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Interaction of *Porphyromonas gingivalis* with low-density lipoproteins: implications for a role for periodontitis in atherosclerosis

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Objective: The association of periodontitis with atherosclerosis has been suggested from epidemiological studies. Recently, we have reported that macrophages stimulated by *Porphyromonas gingivalis* formed foam cells in the presence of low-density lipoproteins (LDL). In this study, we examined the direct interactions between LDL and *P. gingivalis*.

Methods: We investigated the aggregation of LDL with *P. gingivalis* and its outer membrane vesicles (OMVs), degradation of the apo B-100 protein of LDL by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analyses, as well as the effects of protease inhibitors or activators on the mobility of LDL by agarose gel shift assays. The binding of *P. gingivalis* or its OMVs with LDL was demonstrated by western blot analysis. We also examined whether or not the aggregated LDL induced foam cell formation from murine macrophages.

Results: LDL was aggregated in a dose-dependent manner with *P. gingivalis* and its OMVs. Moreover, degradation of the apo B-100 protein of LDL was directly demonstrated in the presence of *P. gingivalis* or its OMVs. Furthermore, the gel shift assays indicated that the mobility of LDL was increased in the presence of *P. gingivalis*. This alteration was attenuated in the presence of the protease inhibitors TLCK and leupeptin and increased in the presence of reducing agents. Moreover, LDL was bound to specific proteins of *P. gingivalis* suggesting that these proteins may also play a role in aggregation. Finally, the aggregated LDL induced murine macrophages to form foam cells.

Conclusions: These results suggest that *P. gingivalis* may stimulate foam cell formation, in part, by aggregating LDL by proteolysis of apo B-100.

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Porphyromonas gingivalis is a major oral pathogen which is involved in periodontitis, a chronic local inflammatory disease that leads to destruction of periodontal tissues and alveolar bone (1, 2). The destruction of host tissues results from the action of various pathogenic factors including proteases and lipopolysaccaride (LPS) released by *P. gingivalis*, as well as factors released from host cells in response to the bacteria (3). Several

reports have suggested that *P. gingivalis* may penetrate the epithelial barrier surrounding the gingival sulcus (4, 5). Moreover, *P. gingivalis* has been observed within gingival tissues *in vivo*, suggesting that *P. gingivalis* may invade deeper structures of the connective tissue (6). These reports are compatible with suggestions that *P. gingivalis* may enter the blood stream following bleeding of the gums which is characteristic of chronic periodontitis. Such transient bacteremia may be a factor in the proposed role for periodontitis in systemic diseases (7, 8).

Several, but not all, reports have supported the association between periodontal disease and atherosclerosis (5, 9–12). Recently, pathological studies have identified periodontopathic bacteria including *P. gingivalis*, *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus* and *Treponema denticola* in atheroma lesions by polymerase chain reactions (13, 14). Moreover, *P. gingivalis* infection of mice has been demonstrated to increase the area of atherosclerosis lesions (15, 16). Furthermore, it has been reported that *P. gingivalis* and its outer membrane vesicles (OMVs) can aggregate platelets *in vitro* (17). Since platelet aggregation is characteristic of atheromas (18, 19), these studies support a role for *P. gingivalis* in the development of atherosclerotic plaque. *P. gingivalis*, as well as many other gram-negative bacteria, shed OMVs into the culture medium during growth (20). Many virulence factors, including LPS, proteases, and adhesins, are present in OMVs (21, 22). Therefore, *P. gingivalis* as well as its OMVs could play significant roles in atherosclerosis.

An important feature in the development of early atherosclerotic lesions is cholesterol uptake into macrophages to form foam cells, as well as lipid accumulation in the subendothelial space (23, 24). We recently reported that murine macrophages could be stimulated by *P. gingivalis* to accumulate low-density lipoproteins (LDL) to form foam cells (25). Although most of the foam cell cholesterol is derived from plasma LDL, native LDL is poorly transported into macrophages and LDL must be initially modified by oxidation or aggregation prior to uptake by the macrophages (24, 26, 27). LDL particles consist of a hydrophobic core with an outer shell consisting of phospholipids, unesterified cholesterol and the apo B-100 protein (28). Modification of the surface structure of LDL particles, which can be induced by chemical agents including proteases, lipases and oxidative compounds, is sufficient to trigger their aggregation and/or fusion (28). Since *P. gingivalis* and its OMVs contain potent protease activity, in this study we examined the hypothesis that the gingipain proteases of *P. gingivalis* (29) may play a role in the modification of LDL to an aggregated form which can be readily transported into macrophages to form foam cells. Previous reports related to modification and aggregation of LDL have been associated with host tissues such as macrophages, smooth muscle cells and epithelial cells (28, 30–33). Therefore, the direct interaction between LDL and infectious agents including bacteria and viruses has not yet been thoroughly investigated.

Materials and methods

Materials

Human LDL was purchased from Intracel Co. (Frederick, MD, USA). Anti apo B-100 antibody was obtained from Calbiochem (La Jolla, CA, USA).

Bacterial growth conditions

P. gingivalis 381 was grown anaerobically (85% N_2, 10% H_2 and 5% CO_2) at 37°C on blood agar plates containing tryptic soy broth (TSB, Difco Co., Detroit, MI, USA) supplemented with 10% sheep blood, 5 μg/ml hemin and 2 μg/ml menadione. Broth cultures were inoculated in TSB containing hemin and menadione anaerobically overnight at 37°C. The cultures were harvested by centrifugation, washed with phosphate-buffered saline (PBS, pH 7.4), and resuspended at the indicated concentrations. Protease mutants of strain 381 including G102 (*rgp*B, Arg-gingipain B, mutant), MT10 (*rgp*A, Arg-gingipain A, mutant), and MT10W (*rgp*A and *kgp*, Lys-gingipain, mutant) were grown in TSB medium including 10 μg/ml of erythromycin. Spontaneous mutant 381 W (*kgp* mutant) was grown as for strain 381 (34).

Cell cultures

The J774 A1 murine macrophage-like cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 5–10% heat inactivated fetal bovine serum (FBS, Gibco BRL) and 50 μg/ml gentamycin (Gibco BRL). The cells were maintained at 37°C in 5% CO_2, and transferred weekly.

Preparation of *P. gingivalis* outer membrane vesicles

OMVs were isolated from *P. gingivalis* 381 essentially as previously described by Kadurugamuwa and Beveridge (35). Briefly, bacterial cultures in early stationary phase were centrifuged at $6000 \times g$ for 15 min. The supernatant fluids were passed through 0.22 μm pore size filters (Millipore Corp., Bedford, MA, USA) to remove residual bacteria. OMVs were collected from the filtrate by centrifugation in a L8-70M ultracentrifuge (Beckman, Palo Alto, CA, USA) at $150{,}000 \times g$ for 3 h at 4°C, resuspended with PBS and stored at −70°C.

Effect of *P. gingivalis* and its OMVs on LDL aggregation

LDL aggregation was determined as previously described by Maor *et al.* (36). Briefly, LDL (200 μg/ml of protein) was incubated with *P. gingivalis* strains (10^6–2×10^8 cells/ml) or OMVs (1–100 μg/ml) in PBS containing 5 mM $MgCl_2$ at 37°C for up to 8 h. The changes in absorbance at 600 nm were measured every hour. Aggregation of LDL was expressed as the difference in absorbance between the bacteria added to LDL and the organism incubated alone.

Electron microscope examination of aggregated LDL

Native LDL or LDL treated with *P. gingivalis* OMVs were incubated at 37°C for 4 h and mixed 1 : 1 with 1% potassium phosphotangstate, pH 7.4. The mixtures were mounted and dried on carbon-coated grids (Ted Pella Inc., Redding, CA, USA). Stained samples were examined under transmission electron microscope (AEM-7100, Hitachi-Hitechnologies, Tokyo, Japan).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting

LDL (200 μg/ml) treated with various concentrations of *P. gingivalis* or its OMVs for 4 h at 37°C were analyzed by SDS–PAGE and western blotting. SDS–PAGE was carried out as described by Laemmli (37) under reducing conditions and stained with Coomassie blue. The proteins separated by SDS–PAGE were also electrotransferred to PVDF membranes, blocked with 1% skim milk in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4), for 1 h at room temperature and then incubated with anti-apo B-100 antibody (1 : 2000) in TBS overnight at 4°C. After washing with 0.05% Tween-TBS, alkaline phosphatase labeled anti-rabbit immunoglobulin (1 : 5000) was added to the membranes, incubated at room temperature for 1 h and the positive bands were detected with CDP-star (Roche Diagnostic Co., Indianapolis, IN, USA).

Relative electrophoresis mobility (REM) shift assays

LDL modifications were also assessed by agarose gel electrophoresis to detect the increase in electrophoretic mobility of the modified LDL relative to native LDL as described previously by Kalayoglu *et al.* (38). In brief, 2–4 μg of native or modified LDL was loaded into 0.6% agarose gels and electrophoresed for 40 min at 100 constant voltage. The gels were fixed in 75% ethanol and 5% acetic acid for 15 min, stained with 1% oil red O (in 60% isopropanol) for 30 min, and rinsed with 30% isopropanol to visualize the LDL bands. Migration of each LDL band was measured and expressed as a REM value compared with native LDL.

Effects of chemical reagents on modification of LDL

The chemical reagents were added to LDL before treatment with OMVs. The chemical reagents used were: leupeptin (Arg-gingipain inhibitor) and TLCK (inhibitors of Arg- and Lys-gingipains), mercaptoethanol and cysteine (activators of *P. gingivalis* cysteine protease activities). LDL was incubated with 5 μg/ml OMVs in the presence or absence of each agent at 37°C for 4 h. After incubation, LDL was analyzed by the REM gel shift assays.

Binding of LDL to *P. gingivalis* proteins

Proteins from *P. gingivalis* 381 and its OMVs were separated under reducing conditions by SDS–PAGE, electrotransferred to PVDF membranes, and incubated in 1% blocking solution overnight at 4°C. The membranes were then incubated with 10 μg/ml LDL in PBS overnight at 4°C, washed with PBS three times for 5 min, and the LDL cross-linked to the proteins with a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA). The membranes were finally incubated with anti-apo B-100 antibody and the complexes detected as described above for western blotting.

Foam cell formation by aggregated LDL

Aggregated LDL was isolated following centrifugation at $20{,}000 \times g$ for 20 min after incubation of LDL with 25 μg/ml OMVs at 37°C for 8 h in DMEM. After centrifugation, the supernatant fluid was stored at 4°C (unaggregated LDL), the aggregates were resuspended in an equal volume of DMEM (aggregated LDL). The macrophages were inoculated at $5–10 \times 10^4$ cells/well in a 48-well plate. The medium was changed to serum-free DMEM after incubation for 2 h and the plates further incubated at 37°C for 20 h. After incubation, the medium was replaced with fresh DMEM medium including native LDL or aggregated LDL or unaggregated LDL and incubated at 37°C for 20 h. The cells were washed with PBS, fixed with 10% formaldehyde for 20 min, stained with 1% oil red O (in 60% isopropanol) for 15 min, and counter-stained with Harris hematoxylin (Sigma, St. Louis, MO, USA). The stained cells were washed with PBS several times, examined by microscopy at 400 × magnification and photographed. Previous results indicated that foam cell formation required both LDL and *P. gingivalis* (25).

Results

Effects of *P. gingivalis* and its OMVs on LDL aggregation

To determine whether LDL can be aggregated in the presence of *P. gingivalis* 381 and its OMVs, various concentrations of the organism and OMVs were mixed with LDL (200 μg/ml), incubated at 37°C, and the O.D. changes assessed. *P. gingivalis* strongly aggregated LDL at concentrations of approximately 10^8 cells/ml. The aggregation was dose-dependent and was maximal after 3–4 h (Fig. 1a). In addition, OMVs at concentrations greater than 10 μg/ml also induced LDL aggregation, but aggregation was not as rapid as with intact cells (Fig. 1b). This apparent difference may result from distinct surface properties of OMVs relative to intact cells. Aggregation induced by both intact cells and OMVs was shown to be heat-sensitive. The aggregation induced by *P. gingivalis* is relatively specific, since two other periodontopathic bacteria, *T. denticola* and *B. forsythus*, did not aggregate LDL under these conditions (data not shown). Since previous results have indicated that proteases such as chymotrypsin can also induce LDL aggregation (39, 40), the role of the major cysteine proteases of *P. gingivalis* in this process was assessed. LDL was aggregated by the *rgp*B mutant G102 and the *rgp*A MT10 mutant but not by two mutants altered in the kgp (Lys-gingipain), MT10W and 381 W (Fig. 2). In addition, the cysteine

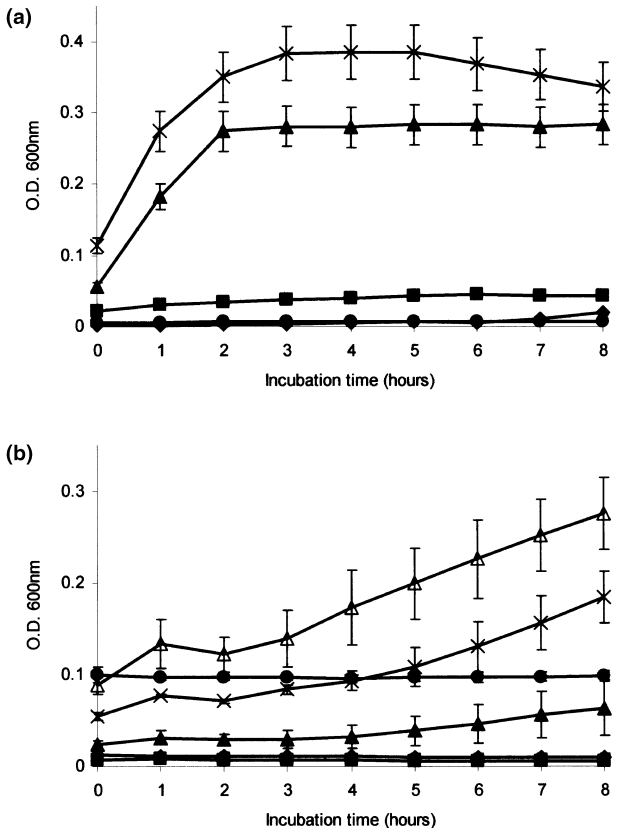

Fig. 1. Effects of *P. gingivalis* 381 and its OMVs on LDL aggregation. LDL (200 μg/ml) was incubated with various concentrations of bacteria (a) or OMVs (b) at 37°C in PBS containing 5 mM $MgCl_2$ and the $O.D._{600\ nm}$ measured at hourly intervals. (a) ●, 10^6 cells/ml; ♦, 10^8 heat-inactivated cells/ml; ■, 10^7 cells/ml; ▲, 10^8 cells/ml; ×, 2×10^8 cells/ml. (b) ■, native LDL alone; ♦, OMVs, 1.0 μg/ml; ▲, 10 μg/ml; ●, heat-inactivated OMVs, 50 μg/ml; ×, OMVs, 25 μg/ml; △, OMVs, 50 μg/ml. The values are means ± standard errors for three independent determinations.

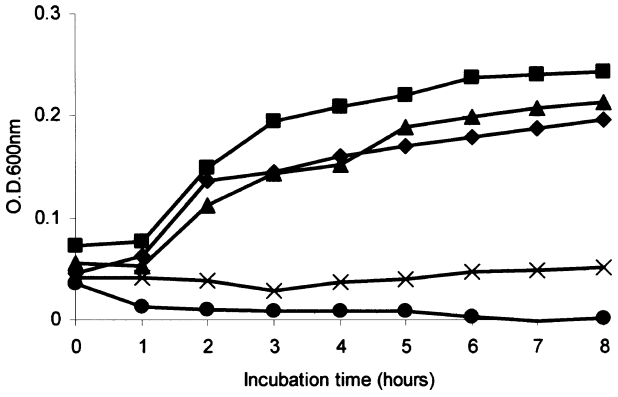

Fig. 2. Comparison of the effects of *P. gingivalis* 381 and protease mutants on LDL aggregation. 200 μg/ml of LDL was incubated with 10^8 cells/ml of strain 381 (♦), G102 (■), MT10 (▲), MT10W (●) and 381 W (×) strains at 37°C in PBS containing 5.0 mM $MgCl_2$ and $O.D._{600\ nm}$ measurements made each hour.

proteinase inhibitors, TLCK and leupeptin, also partially inhibited aggregation induced by OMVs (data not shown). These results suggested an important role for the kgp protease in LDL aggregation. However, since a *rgp*A, *rgp*B double mutant of strain 381 is not available, we cannot rule out a role for Arg-gingipain activity in aggregation and such a role was suggested by the effects of the Arg-gingipain specific inhibitor leupeptin. In addition, aggregated LDL, after incubation with *P. gingivalis* OMVs, was observed under TEM (Fig. 3). As indicated, the OMVs caused aggregation of the darkly stained LDL particles (Fig. 3b) compared to the particles alone (Fig. 3a).

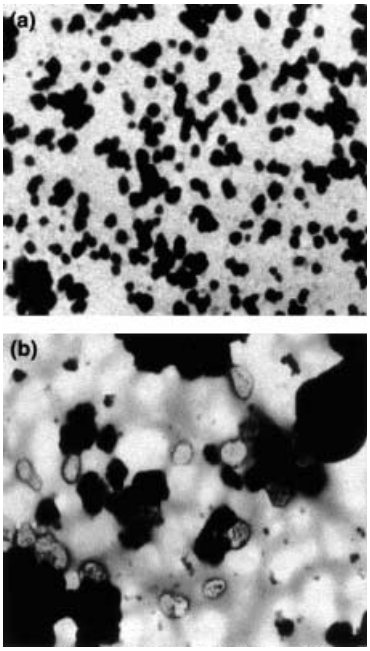

Fig. 3. Electron microscope examination of aggregated LDL. Native LDL (a) or LDL treated with *P. gingivalis* OMVs (b) were incubated at 37°C for 4 h. Dark particles of (a) and (b) are LDL, clear particles of (b) are *P. gingivalis* OMVs. Magnification, 30,000 ×.

Degradation of LDL treated with *P. gingivalis* and OMVs

The demonstration that the *kgp* mutant of strain 381 was attenuated in LDL aggregation suggested that the major protein component of these lipid particles, apo B-100, might be involved in the aggregation phenomena. Therefore, the stability of this protein in LDL was assessed by SDS–PAGE and western blot analysis (Fig. 4). It was demonstrated that at concentrations of bacteria greater than 10^6 cells/ml, apo B-100 was degraded in the LDL particles. The protein was weakly

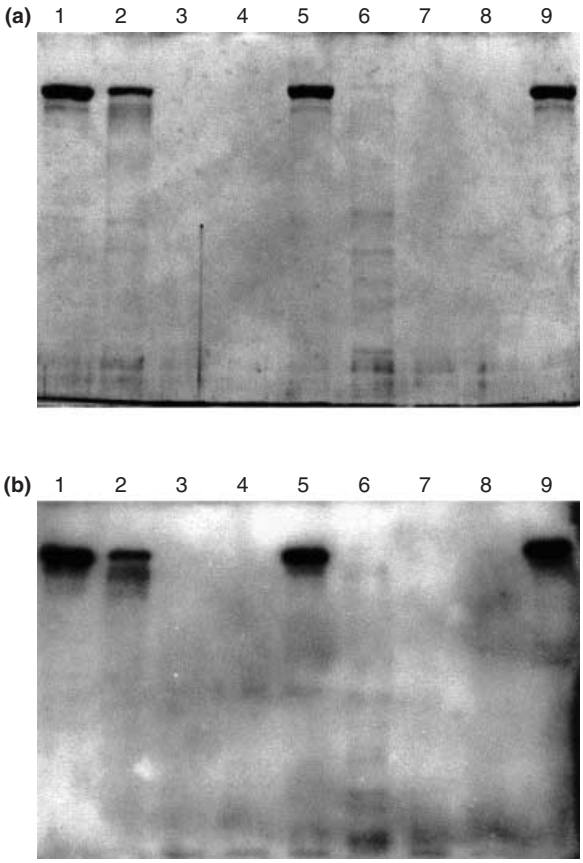

Fig. 4. Degradation of LDL proteins in the presence of *P. gingivalis* and its OMVs. (a) SDS–PAGE and (b) western blot analysis. LDL (200 μg/ml) was incubated with the indicated concentrations of strain 381 and its OMVs at 37°C for 4 h. Samples were then treated with an equal volume of 2 × SDS sample buffer under reducing conditions, applied to SDS–PAGE gels and the proteins transferred to membranes as described in the text. Lanes: 1, native LDL; 2–4; LDL treated with 10^6, 10^7, 10^8 cells/ml; 5; LDL treated with heat-inactivated 10^9 cells/ml; 6–8, LDL treated with 0.1, l.0, and l0 μg/ml of OMVs; 9, LDL treated with 50 μg/ml heat-inactivated OMVs.

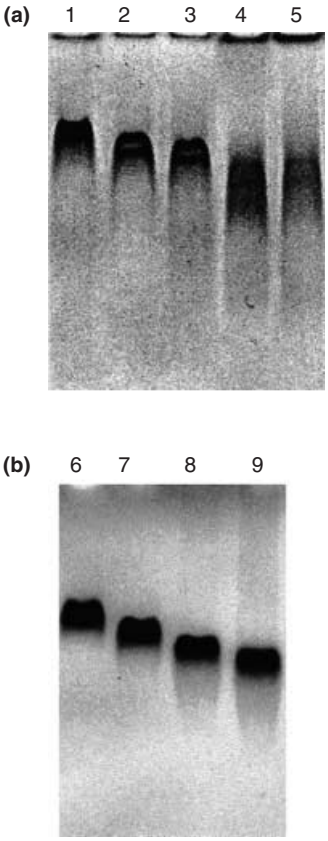

Fig. 5. Relative electrophoresis mobility of LDL following treatment with *P. gingivalis*. LDL was incubated with *P. gingivalis* and its OMVs as described in the text and applied to 0.6% agarose gels. The gels (a, b) were then stained with oil red O. Lanes: 1 and 6, native LDL; 2–5, LDL treated with 10^5–10^8 cells/ml of *P. gingivalis* 381; 7–9 LDL incubated with 1.0, 10, 25 μg/ml OMVs.

degraded in the presence of 10^6 cells/ml of bacteria but could barely be detected following incubation with greater than 10^7 cells/ml following both SDS–PAGE and western blotting (lanes 2–4). Likewise, OMVs also degraded apo B-100 with some lower molecular weight products detected on the gels at lower vesicle concentrations (lane 6). In addition, heat-inactivated bacteria or OMVs did not visibly degrade LDL (lanes 5, 9).

P. gingivalis-treated LDL is altered in electrophoretic mobility

REM shift assays can serve as one index that measures the surface alteration of LDL and is detected by changes in mobility of the LDL following agarose gel electrophoresis. Therefore, the relative mobility of LDL was assessed in the presence and absence of *P. gingivalis* and its OMVs (Figs 5a and b). The mobility of LDL treated with bacteria increased dependent on the concentration of the cells and incubation time but heated bacteria were much less able to alter mobility relative to the unheated cells. Likewise, OMVs, but not heated vesicles, significantly altered the REM values of LDL. This alteration in LDL mobility was not the result of oxidation of the lipid particles since oxidation in the presence of *P. gingivalis* or its OMVs could not be detected using the thiobarbituric acid reactive substance (TBARS) method (data not shown).

Effects of chemical reagents on modification of LDL

It was also of interest to examine the effects of protease modifications in the gel shift assays using the OMVs. These results (Fig. 6) showed that TLCK and leupeptin, two inhibitors of *P. gingivalis* cysteine protease activities, attenuated the increase in LDL mobility on the agarose gels (Fig. 6, lanes 2, 3). Since leupeptin is specific for Arg-gingipain activity, this suggests that one or both of the Arg-gingipains are involved in the modification of LDL. In addition, leupeptin inhibited OMVs induced aggregation of LDL (data not shown). Furthermore, mercaptoethanol and cysteine, two

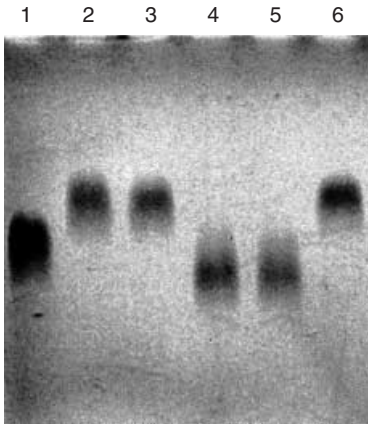

Fig. 6. Effect of protease modifications on LDL mobility. LDL (200 μg/ml) was treated with OMVs (5.0 μg/ml) in the presence or absence of the indicated agents. Lanes: 1, LDL incubated with OMVs; 2–5, LDL treated with OMVs in the presence of TLCK (1.0 mM), leupeptin (1.0 mM), mercaptoethanol (2.0 mM) or cysteine (1.0 mM), respectively; 6, native LDL.

reducing agents which activate the gingipains proteases of *P. gingivalis* (41, 42), increased the mobility of LDL relative to the untreated OMVs (Fig. 6, lanes 4, 5). These results confirm the significant roles of the gingipain proteases in the LDL aggregating activity. However, since a strain 381 *rgp*A, *rgp*B mutant completely devoid of Rgp activity was not available for testing, the relationships between mobility shifting and aggregation relative to specific protease activity could not be demonstrated conclusively. Nevertheless, these results confirm the important role of the gingipains in both surface alterations and aggregation of LDL.

Binding of LDL to proteins prepared from *P. gingivalis* and OMVs

To determine whether or not LDL bound to specific proteins on the surface of *P. gingivalis*, the interaction of LDL with membrane blotted *P. gingivalis* and OMVs proteins was assessed by western blotting (Fig. 7). Four protein bands from the bacteria of 68, 38, 27, and 13 kDa were shown to interact with LDL, and two of these proteins (68 and 38 kDa) were also detected in the OMVs extracts. However, the identity of these proteins still remains to be determined.

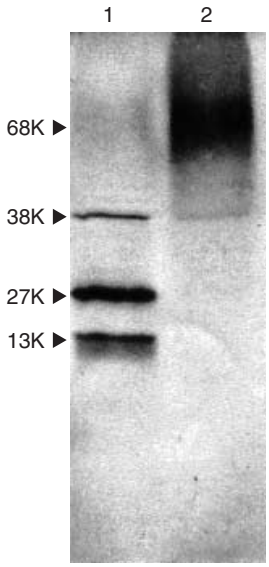

Fig. 7. Binding of LDL to *P. gingivalis* proteins. *P. gingivalis* 381 and OMVs proteins were separated by SDS–PAGE and the proteins transferred to PVDF membranes as described in the text. After blocking, the LDL was detected using anti apo B-100 antibodies. Lanes: 1, bacterial proteins; 2, OMVs proteins. The arrows indicate the positive protein bands at approximately 68, 38, 27, and 13 kDa, respectively.

P. gingivalis aggregated LDL stimulates foam cell formation

It was of interest to determine if the aggregated LDL induced by *P. gingivalis* stimulated murine macrophage transformation into foam cells (Fig. 8). LDL was treated with 25 μg/ml of OMVs at 37°C for 8 h, centrifuged, and separated into supernatant (unaggregated LDL) and sedimented (aggregated LDL) fractions. The aggregated LDL was suspended in an equal volume of DMEM and added to the macrophages. Aggregated LDL strongly induced macrophages transformation into foam cells. However, native LDL and the unaggregated supernatant fraction did not appear to induce alone foam cell formation under these conditions. This suggests that LDL is not sufficiently auto oxidized under these conditions to stimulate detectable foam cell formation. Therefore, the aggregated LDL particles induced by interaction with *P. gingivalis* were taken up by the murine macrophages to form foam cells. Since the OMVs bound to the LDL particles (Fig. 7), it is unlikely that these observations resulted from the interaction of the OMVs with the macrophages.

Discussion

The role of infectious bacteria and viruses in atherosclerosis was originally suggested from epidemiological studies (43–45). Atherosclerosis is a complex disease process with multiple risk factors, and the pathogens associated with atheroma development may constitute one of these factors (25, 44, 46). Elevated LDL serum levels are considered to be one of the important risk factors in the development of atherosclerosis (47). However, a more careful consideration suggests that native LDL is apparently not directly involved in atheroma initiation or progression (24, 26, 27). Specifically, LDL is transported into macrophages following modification of the lipid particles by oxidation or aggregation in the presence of host cells or other factors (28, 30–33). Macrophages accumulate cholesterol resulting in foam cell formation. The accumulation of foam cells in the arterial intima is considered to be an early step in lesion formation in atherosclerosis.

Periodontal diseases are characterized as chronic inflammatory diseases induced by gram-negative bacteria. Recently, several studies have demonstrated that these periodontopathic bacteria, including *P. gingivalis*, *A. actinomycetemcomitans* and *T. denticola*, can be detected in atheroma plaques (13, 14). Moreover, it has been reported recently that *P. gingivalis* can invade human coronary endothelial cells (4, 5), which may be a factor in atherosclerosis development. We have also reported that murine macrophages stimulated by *P. gingivalis* take up LDL to form foam cells (25). However, the possible direct modification of LDL by bacteria to an atherogenic form has not yet been evaluated. We have now demonstrated that at least one periodontopathogenic bacterium, *P. gingivalis,* can directly modify LDL into a form which facilitates its uptake into macrophages. These results dem-

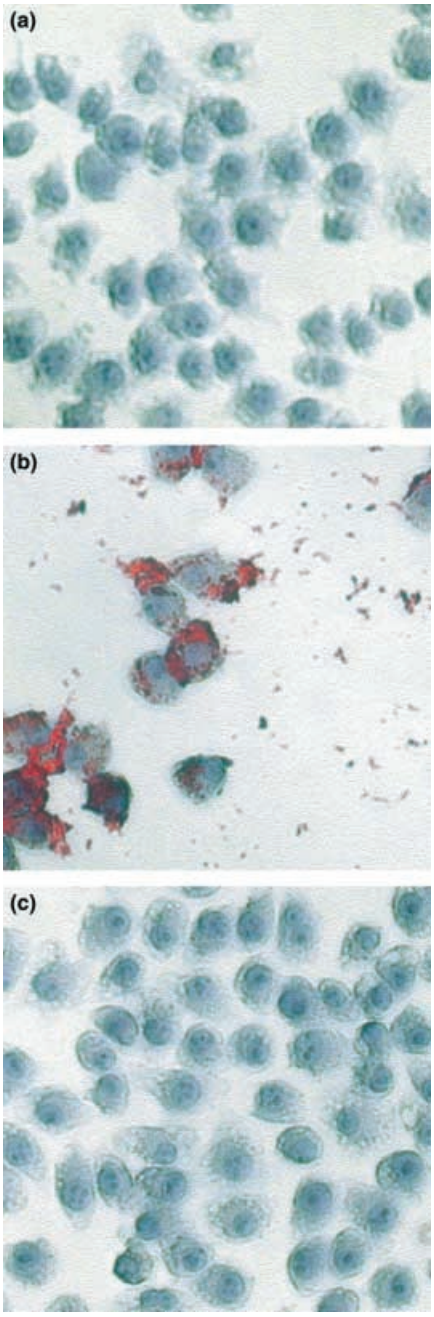

Fig. 8. Foam cell formation by murine macrophages incubated with aggregated LDL. LDL (200 μg/ml) was incubated with 25 μg/ml OMVs at 37°C for 8 h in DMEM. Aggregated LDL was collected by centrifugation and resuspended in an equal volume of DMEM and the supernatant fluids were used as unaggregated LDL. Macrophages were incubated with native LDL (a), aggregated LDL (b), and unaggregated LDL (c) for 20 h, fixed in 10% formaldehyde, and stained with oil red O and hematoxylin.

onstrated that LDL could be aggregated by the organism and its OMVs. Since such aggregation could not be demonstrated with *T. denticola* and *B. forsythus*, it is suggested that neither lipoproteins nor lipopolysaccharides mediate such effects. In addition, it has been reported that *P. gingivalis* and its OMVs can aggregate platelets but other periodontopathic bacteria do not exhibit such activity (17). However, other bacteria in addition to *P. gingivalis* can induce foam cell formation in murine macrophages in the presence of LDL (25). These results suggested the possibility that *P. gingivalis* exhibits a specific mechanism for directly aggregating LDL. Moreover, the results of the utilization of protease inhibitors and specific gingipain mutants suggested a likely role for both Lys-gingipain and one or both Arg-gingipains in such modifications. Furthermore, *P. gingivalis* was demonstrated to degrade apo B-100, which is the major protein component of LDL particles. The degradation of this protein likely alters the surface charge of the particles. Such alterations may be responsible for the altered mobility of LDL exhibited on agarose gels following interaction of the lipid particles with *P. gingivalis* and its OMVs.

It has been established that oxidized LDL is taken up by macrophages (26, 37). Although the interaction of *P. gingivalis* with murine macrophages leads to the oxidation of LDL as measured by the TBARS assay (Qi *et al.* personal communication), the direct oxidation of the lipid particles by the bacteria could not be detected in the present investigation. Therefore, the altered mobility of LDL in the presence of *P. gingivalis* likely results from proteolysis of apo B-100 rather than from oxidation. However, we cannot exclude the possibility that limited oxidation of the lipids occurs at levels which cannot be detected by the TBARS assay. Nevertheless, the attenuation of LDL alterations in mobility by the protease inhibitors and its stimulation by reducing agents known to activate *P. gingivalis* gingipains supports the conclusion that protein degradation is primarily responsible for the altered mobility of the lipid particles. Moreover, since heat-inactivated *P. gingivalis* and OMVs did not alter LDL aggregation and mobility, a role for the gingipains in this atherogenic property is further suggested.

Although the present results suggest an important role for the gingipains in LDL aggregation, the direct addition of purified Lys-gingipain or Arg-gingipain to the LDL particles did not stimulate aggregation (data not shown). This suggests that additional factors, in addition to the proteases, are required for aggregation. Perhaps

one of the *P. gingivalis* proteins identified as interacting with LDL may also be required for this process. The size of the common cellular and OMVs proteins involved in LDL binding, 68 and 38 kDa, are similar to that of the minor and major fimbrae of the organism (48, 49). However, electron micrographs of OMV preparations showed only slight contamination with fimbrae (data not shown). In addition, the smaller protein is similar in size to one of the adhesin peptides of the gingipains (50). Additional investigation will be required to determine which bacterial components are also involved in LDL binding and aggregation. A comparison of the effects of OMVs on apo B-100 levels, gel mobility, and aggregation suggests that the later assay may not be sensitive enough to detect lower levels of aggregation, which may affect the first two parameters. In addition, factors other than surface modifications may also play a role in aggregation.

The present results suggest that LDL modification following interaction with *P. gingivalis* results in aggregated lipid particles that can be taken up by macrophages to form foam cells. This suggests that the non-oxidative modification of LDL by these organisms in addition to the stimulation of LDL oxidation in the presence of macrophages (Qi *et al.* personal communication) can both stimulate foam cell formation. At present, the actual levels of periodontopathogens such as *P. gingivalis* that might be present in the circulation following bacteremia are unknown. Therefore, it is not yet possible to be sure that the levels of these organisms present in atheromas (13) are sufficient to induce the effects demonstrated *in vitro*. *In vivo* approaches will be required to determine if such effects play a significant role in atherosclerosis. For example, the utilization of murine models of atherosclerosis (15, 16) should prove useful in this regard.

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