT-36 (Fig. 4A). Consistent with this finding, the serum LDL and HDL cholesterol levels in mice injected with the WT bacterium were normalized by treatment with KYT-1 but not by that with KYT-36 (Fig. 4B).

Modification of LDL Particles by P. gingivalis in Vivo and in Vitro—Although an increased serum LDL cholesterol level is considered an important risk factor for atherosclerosis, native LDL cholesterol is not directly associated with atherosclerosis progression (*34*). Modification of LDL cholesterol by oxidation or aggregation, followed by its uptake by macrophages, is thus thought to be important for the promotion of atherosclerosis (*35*, *36*). Therefore, we next asked whether LDL cholesterol was modified in the serum of mice injected with *P. gingivalis*. An electrophoretic mobility-shift assay revealed that, whereas LDL cholesterol of mice injected with KDP136 or vehicle for up to 12 weeks exhibited no marked change in mobility, that of mice injected with *P. gingivalis* WT manifested a time-dependent increase in electrophoretic mobility (Fig. 5A), indicating the aggregation of LDL. The serum of mice in all three groups showed no detectable change in the mobility of HDL cholesterol (data not shown). These findings were further substantiated by *in vitro* experiments with human native LDL and HDL

Table 1. **Serum lipid levels in the mice injected with vehicle and *P. gingivalis* WT for 9 weeks.**

Serum lipids	Vehicle	WT	*P* value
Cholesterol	(mg/dl)	(mg/dl)	
Total	1,557.8 ± 67.5	2,047.4 ± 103.5	0.002
HDL	57.4 ± 1.8	38.9 ± 2.6	<0.001
LDL	410.0 ± 24.0	614.6 ± 36.2	0.001
VLDL	1,087.6 ± 44.3	1,387.5 ± 66.3	0.003
CM	2.68 ± 0.75	6.38 ± 0.93	0.006
Triglyceride	13.8 ± 3.2	7.8 ± 1.2	0.039

Values were represented as means ± SD ($n = 3$). The p values for the differences between vehicle- and *P.g.*-injected mice were calculated by Student's t-test.

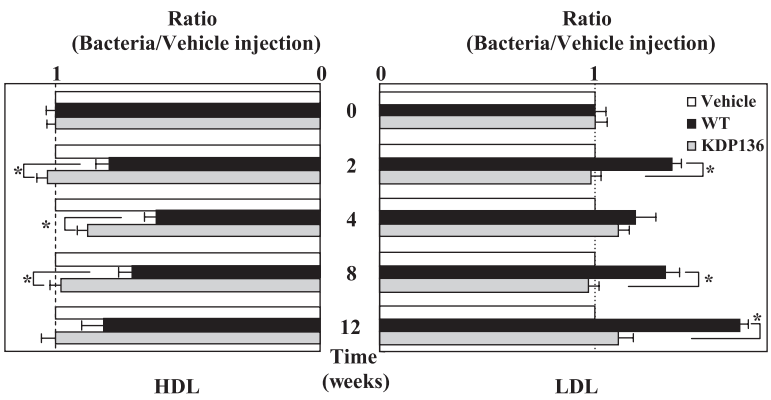

Fig. 2. **Effect of infection with *P. gingivalis* WT or KDP136 on serum cholesterol levels in apoE-knockout mice fed a high-fat diet.** Male apoE-null mice were fed a high-fat diet from 4 weeks of age and injected once a week for up to 12 weeks with *P. gingivalis* (black), KDP136 (grey), or vehicle (open) beginning at 8 weeks of age. The serum levels of LDL cholesterol and HDL cholesterol were determined at the indicated times relative to the initiation of injections. Values were shown as the relative LDL concentrations in *P. gingivalis* WT– or KDP136– and vehicle-injected mice. Data are means ± SD of values from six mice per group. *$P <$ 0.01 *versus* corresponding values for vehicle-treated mice.

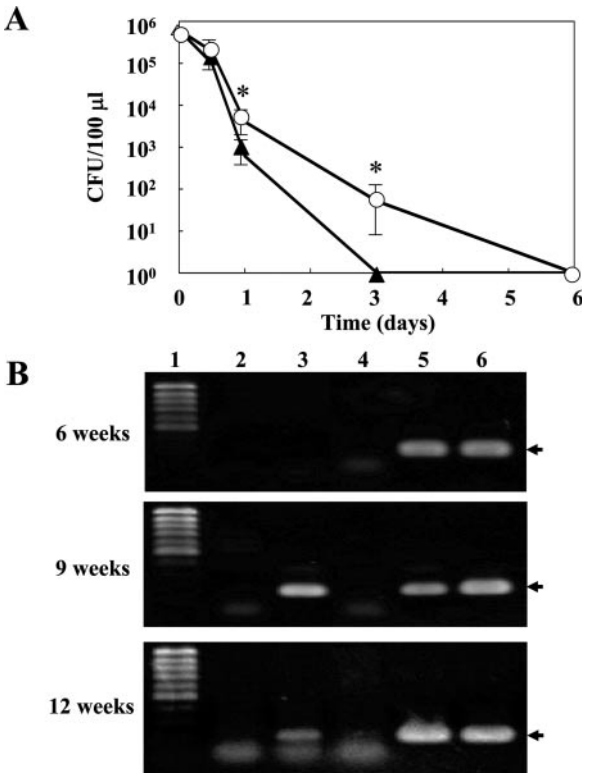

Fig. 3. **Detection of *P. gingivalis* bacteria in apoE-knockout mice.** A: Whole blood was collected from mice at the indicated times after the first injection with *P. gingivalis* WT (open circles) or KDP136 (closed triangles). Time 0 corresponds to immediately after injection with 1×10^7 CFU of bacteria in 100 µl. The blood was then plated on CDC Anaerobe Blood Agar. The number of generated colonies was counted and is presented as CFU per 100 µl of blood. Data are means ± SD of values from six mice per group. $^*P < 0.05$ *versus* corresponding value for KDP136-injected mice. B: Male apoE-knockout mice were fed a high-fat diet and injected weekly for 6, 9, or 12 weeks with vehicle (lane 2), *P. gingivalis* WT (lane 3), or KDP136 (lane 4). DNA was then extracted from the heart with the use of an RNeasy Mini Kit and then subjected to PCR with primers specific for a 197-bp region of the gene for 16S ribosomal RNA of *P. gingivalis* (5′-TGTAGATGACTGATGGTGAAA-3′, 5′-ACGTCATCCCCACCTTCCTC-3′). PCR products (arrows) were detected by agarose gel electrophoresis. Genomic DNA extracted from *P. gingivalis* (lane 5) or KDP136 (lane 6) was also subjected to PCR as a positive control. Lane 1, DNA size markers.

cholesterols. While incubation with KDP136 had no effect on the electrophoretic mobility of LDL cholesterol, exposure to *P. gingivalis* WT resulted in a time-dependent increase in LDL cholesterol mobility (Fig. 5B). Again, neither WT nor mutant bacteria affected the electrophoretic mobility of native HDL cholesterol even *in vitro*. In addition, LDL cholesterol treated with culture supernatants of *P. gingivalis* WT or of the Kgp-null mutant (KDP129) exhibited an increased electrophoretic mobility, with the effect of the former supernatants being greater than that of the latter (Fig. 5C). In contrast, culture supernatants of an Rgp-null (*rgpA rgpB*–double deficient) mutant (KDP133) or of KDP136 had no such effect. These results strongly suggest that Rgp plays an important role in modification of serum LDL cholesterol in mice infected with *P. gingivalis*.

The progressive accumulation of modified cholesterol in macrophages, resulting in the formation of foam cells in the vessel intima, is an important step in the early development of atherosclerotic lesions (*37*). Given that modification of LDL cholesterol by events such as aggregation or oxidation is required for its uptake by macrophages (*34–36*), we explored the effect of treatment of LDL cholesterol with *P. gingivalis* on its uptake by macrophages and consequent foam cell formation. Incubation of mouse macrophages with native LDL cholesterol treated with *P. gingivalis* WT, but not with that treated with KDP136, induced foam cell formation (Fig. 5D). Given that an increased serum concentration of HDL cholesterol protects against the development of atherosclerosis in mice and humans (*38*, *39*), we examined the effect of native HDL cholesterol on foam cell formation induced by *P. gingivalis*–treated LDL cholesterol. We found that HDL cholesterol inhibited the formation of foam cells from mouse macrophages induced by *P. gingivalis*–treated LDL cholesterol.

Role of Rgp in P. gingivalis–Induced LDL Modification—To characterize the LDL modification mediated by *P. gingivalis*, we examined the effects of Rgp or Kgp deficiency *in vivo* as well as of proteinase inhibitors specific for Rgp and Kgp *in vitro* on apoB-100, which is the major protein component of LDL implicated in maintenance of cellular cholesterol homeostasis as well as pathogenesis of atherosclerosis (*40*, *41*). Whereas apoE-knockout mice injected with KDP136 or vehicle manifested a time-dependent increase in the amount of apoB-100 in serum, repeated injection with *P. gingivalis* WT resulted in marked degradation of apoB-100 (Fig. 6A). An unchanged immunoreactive band detected below apoB-100 protein was estimated to be apoB-48 protein, as the antibodies used can recognize a common NH_2-terminal sequence of these two proteins. In contrast, the serum level of apoA-I, the major protein component of HDL particles, did not differ among the three groups of mice (Fig. 6B). Consistently, the *in vitro* treatment of sera from apoE-null mice fed a high-fat diet with *P. gingivalis* WT also resulted in a rapid degradation of apoB-100 and a time-dependent generation of its degradation product of 62 kDa (Fig. 7A) and the concurrent aggregation of LDL (Fig. 7B). These alterations were strongly inhibited in the presence of KYT-1 but not KYT-36. However, no significant degradation of apoA-I was observed with the same serum (Fig. 7C). These results support the notion that Rgp is responsible for the modification of LDL cholesterol by *P. gingivalis* and its subsequent uptake by macrophages to form foam cells.

Different Modification of LDL with P. gingivalis Infection and Oxidation—It is well known that LDL is heterogenous particles varying in size, composition, and structure. LDL particles are composed of apoB-100 protein of 4,536 amino acids residues and approximately 3,000 lipid molecules (*40*). Previous studies demonstrated that LDL particles were extensively modified when treated with proteolytic or lipolytic enzymes and extensive oxidation *in vitro* (*42–46*). Therefore, to further clarify the nature of *P. gingivalis*–modified LDL, we compared the structural and functional features of LDL particles treated with *P. gingivalis* WT or the oxidant $CuSO_4$. The mobility in

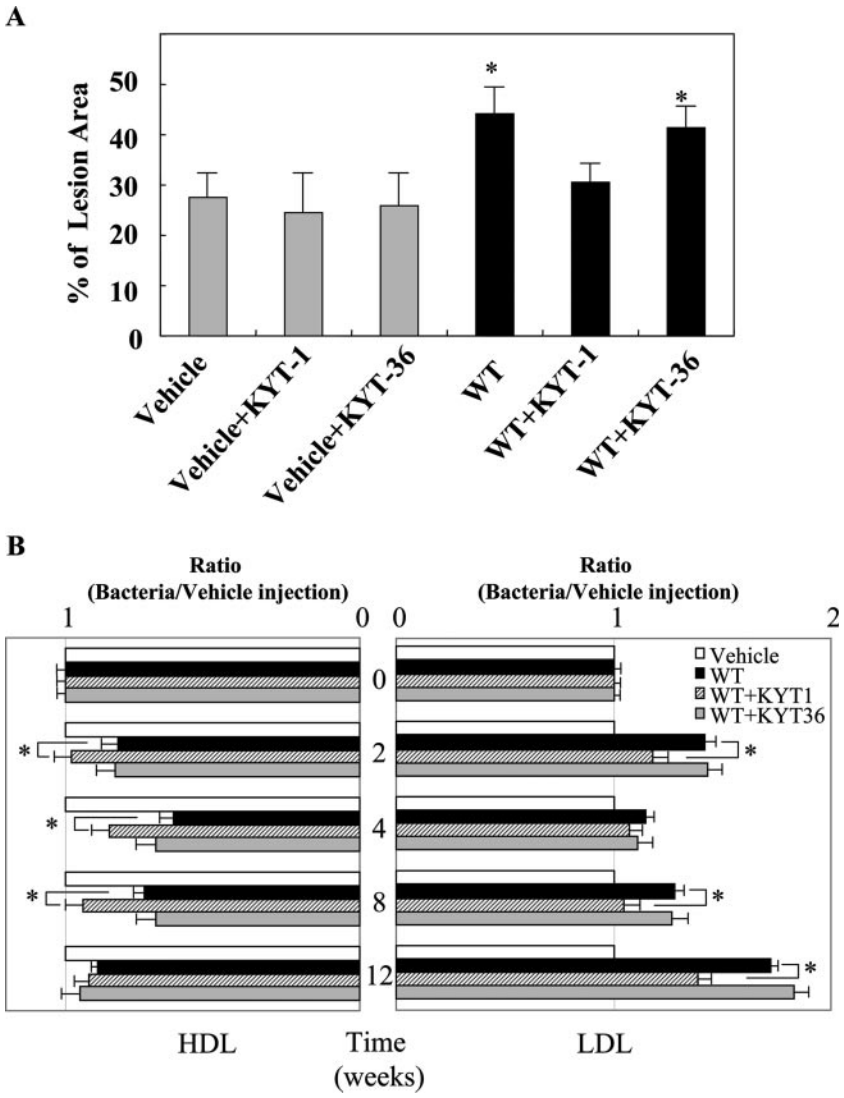

Fig. 4. **Effects of proteinase inhibitors specific for Rgp and Kgp on the development of arterial lesions in apoE knockout mice fed a high-fat diet, injected with *P. gingivalis* WT.** A: Morphometric analysis of the total area of atherosclerotic lesions in animals treated 9 weeks after the initiation of weekly injections with vehicle or *P. gingivalis* WT either alone or together with KYT-1 or KYT-36, as indicated. Data are means ± SD of values from at least six mice per group. *$P < 0.001$ *versus* corresponding value for vehicle-injected mice. B: Serum levels of LDL and HDL cholesterol in apoE-knockout mice fed a high-fat diet and injected once a week for up to 12 weeks with *P. gingivalis* WT in the absence (black) or presence of KYT-1 (hatched) or KYT-36 (grey). Values were shown as the relative LDL concentrations in each inhibitor- and vehicle-injected mice. Data are means ± SD of values from six mice per group. *$P < 0.01$ *versus* the corresponding values for apoE knockout mice injected with *P. gingivalis* without inhibitors.

agarose gel electrophoresis of native LDL treated with 0.1 mM $CuSO_4$ at 37°C for 14 h remarkably increased (Fig. 8A). The extent of increase in the mobility was dependent on incubation time and oxidant concentrations (data not shown). Likewise, *P. gingivalis* WT, but not KDP136, significantly increased the mobility of LDL, although its extent was much smaller than that treated with the oxidant under the conditions used. The stability of apoB-100 in LDL was further examined by SDS-PAGE and western blot analysis with antibodies to this protein. Intriguingly, while *P. gingivalis* WT, but not KDP136, extensively degraded apoB-100, oxidation seemed to mostly retain the structural stability of this protein, although the mobility of the original apoB-100 slightly increased and its fragment during storage disappeared during oxidation (Fig. 8B). We further determined whether the ability to induce macrophage transformation into foam cells was different between these differentially modified LDL particles. As demonstrated in Fig. 8C, LDL particles modified with either *P. gingivalis* or the oxidant strongly induced foam cell formation of macrophages, although the potency for macrophage foam cell formation was not significant between the two.

DISCUSSION

Recent epidemiologic evidence suggests that periodontal infections increase the risk of atherosclerosis and related cardiovascular and cerebrovascular events in humans (*1*, *2*, *47–49*). Although the presence of periodontal pathogens in atherosclerotic lesions strongly supports an association between periodontal disease and athrosclerosis, it appears to be difficult to account for the etiology of the accelerated atheroma formation by the bacterial presence alone. Therefore, it seems reasonable to raise the question whether periodontal pathogens actually have a role in the pathogenesis of atherosclerosis *in vivo* and, if so, how the bacteria contribute to this pathological process. To our knowledge, this is the first study indicating the crucial role of Rgp in the promotion of atherosclerosis induced by infection with the major periodontal pathogen

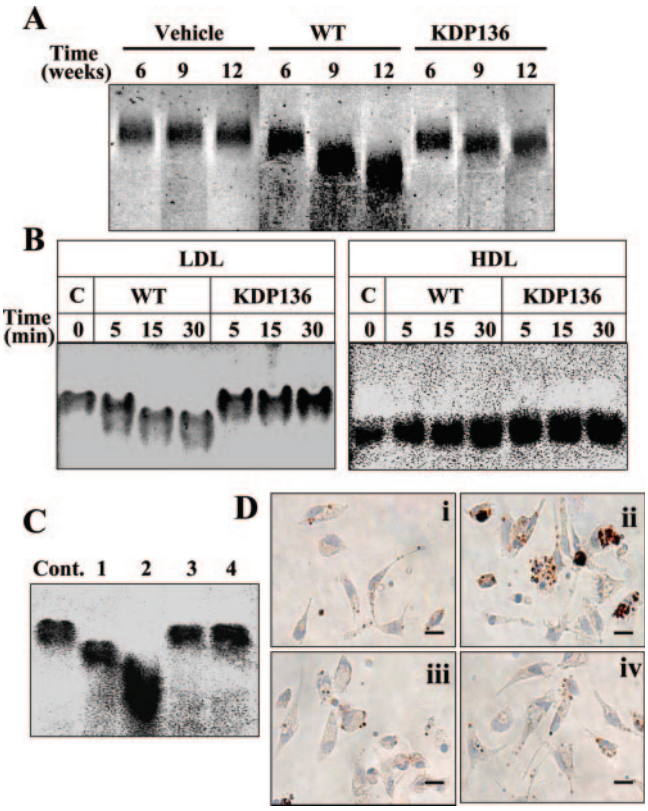

Fig. 5. **Modification of LDL particles by *P. gingivalis*.** A: Serum from apoE-knockout mice fed a high-fat diet and injected weekly for 6, 9, or 12 weeks with vehicle, *P. gingivalis* WT, or KDP136 was subjected to agarose gel electrophoresis, after which the gel was stained with Sudan IV. B: Human native LDL or HDL was incubated for the indicated times *in vitro* in the absence (C) or presence of *P. gingivalis* WT or KDP136. The lipoproteins were then subjected to agarose gel electrophoresis. C: Human native LDL was incubated for 30 min *in vitro* with culture supernatants of either *P. gingivalis* WT (lane 1), a Kgp-null mutant (lane 2), an Rgp-null mutant (lane 3), or KDP136 (lane 4) before analysis by agarose gel electrophoresis. Nontreated LDL was similarly analyzed as a control (Cont.). D: Mouse macrophages were incubated with human native LDL that had been either left untreated (panel i) or treated with *P. gingivalis* WT (panel ii), with KDP136 (panel iii), or with *P. gingivalis* WT plus human native HDL (panel iv). The cells were then fixed, stained with Sudan IV, and counterstained with hematoxylin. Scale bars, 20 μm.

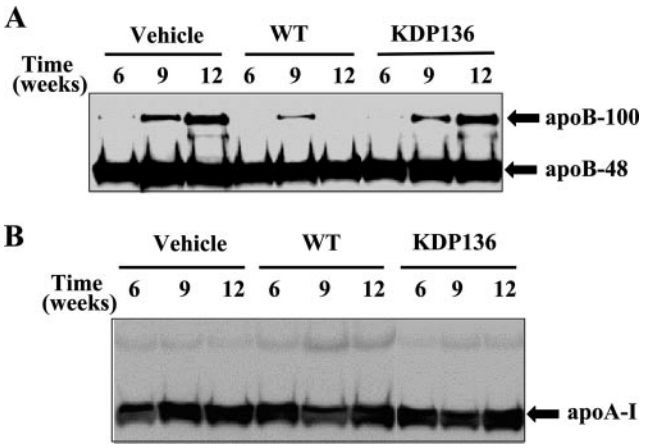

Fig. 6. ***In vivo* degradation of apoB-100 induced by *P. gingivalis* infection.** Serum from apoE-knockout mice fed a high-fat diet and injected weekly for 6, 9, or 12 weeks with vehicle, *P. gingivalis* WT, or KDP136 was subjected to SDS-PAGE and immunoblot analysis with antibodies to apoB (A) or to apoA-I (B).

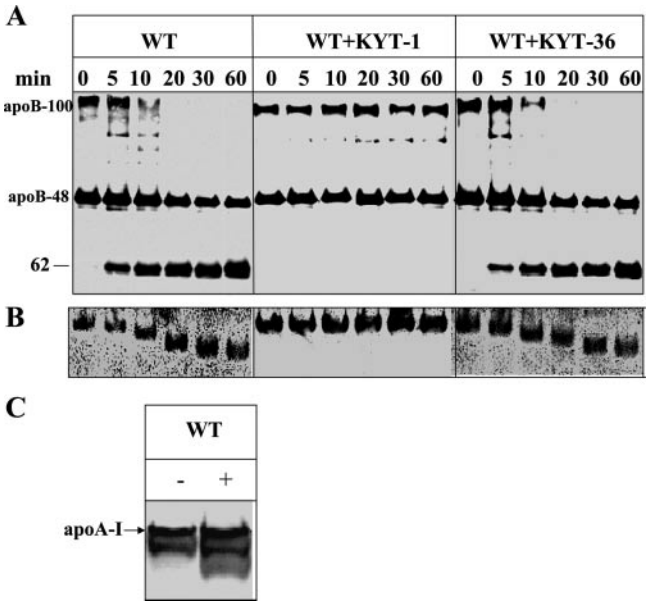

Fig. 7. ***In vitro* degradation of apoB-100 by *P. gingivalis*.** A: Serum from apoE-knockout mice fed a high-fat diet for 9 weeks was incubated at 37°C for the indicated times with *P. gingivalis* WT (5×10^4 CFU) in the absence or presence of KYT-1 (1 μM) or KYT-36 (1 μM). The samples were then subjected to SDS-PAGE and western blot analysis with antibodies to apoB-100. B: The same samples as described in A were subjected to agarose gel electrophoresis. C: Serum from apoE-knockout mice fed a high-fat diet for 9 weeks was incubated at 37°C for 30 min with *P. gingivalis* WT or vehicle and then subjected to SDS-PAGE and western blot analysis with antibodies to apoA-I.

P. gingivalis in vivo, thus illuminating a previously unidentified function for this multi-faceted virulence proteinase. Using apoE-knockout mice as an animal model for assessment of accelerated atherosclerosis, we first evaluated the impact of intravenous inoculation of *P. gingivalis* on the onset and development of atherosclerosis, and then added strong support for the notion that *P. gingivalis* can accelerates the progression of atherosclerosis and provide evidence indicating that the acceleration of atherosclerosis progression by *P. gingivalis in vivo* is mainly mediated by selective proteolytic cleavage of apoB-100 in LDL particles by Rgp.

Serum LDL cholesterol levels in apoE-knockout mice weekly injected with *P. gingivalis* WT increased 1.3-folds those with vehicle or KDP136 at 8 weeks after initial injection. In contrast, serum apoB-100 levels markedly diminished in *P. gingivalis*–injected mice, implying the increase of modified LDL devoid of apoB-100. These structural and quantitative alterations of LDL were most likely to induce enhanced atherosclerotic lesions in these mice. Meanwhile, serum HDL levels in apoE-knockout mice injected with *P. gingivalis* WT reversely decreased to 70% of those with vehicle or KDP136 at 8 weeks, while

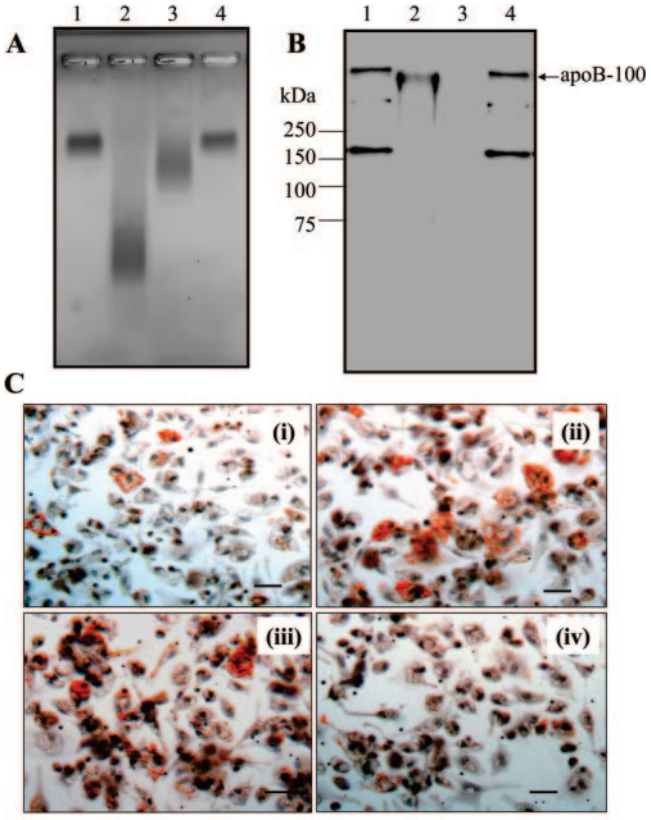

Fig. 8. **Modification of native LDL with oxidation and *P. gingivalis* infection.** A: Human LDL (50 μg) was incubated with $CuSO_4$ (0.1 mM) (lane 2) at 37°C for 14 h and *P. gingivalis* WT(10^7 CFU) (lane 3) or KDP136 (10^7 CFU) (lane 4) at 20°C for 30 min, and then each sample (10 μg) was subjected to 0.6% agarose gel electrophoresis. Untreated LDL (10 μg) as a control was also subjected to 0.6% agarose gel electrophoresis (lane 1). The gel was then stained with Sudan IV. B: SDS-PAGE and western blot analysis of apoB-100 in native and modified LDL. The same samples as A were applied to SDS-PAGE and western blot analysis with antibodies to apoB-100. C: Foam cell formation by modified LDL. Mouse macrophages were incubated with native LDL (i) oxidized LDL (ii), wild-type *P. gingivalis*-treated LDL (iii), and KDP136-treated LDL (iv), which were prepared as described in A, at 37°C for 18 h. The cells were then fixed, stained with Sudan IV, and counterstained with hematoxylin. Scale bars, 20 μm.

serum apoA-I levels were not significantly different between the animals injected with wild-type *P. gingivalis* and vehicle or KDP136, suggesting that such an extent of decrease in serum HDL levels is unlikely to reflect differences in apoA-I levels.

Based on both *in vivo* and *in vitro* experiments using selective inhibitors for Rgp and Kgp, as well as genetically defined Rgp and/or Kgp mutants, we could clearly demonstrate that Rgp is responsible for the acceleration of atherosclerosis progression in apoE-null mice infected with *P. gingivalis*, while the analogous virulent cysteine proteinase Kgp is not directly involved in this process. Miyakawa *et al*. (*13*) recently reported in the *in vitro* study that the *rgpB* or *rgpA* mutants, but not by the *rgpA/kgp* mutant or the spontaneous *kgp* mutant, caused aggregation of LDL and that a broad spectrum of cysteine and serine proteinase inhibitors, leupeptin and TLCK, partially inhibited aggregation induced by *P. gingivalis* outer membrane vesicles, thereby suggesting the importance of Kgp in this phenomena. Our data seemed inconsistent with their results. Although the reason for the inconsistent results is not clear at this moment, it may be due to the different experimental systems used. However, considering the fact that the structural and functional integrity is sometimes disrupted in genetically defined bacterial mutants, indeed, we reported the alterations in the structural and functional integrity of *P. gingivalis* in its mutants (*19–21*), the more detailed research for evaluating the data concerning the efficacy of the genetically defined bacterial mutants in the proposed mechanisms may be required.

Degradation of apoB-100 is most likely to modify the surface structure of LDL particles and thereby promotes their uptake by macrophages. Indeed, *in vitro* studies demonstrated that LDL particles modified by *P. gingivalis* via Rgp-induced degradation of apoB-100 were efficiently taken up by macrophages and induced the foam cell formation. In normal lipoprotein metabolism, approximately 80% of LDL as well as VLDL are removed from the circulation by interaction of apoB-100 with LDL receptor which is also known as the apoB/apoE receptor in liver. The apoB-degraded LDL by *P. gingivalis*, however, is not recognized by this receptor and accumulated in the circulation. The modified LDL is thought to interact with scavenger receptors expressed on the phagocytes such as macrophages. We also demonstrated that LDL modified with $CuSO_4$ were strongly aggregated and thereby stimulated macrophage foam cell formation. Although bacterial infection *in vivo* is known to generate reactive oxygen species that likely induce oxidation of LDL cholesterol, our data indicated that oxidation of LDL with $CuSO_4$ did not induce extensive degradation of apoB-100 under the conditions employed. Therefore, it is most likely that LDL particles oxidized with $CuSO_4$ are structurally different from those modified by *P. gingivalis*. Despite the difficulty in interpreting changes in the structure of LDL particles during oxidation, a mild degree oxidation has already been shown to induce aggregation of the particles, most probably due to cross-linking of the particles by lipid aldehydes (*50*). Extensive oxidation of LDL has also been shown to induce the loss of particle integrity. Therefore, the present data indicate that LDL particles modified by either *P. gingivalis* or oxidation are capable of transforming macrophages into foam cells, although the structural effects of these modifications on LDL particles are different.

Proteolysis of LDL particles has been studied with several different proteases, including plasmin, kallikrein, thrombin, trypsin, and α-chymotrypsin (*43*, *44*). To our knowledge, however, this is the first study clearly indicating that Rgp is primary responsible for degradation of apoB-100 of LDL after *P. gingivalis* infection in both *in vivo* and *in vitro*. Rgp is known to contribute to platelet aggregation through activation of protease-activated receptors 1 and 4 expressed on the cell surface (*11*), and it is also indispensable for the processing and maturation of fimbrilin A (*21*, *33*), a major component of the fimbriae responsible for facilitation of the initial interaction between *P. gingivalis* and host cells. Given that a *P. gingivalis* mutant deficient in fimbriae did not induce acceleration of atherosclerosis via up-regulation of Toll-like receptor-2

and -4 by the wild-type strain (*51*), Rgp may also contribute to atherosclerosis progression through the generation of mature fimbriae. Rgp thus appears to play a role in multiple steps in the promotion of atherosclerosis by *P. gingivalis* infection, indicating a newly identified function for the multi-faceted proteinase Rgp. Rgp is produced as secretory and membrane-associated forms on cell-surface by *P. gingivalis* (*52–56*), and the membrane-associated Rgp comprises the majority of Rgp activity (*56*). Therefore, the cell-associated enzyme is believed to be responsible for the virulence of the bacterium. It remains unclear to what extent either the secretory or membrane-associated Rgp contributes to the promotion of atherosclerosis by *P. gingivalis* infection, however, the membrane-associated Rgp is most likely to play a crucial role in this process, probably due to its large quantity and the need of prolonged or sustained action between Rgp and apoB-100 protein for the atherosclerosis progression.

Our murine model is distinct from previous models of periodontal infection in which *P. gingivalis* is introduced into the oral cavity. Oral infection with this bacterium has also been shown, however, to accelerate early atherosclerosis in apoE knockout mice (*57*, *58*). Given that oral microorganisms are readily introduced into the systemic circulation through perturbations of periodontal tissue in individuals with periodontitis (*15*), our murine model based on intravenous infection should prove informative in mechanistic studies of the modulation of atherosclerosis progression by *P. gingivalis* infection.

In conclusion, the present study strongly suggests that LDL modification induced by selective degradation of the apoB-100 protein by Rgp of *P. gingivalis* results in aggregated lipid particles that can be taken up by macrophages to form foam cells and consequently accelerates the progression of atherosclerosis in apoE-knockout mice fed with a high-fat diet.

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