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Involvement of adhesion molecule in *in vitro* plaque-like formation of macrophages stimulated with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide

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Background and Objective: Inflammatory agents, such as lipopolysaccharide (LPS), in periodontal pockets may promote atherogenesis by activating leukocytes. In our previous study, we developed a microchannel chip to observe the cell adhesion process in a fluid system. The objective of this investigation was to examine the mechanism by which periodontopathic bacterial LPS enhances plaque-like formation on a microchannel chip.

Material and Methods: To evaluate the effect of *Aggregatibacter actinomycetemcomitans* LPS on the expression of adhesion molecules, e.g. intercellular adhesion molecule 1 (ICAM-1), lymphocyte function-associated antigen 1 (LFA-1) and L-selectin, on the surface of murine macrophage RAW264.7 cells, the expression of each adhesion molecule was examined by flow cytometry and western blot analysis. Moreover, a flow test on the microchannel chip involving anti-adhesion molecule antibodies was conducted to clarify which adhesion molecule is related to plaque-like formation of RAW264.7 cells.

Results: The expressions of ICAM-1 and LFA-1 on the surface of RAW 264.7 cells increased following 12 h culture with LPS; L-selectin expression was unaffected. An increase in ICAM-1 expression was also confirmed by western blot analysis. The flow test revealed that anti-ICAM-1 antibody inhibited plaque-like formation of LPS-stimulated macrophages on the micropillars of the microchannel chip.

Conclusion: These findings indicate that ICAM-1 plays an important role in plaque-like formation of LPS-stimulated macrophages. Our microchannel chip is a suitable tool for the investigation of etiological factors of atherosclerosis, including periodontitis, *in vitro*.

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Chronic bacterial infections, including periodontitis, are associated with an increased risk of coronary heart disease (CHD), e.g. atherosclerosis (1–4). The mechanisms via which chronic infections increase the likelihood of atherosclerosis are poorly defined. Inflammatory agents, such as lipopolysaccharide (LPS), in periodontal pockets may promote atherogenesis by activating leukocytes. Atherogenic properties of LPS derived from *Aggregatibacter actinomycetemcomitans* on macrophage-like cells, namely RAW264.7, have been examined in several studies (5,6). In addition, recent experimental and clinical investigations indicated that adhesion of monocytes to vascular endothelial cells is an important step in atherosclerosis (7).

Adhesion molecules, which are specific proteins located on the surface of endothelial cells and leukocytes, regulate the different steps in leukocyte migration from the bloodstream into the vessel wall. This migration is mediated by adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1; CD54), lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) and L-selectin (CD62L). Intercellular adhesion molecule 1 is clearly expressed on monocytes in inflammatory conditions (8–10). Elevated concentrations of soluble adhesion molecules have been noted in patients with CHD, although their pathological role remains unclear (8–10).

We fabricated a microchannel chip to observe both the cell adhesion process and plaque-like formation (11). We also established a method for the measurement of the difference in adhesion between unstimulated and LPS-simulated macrophages on a microchannel chip in order to evaluate cell adhesion in a fluid system; this system revealed that LPS-stimulated macrophages rapidly adhere following a short incubation period in a flowing liquid stream.

The present study examined whether LPS regulates adhesion molecules, namely ICAM-1, LFA-1 and L-selectin, on macrophages, thereby promoting cell-to-cell adhesion during inflammatory conditions. The role of these adhesion molecules in the development of atherosclerotic plaques was also evaluated.

Material and methods

Cells and LPS preparation

Murine macrophage-like cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured at 37°C in α-minimal essential medium (α-MEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/mL) and streptomycin (100 μg/mL) in an atmosphere of 5% CO_2 in air. Lipopolysaccharide was extracted from lyophilized cells of *A. actinomycetemcomitans* Y4 employing the hot phenol–water method. The extract was treated with nuclease and washed extensively with pyrogen-free water via a series of ultracentrifugations (12,13). The chemical composition of the extract was 41% neutral sugar, 8% hexosamine, 31% fatty acid, 2% protein and 2% phosphorus.

Flow test on the microchannel chip

The microchannel chip was fabricated from silicon (Sumitomo 3M, Ltd, Tokyo, Japan) and acrylic resin as described previously (11). In brief, five channels (1.4 mm width, 10 mm length, 200 μm depth) were located on the chip; each channel consisted of 20 micropillars. Channel walls and micropillars were coated with a layer of silicon rubber (50 μm thickness). A Teflon film (80 μm, PTFE tape; Sumitomo 3M, Ltd), which was fixed on the chip, functioned as the upper substrate. A microchannel chip was placed on the stage of an optical microscope and connected to both the reservoir containing sample solution and a peristaltic pump with a tube. The RAW264.7 cells were cultured in the presence or absence of 1 μg/mL LPS for 12 h. Prior to the flow test, cells were prepared at a concentration of 1.5×10^6 cells/mL. Each flow test involved 3 mL of the sample solution. The flow rate at the exit of the chip was set to 5 mL/min. The culture medium was circulated for 2 h at room temperature by a pump. Digital images of macrophages adherent to micropillars in the channels were obtained using a CCD camera connected to the optical microscope. As described in our previous study (11), the side wall of the micropillar, where an impact area with the liquid stream and plaque-like formation occurred, was defined as the analysis area (9.0×10^4 μm^2). Areas of plaque-like formation were measured for 100 analysis areas with image analysis software (DP2-BSW; Olympus Co., Tokyo, Japan) to calculate the percentage of plaque-like formation area in each analysis area as an adhesion rate (11).

Flow cytometry analysis

The RAW264.7 cells were cultured in the presence or absence of 1 μg/mL LPS. The cells were incubated for 30 min with one of the following monoclonal antibodies: anti-ICAM-1 (Clone 2Q710; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LFA-1 (Clone I21/7; Southern Biotech, Birmingham, AL, USA) or anti-L-selectin (Clone Iam1-116; Santa Cruz Biotechnology). The cells were washed twice with phosphate-buffered saline (pH 7.2), followed by a 30 min incubation with fluorescein isothiocyanate-conjugated immunoglobulin G antibody (Santa Cruz Biotechnology). Expressions of ICAM-1, LFA-1and L-selectin on RAW264.7 cells were measured with a flow cytometer (EPICS XL; Beckman Coulter, Fullerton, CA, USA) and Multicycle® for Windows (Phoenix Flow Systems, San Diego, CA, USA).

Immunoblot analysis

The RAW264.7 cells were cultured in the presence or absence of LPS for 2, 6 or 12 h. The cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris–HCl, 2% SDS, pH 6.8). The protein concentration of the samples was determined with a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Each sample was separated by electrophoresis on 10% SDS–polyacrylamide gels and transferred to polyvinylidine fluoride

(PVDF) membranes (Millipore Co., Bedford, MA, USA). The membranes were incubated with a polyclonal antibody against ICAM-1 (CD54; Santa Cruz Biotechnology) overnight at 4°C after blocking non-specific binding sites with 5% non-fat skim milk in phosphate-buffered saline containing 0.1% Tween 20 for 1 h. Immunodetection was performed using the ECL-Plus western blotting detection system (Amersham Biosciences UK Ltd, Chalfont St Giles, UK). Blots were also stained with Coomassie brilliant blue to confirm that all lanes contained similar amounts of protein extract.

Inhibition experiment with anti-adhesion molecule antibody

The RAW264.7 cells were incubated in the presence or absence of 1 μg/mL LPS for 12 h. Subsequently, the cells were incubated for 60 min with control immunoglobulin G (normal rat IgG; Santa Cruz Biotechnology), anti-ICAM-1 antibody (CloneYN1/1.7.4; Beckman Coulter) or anti-LFA-1 antibody (Clone I21/7; Beckman Coulter). The flow test was performed using the aforementioned cells. Statistical analyses were conducted with a software program (JMP6.0.3; SAS Institute Inc., Cary, NC, USA).

Results

Plaque-like formation on the microchannel chip

A representative image of forming plaques attached to a micropillar during the flow test is presented in Fig. 1A. In unstimulated cells [LPS (−)], the flow test was not conducted for plaque-like formation at 0 and 20 min; however, plaque-like formation was noted at 60 and 120 min. In LPS-stimulated cells [LPS (+)], plaque-like formation was observed at 20 min; moreover, plaque expanded during the flow test. Plaque-like formations in LPS-stimulated cells were greater than those in unstimulated cells at 60 and 120 min. Adhesion rates in unstimulated and stimulated cells were increased in a time-dependent manner (Fig. 1B). Adhesion rates in the stimulated cells were significantly greater compared with unstimulated cells.

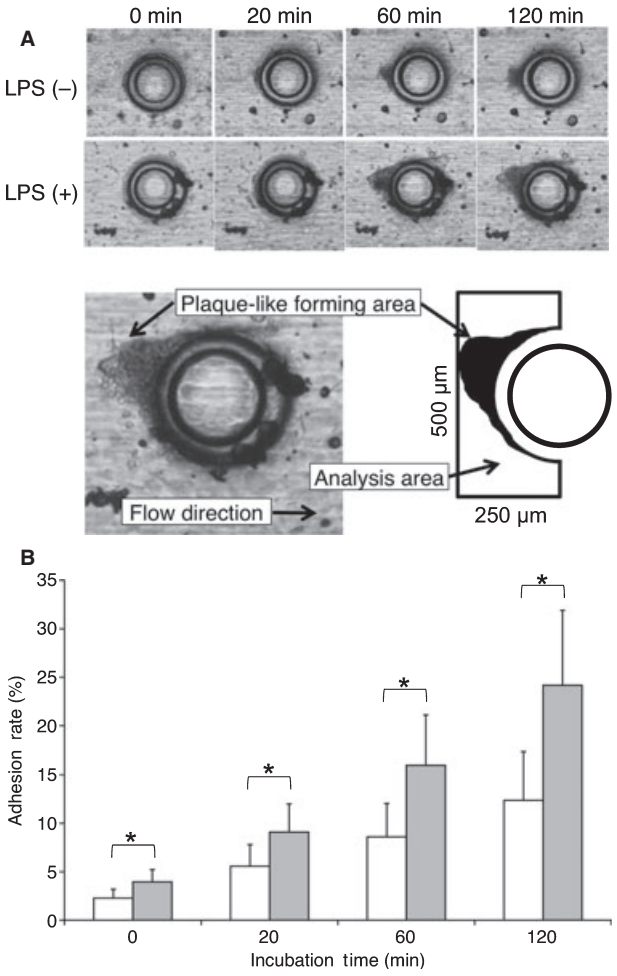

Fig. 1. (A) Representative microscope images of plaque-forming RAW264.7 cells on a microchannel chip. The RAW264.7 cells were cultured in the absence or presence of 1 μg/mL LPS at 37°C for 12 h. The flow test was performed for 0, 20, 60 or 120 min with unstimulated and LPS-stimulated cells, expressed as LPS (−) and LPS (+), respectively. The side wall of the micropillar was defined as the analysis area (subtracted area from 250 μm × 500 μm to a half area of micropillar; 9.0×10^4 μm^2; 11). (B) Adhesion rates in unstimulated and LPS-stimulated RAW264.7 cells. The RAW264.7 cells were cultured in the absence or presence of 1 μg/mL LPS at 37°C for 12 h. The flow test was performed for 0, 20, 60 or 120 min with unstimulated and LPS-stimulated cells. Data are expressed as means (bar) with standard deviation (continuous line) of three experiments, with similar results obtained in each experiment. White and grey bars indicate unstimulated and LPS-stimulated cells, respectively. * Significant difference between means of adhesion rate in two groups assessed by Tukey–Kramer honestly significant difference (HSD) test ($p < 0.05$).

Expression of adhesion molecules

Expressions of ICAM-1, LFA-1 and L-selectin on RAW264.7 cells were measured with a flow cytometer. Unstimulated and LPS-stimulated cells at 0 h of stimulation displayed identical ICAM-1 peaks with very low fluorescence intensity; these peaks were slightly higher than that of the negative control, which consisted of unstimulated cells without anti-ICAM-1 antibody (Fig. 2). However, peaks of LPS-stimulated cells at 6 and 12 h of stimulation shifted to the right. The fluorescence intensity of the peak of LPS-stimulated cells at 12 h of stimulation was 1.5 times greater in comparison with unstimulated cells.

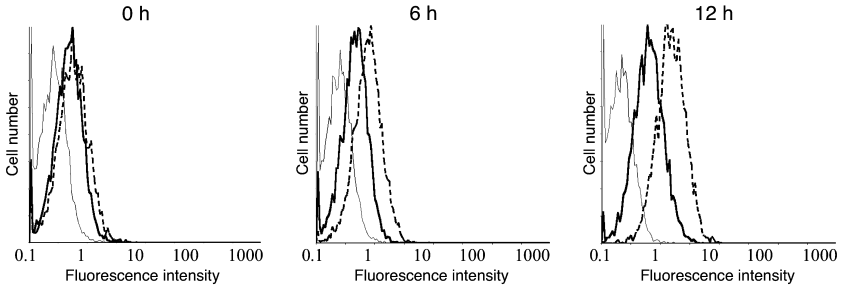

Fig. 2. Expression of ICAM-1 in unstimulated and LPS-stimulated RAW264.7 cells. The RAW264.7 cells were cultured in the absence or presence of 1 μg/mL LPS at 37°C for 0, 6 or 12 h. Distributions of fluorescence intensity were measured by flow cytometry after incubation with anti-mouse ICAM-1 antibody. Negative control cells without anti-ICAM-1 antibody, unstimulated cells and LPS-stimulated cells are depicted as grey, continuous and dashed lines, respectively.

Unstimulated and LPS-stimulated cells at 0 h of stimulation exhibited peaks identical to those of LFA-1 with medium fluorescence intensity; these peaks were higher than that of the negative control, which consisted of unstimulated cells without anti-LFA-1 antibody (Fig. 3). However, the peak of LPS-stimulated cells at 12 h of stimulation shifted to the right. The fluorescence intensity of the peak of LPS-stimulated cells at 12 h of stimulation was 1.3 times greater in comparison with unstimulated cells.

Unstimulated and LPS-stimulated cells at 0 h of stimulation demonstrated peaks identical to those of L-selectin with medium fluorescence intensity; these peaks were higher than that of the negative control, which consisted of unstimulated cells without anti-L-selectin antibody (Fig. 4). Peaks of LPS-stimulated cells at 6 and 12 h of stimulation did not shift to the right.

Immunoblot analysis

The expression of ICAM-1 on RAW264.7 cells was confirmed using immunoblot analysis (Fig. 5). The ICAM-1 was detected as a protein band with molecular mass of 85–110 kDa following stimulation of the cells with 0.1, 1 and 5 μg/mL LPS; in contrast, this band was faintly detected in the absence of LPS stimulation. Levels of ICAM-1 were elevated at 2 h in LPS-stimulated cells; moreover, ICAM-1 continued to increase up to 6 h. The level of ICAM-1 was maximal at 1.0 μg/mL of LPS, and there was no further significant increase at the higher concentration (5.0 μg/mL).

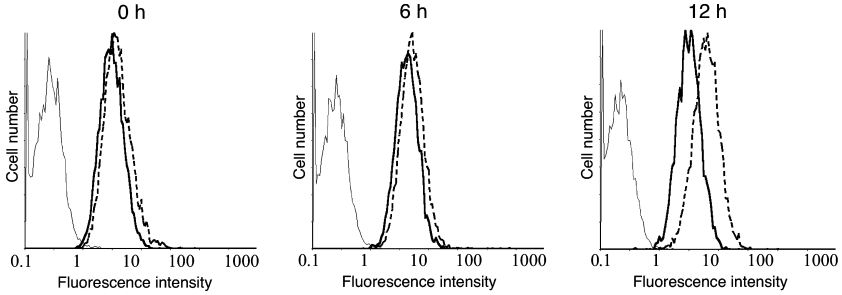

Fig. 3. Expression of LFA-1 in unstimulated and LPS-stimulated RAW264.7 cells. The RAW264.7 cells were cultured in the absence or presence of 1 μg/mL LPS at 37°C for 0, 6 or 12 h. Distributions of fluorescence intensity were measured by flow cytometry after incubation with anti-mouse LFA-1 antibody. Negative control cells without anti-LFA-1 antibody, unstimulated cells and LPS-stimulated cells are depicted as grey, continuous and dashed lines, respectively.

Effects of anti-ICAM-1 and anti-LFA-1 antibodies on plaque-like formation

The RAW264.7 cells were incubated with control IgG, anti-ICAM-1 antibody or anti-LFA-1 antibody to elucidate the role of these adhesion molecules in plaque-like formation following stimulation of the cells in the presence or absence of LPS. Mean adhesion rates of cells incubated with control IgG decreased slightly in both unstimulated and LPS-stimulated cells; however, the differences were not meaningful (Fig. 6). In contrast, mean adhesion rates of cells incubated with anti-ICAM-1 antibody decreased significantly in both unstimulated and LPS-stimulated cells. Mean adhesion rates of LPS-stimulated cells incubated with anti-ICAM-1 antibody were markedly higher than those of unstimulated cells incubated with the same antibody. Mean adhesion rates of cells incubated with anti-LFA-1 antibody declined significantly in both unstimulated and LPS-stimulated cells relative to control IgG.

Discussion

Leukocyte adhesion molecules play important roles in immune regulation and host defense. The major families of cell adhesion molecules include immunoglobulins, integrins and selectins. Intercellular adhesion molecule 1, which is a member of the immunoglobulin family, is expressed on leukocytes and endothelial cells. Intercellular adhesion molecule 1 is responsive to numerous inflammatory mediators; moreover, it mediates both leukocyte firm adhesion and migration through the endothelium into tissues (14). Lymphocyte function-associated antigen 1, a β2-integrin that functions as the counter-receptor of ICAM-1, is expressed on monocytes and aids in the regulation of adhesion (15,16). L-Selectin belongs to the selectin family of proteins, which recognize sialylated carbohydrate groups; L-selectin is regularly expressed on the surface of leukocytes. L-Selectin slows leukocytes by mediating rolling, which is followed by firm adhesion between leukocytes and endothelial cells (17).

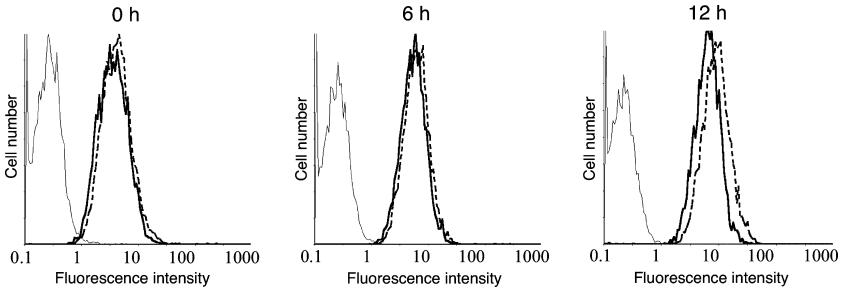

Fig. 4. Expression of L-selectin in unstimulated and LPS-stimulated RAW264.7 cells. The RAW264.7 cells were cultured in the absence or presence of 1 μg/mL LPS at 37°C for 0, 6 or 12 h. Distributions of fluorescence intensity were measured by flow cytometry after incubation with anti-mouse L-selectin antibody. Negative control cells without anti-L-selectin antibody, unstimulated cells and LPS-stimulated cells are illustrated as grey, continuous and dashed lines, respectively.

We established the method involving the evaluation of *in vitro* plaque-like formation by macrophage cells in a fluid system via development of the microchannel chip (11). In the microchannel, plaque-like formation by RAW264.7 cells increased significantly following LPS stimulation. The present study attempted to elucidate the role of adhesion molecules in plaque-like formation. Initially, the time-dependent increase in plaque-like formation in LPS-stimulated RAW264.7 cells was confirmed (Fig. 1). Expression of adhesion molecules on RAW264.7 cells was examined with flow cytometry and western blotting analysis. Expression levels of ICAM-1 were very low in LPS-stimulated cells at 0 h of stimulation; however, these levels were markedly elevated in LPS-stimulated cells at 6 and 12 h of stimulation (Fig. 2). Slight ICAM-1 expression was observed in unstimulated RAW264.7 cells in the present investigation, a finding consistent with those of previous reports (18–20). Intercellular adhesion molecule 1 was not expressed in unstimulated J774.1 cells, murine macrophage cell line obtained from the Japanese Cancer Research Resources Bank; therefore, expression of ICAM-1 on macrophages in unstimulated conditions may depend on the cell lines used in the experiment (data not shown). Expression levels of LFA-1 and L-selectin were medium in unstimulated and LPS-stimulated cells at 0 h of stimulation. Levels of LFA-1 were elevated significantly in LPS-stimulated cells at 12 h of stimulation; however, L-selectin levels did not increase in LPS-stimulated cells (Figs 3 and 4). Western blot analysis detected ICAM-1 in RAW264.7 cells stimulated with LPS for 2 h (Fig. 5). These data indicate that LPS increases plaque-like formation and upregulates expression of ICAM-1 and LFA-1, but not that of L-selectin, in RAW264.7 cells. These findings are in accord with the results of the previous report showing the increased expression of monocyte adhesion molecules, such as LFA-1, Mac-1 (CD11b/CD18) and ICAM-1 (9). In the previous report, Meisel *et al.* (9) suggest that the increased expression of monocyte ICAM-1 points to a possible involvement in leukocyte microaggregation through association with upregulated ligands LFA-1 and Mac-1 on neutrophils and monocytes.

We examined the roles of ICAM-1 and LFA-1 with respect to increased

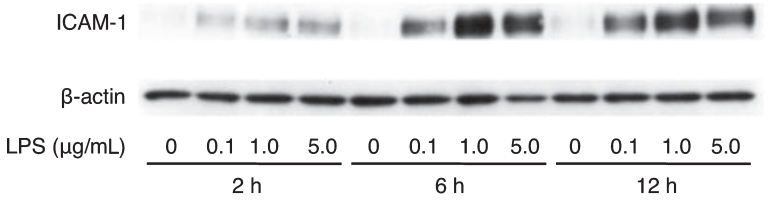

Fig. 5. Expression of ICAM-1 in unstimulated and LPS-stimulated RAW264.7 cells. The RAW264.7 cells were cultured in the absence or presence of LPS (0.1, 1.0 or 5.0 μg/mL) at 37°C for 2, 6 or 12 h. Protein contents from cells (20 μg protein) were separated on a 10% SDS–polyacrylamide gel and electroblotted onto PVDF membranes. Immunodetection was performed with the ECLPlus western blotting detection system following incubation with anti-ICAM-1 polyclonal antibody.

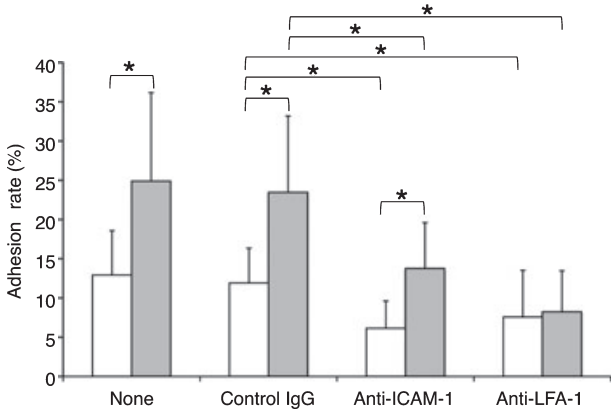

Fig. 6. Effect of anti-ICAM-1 and anti-LFA-1 antibodies on adhesion rates. The RAW264.7 cells were cultured in the absence or presence of 1 μg/mL LPS at 37°C for 12 h. Subsequently, cells were incubated with control IgG, anti-ICAM-1 antibody or anti-LFA-1 antibody, after which the flow test was performed. Data are expressed as means (bar) with standard deviation (continuous line) of three experiments, with similar results obtained in each experiment. White and grey bars indicate unstimulated and LPS-stimulated cells, respectively. * Significant difference between means of adhesion rate in two groups assessed by Tukey–Kramer HSD test ($p < 0.05$).

plaque-like formation by RAW264.7 cells on the microchannel chip (Fig. 6). Control IgG did not affect adhesion rates of unstimulated and LPS-stimulated cells. Adhesion rates of cells incubated with anti-ICAM-1 antibody were significantly lower in comparison with cells incubated with control IgG, a finding indicative of ICAM-1 involvement in plaque-like formation. Higher adhesion rates in LPS-stimulated cells in comparison with unstimulated cells may be due to a relative decrease in the ratio of anti-ICAM-1 antibody to ICAM-1 molecules. Adhesion rates of cells incubated with anti-LFA-1 antibody were significantly lower in comparison with cells incubated with control IgG; this finding indicated that LFA-1 might participate in plaque-like formation. Lymphocyte function-associated antigen 1, which is constitutively expressed on macrophages, is related to firm adhesion to ICAM-1-expressing macrophages (21). In the present study, flow test analysis revealed that difference in adhesion rates between unstimulated and LPS-stimulated RAW264.7 cells incubated with anti-LFA-1 antibody. We have no ready explanation for this issue, but think it is caused by the high affinity of anti-LFA-1 antibody for the LFA-1 molecule.

L-Selectin reportedly plays a role in cell-to-cell adhesion (22–24). Furthermore, constant L-selectin expression on the surface of macrophages and the important functions of L-selectin in rolling and subsequent firm adhesion by binding to sugar chains on the endothelium were demonstrated (17). However, results of this study indicated that while L-selectin is not involved in increased plaque-like formation in LPS-stimulated RAW264.7 cells, it might participate in plaque-like formation in unstimulated cells. Previous studies have shown that another adhesion molecule, Mac-1, functions in the attachment of monocytes to glass and plastic slides (25,26).

Atherosclerosis is a focal inflammatory disease characterized initially by the recruitment of mononuclear cells into the arterial wall (7). Leukocyte accumulation on activated endothelium is initiated, augmented and prolonged by adhesion events that mediate leukocyte–endothelial cell and leukocyte–leukocyte interactions. Early events in lesion development include endothelial activation, which can be triggered by risk factors such as hypercholesterolemia. This situation results in leukocyte recruitment to the endothelium and migration into the subendothelium (7). The interaction of monocytes/macrophages with the endothelium is promoted by the expression of receptors for adhesion molecules on monocytes/macrophages, which mediate adherence to the corresponding adhesion molecules on endothelial cells, e.g. ICAM-1, vascular adhesion molecule 1, E-selectin and P-selectin (27). Upregulation of adhesion molecules, which is mediated by monocyte and endothelial cell activation, is correlated with enhanced monocyte adherence (28–30). A recent study indicated that the infected/inflamed area in periodontitis in systemically healthy patients is associated with macrophage activation via increased serum LPS concentration (5,31).

The present investigation indicates that ICAM-1 plays an important role in cell aggregation of LPS-stimulated macrophages. Our microchannel chip is a suitable tool for the *in vitro* evaluation of etiological factors of atherosclerosis, including periodontitis.

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