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Subcutaneous and continuous administration of lipopolysaccharide increases serum levels of triglyceride and monocyte chemoattractant protein-1 in rats

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Objectives: Previous studies have suggested an association between chronic infection and cardiovascular disease, and combined infection/inflammation is frequently related with hypertriglyceridemia, which may be a risk factor of cardiovascular disease. In fact, experimental transient or intermittent administration of lipopolysaccharide (LPS) is known to cause hypertriglyceridemia. We investigated the effects of subcutaneous and continuous administration of LPS in rats, which was considered to mimic chronic infection such as periodontal disease, on the serum levels of lipids [triglyceride (TG), total cholesterol (TC) and free fatty acid (FFA)] and cytokines [tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1)], all of which are thought to be etiological factors of atherosclerotic cardiovascular disease.

Methods: Ten fasted Wistar rats were subcutaneously injected with LPS (500 μg/100 g of body weight) (LPS-injected rats; $n = 5$) or saline (saline-injected rats; $n = 5$) as a model of transient infection. Further, mini-osmotic pumps containing the same concentration of LPS (LPS-implanted rats; $n = 5$) or saline (saline-implanted rats; $n = 5$) were subcutaneously implanted into an additional 10 rats as a model of chronic infection. After the injection or implantation, time-dependent changes in serum levels of TG, TC, FFA, TNF-α, MCP-1 and LPS were determined.

Results: LPS was detected in serum until 24 h in LPS-injected rats and until day 5 in LPS-implanted rats. Serum TG levels significantly increased from 6 to 24 h in LPS-injected rats and from day 5 to 14 in LPS-implanted rats. In both LPS-injected and LPS-implanted rats, serum FFA levels increased only slightly, whereas serum TC levels did not appreciably change. Both types of LPS administration increased serum TNF-α levels transiently and MCP-1 levels continuously.

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Conclusions: These findings suggest that chronic infection such as periodontal disease induces hypertriglyceridemia and increases serum MCP-1 levels in a manner that increases the risk of atherosclerotic cardiovascular disease.

Cardiovascular disease, which is primarily associated with atherosclerosis, remains one of the primary causes of death in the United States, as well as in Europe and Asia. Recent reports (1, 2) have focused on the role of infection as an additional etiological factor in the development of atheroma, and the participation of infection with cardiovascular disease is well established (3, 4). On the other hand, previous studies (5–9) investigated whether elevated levels of serum triglyceride (TG) are associated with an increased risk of cardiovascular disease and, though the results were not consistent, suggested that hypertriglyceridemia is an independent risk factor for atherosclerotic cardiovascular disease.

Various potential causative mechanisms have been proposed for the reported associations between infection and atherosclerotic disease, with classical cardiovascular risk factors [increase of low density of lipoprotein (LDL), fibrinogen and TG levels] thought to be involved in those mechanisms (3). Acute infection is known to interfere with lipid metabolism, and elevated levels of plasma TG have been especially observed in infection with gram-negative bacteria (10). Further, hypertriglyceridemia induced by experimental administration of lipopolysaccharide (LPS) to animals, used to mimic infection, has been attributed to an increase in serum TG levels and/or a decrease in activity of adipose tissue lipoprotein lipase, a key enzyme in TG catabolism, along with a decrease of clearance of TG-rich lipoproteins (11–19). Therefore, chronic infection may also modify the serum profile of TG and TG-rich lipoproteins in a way that increases the risk of atherosclerotic cardiovascular disease. However, experimental hypertriglyceridemia in those studies was induced when LPS was transiently or intermittently injected via a vein or intraperitoneally, and there are few reports regarding the effect of subcutaneous and continuous administration of LPS, as a model for chronic infection such as periodontal disease, on lipid metabolism.

Atherosclerosis is recognized to involve chronic inflammatory and immune responses, and a considerable body of evidence supports the notion that various mediators such as adhesion molecules, cytokines and chemokines are associated with the early initiation of atherosclerotic lesions (1, 20). Of these mediators, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) induce a rapid increase in serum TG levels, due to increased amounts of very-low-density lipoprotein (VLDL), followed by a later rise in serum cholesterol levels, due to increased amounts of LDL (21–23). TNF-α has a potential to promote atherogenesis and thromboembolic events, as well as hypertriglyceridemia. Previous findings (24–29) suggested that monocyte chemoattractant protein-1 (MCP-1), a chemokine, also contributes to plaque destabilization and thrombosis, as well as vascular inflammation.

To further clarify the mechanisms underlying the association between chronic infection and atherosclerotic cardiovascular disease, we investigated the effects of subcutaneous and continuous administration of LPS in rats, which was considered to mimic chronic infection such as periodontal disease, on serum levels of lipids such as TG, free fatty acid (FFA) and total cholesterol (TC) and cytokines such as TNF-α and MCP-1, all of which are considered to be etiological factors of atherosclerotic cardiovascular disease, and compared the results with those from a transient administration of LPS.

Material and methods

Animals

All experimental procedures were approved by the Animal Ethics Committee of Showa University (Tokyo, Japan). Male Wistar strain rats weighing approximately 180–200 g were purchased from Saitama Animal Laboratories (Saitama, Japan). The animals were maintained in a reverse-light-cycle room (light from 8.00 a.m. to 8.00 p.m., dark from 8.00 p.m. to 8.00 a.m., ambient temperature 21 ± 3°C), with access to standard rat chow (Nosan Co., Yokohama, Japan) and water *ad libitum*. They were allowed to adapt to the environment for at least 1 week before commencement of the experiments.

Continuous administration of LPS

Animals were fasted for 12 h before beginning the experiment. An Alzet mini-osmotic pump (Alza Corporation, Palo Alto, CA, USA) containing LPS from *Escherichia coli* 055:B5 (500 μg/100 g of body weight; Sigma Chemical Company, St. Louis, MO, USA) in 200 μl of 0.9% saline buffer was subcutaneously implanted into the back area just behind the rib cage of five rats (LPS-implanted), under brief halothane anesthesia. For the control group, a same type of pump containing the same volume of saline buffer alone was subcutaneously implanted in another five rats (saline-implanted). The pump had a nominal flow rate of approximately 0.5 μl/h. At 0, 1, 3, 5, 7, 10, 14 and 15 days after implantation, blood samples were obtained under ether anesthesia. The animals were fasted for 12 h prior to each blood sample collection to avoid any confounding nutritional effects between the two groups. Serum samples were immediately separated by centrifugation at 1500 ***g*** for 10 min at 4°C, and then stored at −80°C until each assay. Liver, abdominal aorta, brain, heart, spleen, kidney, tongue and gingiva specimens were also collected from some animals in both groups on day 10 for histological analysis.

Transient administration of LPS

Animals were fasted for 12 h before beginning the experiment. Five rats

were subcutaneously injected with *E. coli* LPS (500 μg/100 g of body weight) in 2.5 ml of 0.9% saline into the back area just behind the rib cage (LPS-injected), under ether anesthesia. Another five rats were subcutaneously injected with the same volume of saline alone (saline-injected), as the control group. At 0, 2, 6, 12 and 24 h after the injection, blood samples were collected. Food was withheld from all rats throughout the experimental period. Serum samples were immediately separated and stored at −80°C until analysis.

Detection of LPS in serum

Serum LPS levels were measured with Limulus Amebocyte Lysate using a procedure based on an Limulus ES-II Test Wako (Wako Pure Chemical Industries, Osaka, Japan). Briefly, for pretreatment of the samples, 0.45 ml of Sample Pretreatment Solution (Wako) and 0.05 ml of sample serum were mixed in a test tube and incubated for 10 min (min) at 70°C, then the sample was cooled on ice and used for the assay. Each of the pretreated samples (0.1 ml) and Limulus Amebocyte Lysate were mixed in a test tube and incubated at 37°C in a tube reader (Toxinometer ET-301, Wako). Gelation time (T_g) was defined as the time needed for transmittance of the reaction mixture to reach a decreased value of 8%. LPS concentration was obtained using the T_g of the sample in relation to the calibration curve.

Measurement of lipid and cytokine levels in serum

Serum levels of TG, TC and FFA were measured using an enzymatic method with the chemical reagents L-Type Wako TG-H (Wako), L-Type Wako CHO-H (Wako) and NEFA-SS 'Eiken' (Eiken Chemical Co. Ltd, Tokyo, Japan), respectively. Serum TNF-α and MCP-1 levels were determined using enzyme-linked immunosorbent assay kits for rat TNF-α and rat MCP-1 (Biosource International Inc., Camarillo, CA, USA), respectively.

Histology

Liver, abdominal aorta, brain, heart, spleen, kidney, tongue and gingiva specimens were dissected, then fixed in 4% paraformaldehyde, embedded in paraffin and sectioned (3 μm). The sections were deparaffinized in xylene and rehydrated with graded alcohol, then stained with hematoxylin and eosin.

Immunohistochemical staining

Immunostaining was carried out using a Histofine SAB-PO (G) kit (Nichirei Corporation, Tokyo, Japan). After they were deparaffinized and rehydrated, each liver section was washed three times for 5 min each time in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl). Endogenous peroxidase activity was then blocked with 0.3% hydrogen peroxide for 30 min. After washing with TBS, the sections were incubated with 10% normal rabbit serum for 10 min at room temperature and then incubated with goat anti-rat TNF-α polyclonal antibody (Genzyme, Cambridge, MA, USA) overnight at 4°C in a humid atmosphere. After rinsing with TBS, the sections were incubated with horseradish biotinylated rabbit anti-goat IgG for 10 min. After rinsing with TBS, the sections were incubated with peroxidase-conjugated streptavidin for 5 min and then rinsed with TBS again. Color was developed with 0.025% 3,3′-diaminobenzidine tetra-hydrochloride in Tris-HCl buffer with hydrogen peroxide (Dako, Carpinteria, CA, USA).

Statistical analysis

Data are shown as means ± standard deviations (SD). The statistical significance of differences among groups was examined by one-way ANOVA and a two-tailed Student's *t*-test. The two-tailed Student's *t*-test was performed when the ANOVA test indicated significance, which was determined at $p < 0.05$.

Results

Rats in all four groups showed slightly lower body weights on day 1 after injection or implantation; however, the loss was greater in LPS-implanted and LPS-injected rats as compared with the respective control groups (mean percentage against initial body weight was 90% in LPS-implanted rats vs. 93% in saline-implanted rats and 95% in LPS-injected rats vs. 97% in saline-injected rats). Further, the reduction in body weight was more prolonged in the LPS-implanted than the saline-implanted rats, as the latter recovered their initial weight on day 3, whereas it took 5 days to recover in the LPS-implanted rats. None of the animals died during the experimental periods, though LPS-implanted rats showed some apparent signs of disease until day 5 (data not shown).

Changes in serum LPS levels after administration of LPS

Following injection of LPS, LPS was detected in serum at 2 h, and its level increased until 24 h (Fig. 1A). In LPS-implanted rats, serum LPS levels markedly increased to reach maximum on day 1 after implantation and then gradually declined to baseline on day 7 (Fig. 2A). In both saline-injected and saline-implanted rats, LPS could not be detected in serum throughout the experimental periods (Fig. 1A and Fig. 2A).

Effects of LPS administration on serum levels of lipids

Following injection of LPS, an increase in serum TG level was first detected at 2 h and gradually increased until 24 h (2.3-fold increase vs. 0 h) (Fig. 1B). No appreciable changes in serum TG levels were seen in saline-injected rats throughout the experimental period, whereas serum TG levels in LPS-injected rats were significantly elevated from 6 to 24 h after injection (Fig. 1B). As shown in Fig. 2(B), an increase in serum TG level was first observed on day 5 in LPS-implanted rats, and then it gradually increased to reach maximum on day 14 (3.7-fold increase vs. day 0) and declined thereafter. However, serum TG levels did not appreciably change throughout the experimental period in saline-implanted

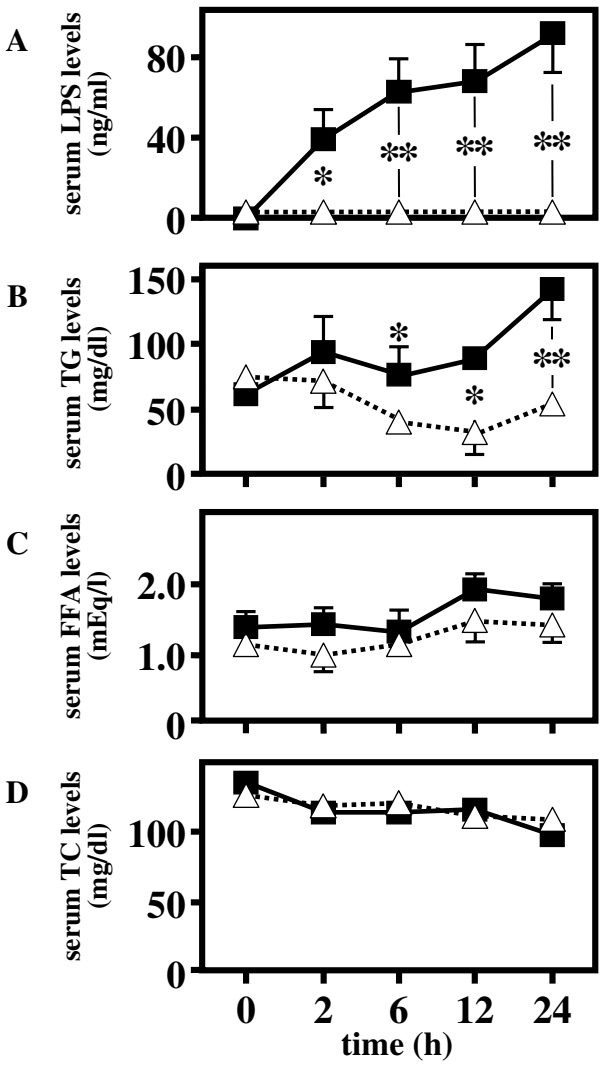

Fig. 1. Effects of subcutaneous and transient administration of lipopolysaccharide (LPS) on serum levels of LPS and lipids. Animals were fasted for 12 h before starting the experiment, and then injected subcutaneously with LPS (500 μg/100 g of body weight) in 2.5 ml of 0.9% saline buffer (LPS-injected; ■) or with saline buffer alone (saline-injected; △). At 0, 2, 6, 12 and 24 h after the injection, blood samples were collected. Food was further withheld from all groups throughout the experimental period. Serum LPS (A), triglyceride (B), free fatty acid (C) and total cholesterol (D) levels were measured as described in Material and methods. Values are shown as means ± SD of data obtained for five animals in each group. The difference from the value as compared to the saline-injection group was significant at $*p < 0.05$ or $**p < 0.01$.

rats. Further, LPS implantation significantly increased serum TG levels from day 5–15 as compared with saline implantation.

FFA levels in serum were slightly increased from 6 to 12 h after LPS injection (Fig. 1C). In LPS-implanted rats, a slight increase of serum FFA levels was seen on days 1 and 14 as compared with saline-implanted rats (Fig. 2C).

Conversely, serum TC levels did not change in any of the four groups throughout the experimental periods (Fig. 1D and Fig. 2D).

Effects of LPS administration on serum levels of cytokines

In both saline-injected and saline-implanted rats, very low levels of TNF-α and MCP-1 were detected; however, they did not significantly change throughout the experimental periods (Figs 3A and B). Following injection of LPS, serum TNF-α levels rapidly increased up to 2 h and subsequently declined to baseline at 24 h (Fig. 3A). In contrast, an increase of serum TNF-α levels could not be detected from days 1–15 in LPS-implanted rats (Fig. 3B).

In LPS-injected rats, serum MCP-1 levels remarkably increased to reach a peak at 2 h, and then slowly decreased until 24 h (Fig. 3A). A maximal increase in serum MCP-1 levels was also observed on day 1 in LPS-implanted rats and slowly declined thereafter, though remained significantly higher than that in saline-implanted rats until day 15 (Fig. 3B).

Histological and immunohistochemical assessments

The upper panels of Fig. 4 show histological changes in the liver on day 10 in LPS-implanted and saline-implanted rats. Liver sections from saline-implanted rats showed no tissue damage or inflammatory changes. In those from LPS-implanted rats, hepatocytes with nuclear morphological changes were slightly detected, though other apparent pathological changes could not be observed. In both groups, TNF-α-positive hepatocytes were detected along the central vein of the liver on day 10; however, the numbers of TNF-α-positive cells were nearly the same (lower panels of Fig. 4).

We did not detect any tissue damage or inflammatory changes in sections from the brain, heart, spleen, kidney, tongue or gingiva, as well as in sections from the abdominal aorta on day 10 in both LPS-implanted and saline-implanted rats (data not shown).

Discussion

To clarify the mechanisms underlying the association between chronic infection and atherosclerotic cardiovascular disease, we first investigated the effects of subcutaneous and continuous administration of LPS in rats, which was considered to mimic chronic infection such as periodontal disease, on the induction of hypertriglyceridemia. Previous studies [for a review, see (30)] have reported that transient or

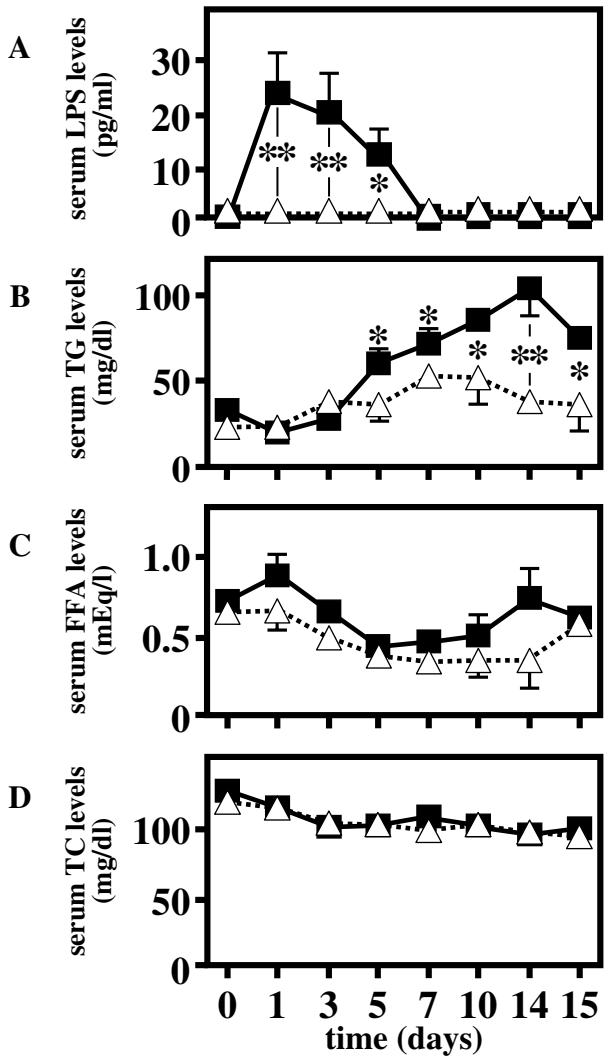

Fig. 2. Effects of subcutaneous and continuous administration of lipopolysaccharide (LPS) on serum levels of LPS and lipids. Animals were fasted for 12 h before starting the experiment. Mini-osmotic pumps containing LPS from *Escherichia coli* 055:B5 (500 μg/100 g of body weight) in 200 μl of 0.9% saline (LPS-implanted; ■) or saline alone (saline-implanted; △) were implanted subcutaneously. On 0, 1, 3, 5, 7, 10, 14 and 15 days after implantation, blood samples were obtained under ether anesthesia. The animals were fasted for 12 h prior to the collection of each blood sample. Serum LPS (A), triglyceride (B), free fatty acid (C) and total cholesterol (D) levels were measured as described in Material and methods. Values are shown as means ± SD of data obtained for five animals in each group. The difference from the value as compared to the saline-implanted group was significant at $*p < 0.05$ or $**p < 0.01$.

intermittent administration of LPS via a vein or intraperitoneally induced hypertriglyceridemia *in vivo*. In the present study, subcutaneous and continuous administration of LPS also induced hypertriglyceridemia, as it increased serum TG levels significantly and serum FFA levels slightly. However, the kinetics of the increase of serum TG levels with continuous administration of LPS were different from those seen with transient administration. In LPS-injected rats, a model of transient infection, serum LPS levels increased until 24 h after the injection, and those of TG also rapidly increased concomitantly with serum LPS levels. On the other hand, in LPS-implanted rats, a model of chronic infection, serum LPS levels increased to reach a maximum on day 1 after implantation and then gradually declined to the baseline by day 7. In contrast, an increase in serum TG level in these rats was first observed on day 5, after which it gradually increased to reach a maximum on day 14 and declined thereafter. These differences between LPS-implanted and LPS-injected rats may have been due to the remarkably lower levels of serum LPS in the former group as compared with the latter (25 pg/ml at 24 h after implantation vs. 90 ng/ml at 24 h after injection). Thus, the present results suggest that chronic infection increases the risk of hypertriglyceridemia.

Hypertriglyceridemia that frequently accompanies infections and inflammatory diseases is attributed to both an increase in lipoprotein production and a decrease in lipoprotein clearance. Previous reports have demonstrated that LPS-induced hypertriglyceridemia is due to a delay in catabolism of circulating lipids, and secondarily, to a decrease in adipose tissue lipoprotein lipase activity (14–18). On the other hand, Feingold *et al.* (19) reported that the mechanism of LPS-induced hypertriglyceridemia is dependent on the concentration of LPS, as an intraperitoneal administration of a low dose of LPS (100 ng/100 g of body weight) stimulated hepatic *de novo* fatty acid synthesis and lipolysis, both of which provided a source of fatty acids for an increase in hepatic *de novo* TG production. In contrast, administration of a high dose of LPS (50 μg/100 g of body weight) did not increase hepatic TG secretion or lipolysis in their study, but rather induced hypertriglyceridemia by decreasing TG lipoprotein clearance and post-heparin lipoprotein lipase activity. Those results supported a previous *in vitro* study (31), which demonstrated that low concentrations of LPS increased lipid synthesis in hepatocytes and the secretion of lipids into culture medium, whereas high doses of LPS did not affect lipid homeostasis. In the present study, LPS at 500 μg/100 g of body weight was subcutaneously administrated by injection as well as with mini-osmotic pumps. This dose is higher than that used by Feingold *et al.* (19), but far below the dose required to cause death in their laboratories (LD_{50}: approximately 5 mg/100 g of body weight). In our experiment, none of the animals died

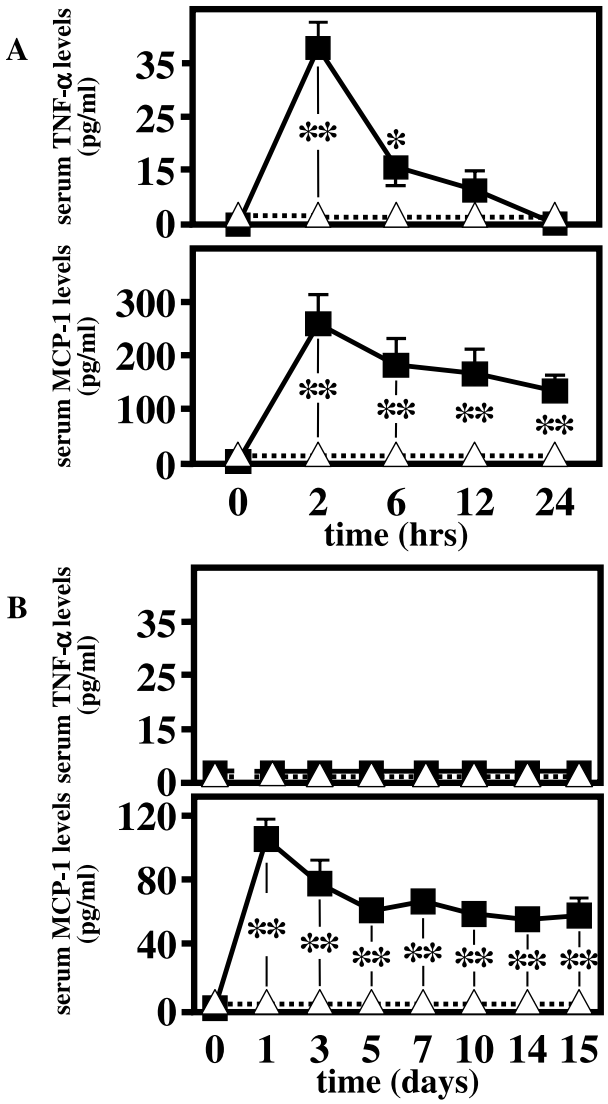

Fig. 3. Changes in serum tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) levels by transient or continuous administration of lipopolysaccharide (LPS). Following injection (A) or implantation (B) of LPS, blood samples were obtained at the time points indicated. The animals in each group were fasted as described in the legend to Fig. 2. Serum TNF-α and MCP-1 levels were measured using enzyme-linked immunosorbent assay kits for rat TNF-α and rat MCP-1, respectively. Values are shown as means ± SD of data obtained for five animals in each group. The difference from the value as compared to the saline-injected or saline-implanted groups was significant at $*p < 0.05$ or $**p < 0.01$.

following an administrations of LPS. In addition, our histological findings showed that some hepatocytes with nuclear morphological changes were detected in the livers of LPS-implanted rats, whereas no other apparent tissue damage or inflammatory changes were observed in the abdominal aorta, brain, heart, spleen, kidney, tongue or gingiva of any of the animals. Furthermore, maximum serum LPS levels were relatively low in both LPS-injected and LPS-implanted rats. However, it is not clear at present which mechanisms (increase of hepatic *de novo* fatty acid and TG synthesis or decrease of TG lipoprotein clearance) are predominantly involved in the hypertriglyceridemia caused by subcutaneous administration of LPS at the dose used in the present study.

Previous studies (5–9) suggested that hypertriglyceridemia is an independent risk factor for atherosclerotic cardiovascular disease, because elevated levels of serum TG are associated with a decrease in serum high density lipoprotein (HDL) cholesterol levels and increases of remnant lipoproteins, LDL and thrombogenic conditions, all of which are pathogenic conditions considered to be related to atherosclerosis. The results of primary and secondary prevention studies (32–34) also support the concept that some types of hypertriglyceridemia cause athrosclerosis. On the other hand, many investigators (35–49) have suggested a possible contribution of periodontal pathogens and periodontal disease to cardiovascular disease. In addition, recent human studies (41, 50–53) have demonstrated positive significant correlations between serum lipid profiles, such as serum TG, LDL and/or TC levels, and periodontal conditions. The present data as well suggest an association between chronic infection, such as periodontal disease, and cardiovascular disease via hypertriglyceridemia.

Feingold *et al.* (19) found that administration of anti-TNF-α or IL-1 receptor antagonist blocked an increase in serum TG levels induced by TNF-α or IL-1, respectively; however, they did not prevent the increase induced by LPS. These results suggest that neither TNF nor IL-1 is essential for the increase in serum TG levels induced by LPS, and that other cytokines, hormones, small molecular mediators or LPS itself participates with it. Thus, we next examined the participation of TNF-α with the increase of TG levels induced by subcutaneous and continuous or transient administration of LPS. After injection of LPS, serum levels of TNF-α rapidly increased up to 2 h, and then declined to the baseline by 24 h, while the increase of serum TG levels, which first occurred at 2 h, increased up to 24 h. These results suggest that when using a transient administration of LPS as a model of transient infection, TNF-α is partially involved in the initial stage of up-regulation of serum TG levels by LPS. In contrast, after implantation of LPS, an increase in serum TG levels was first observed on day 5 and gradually

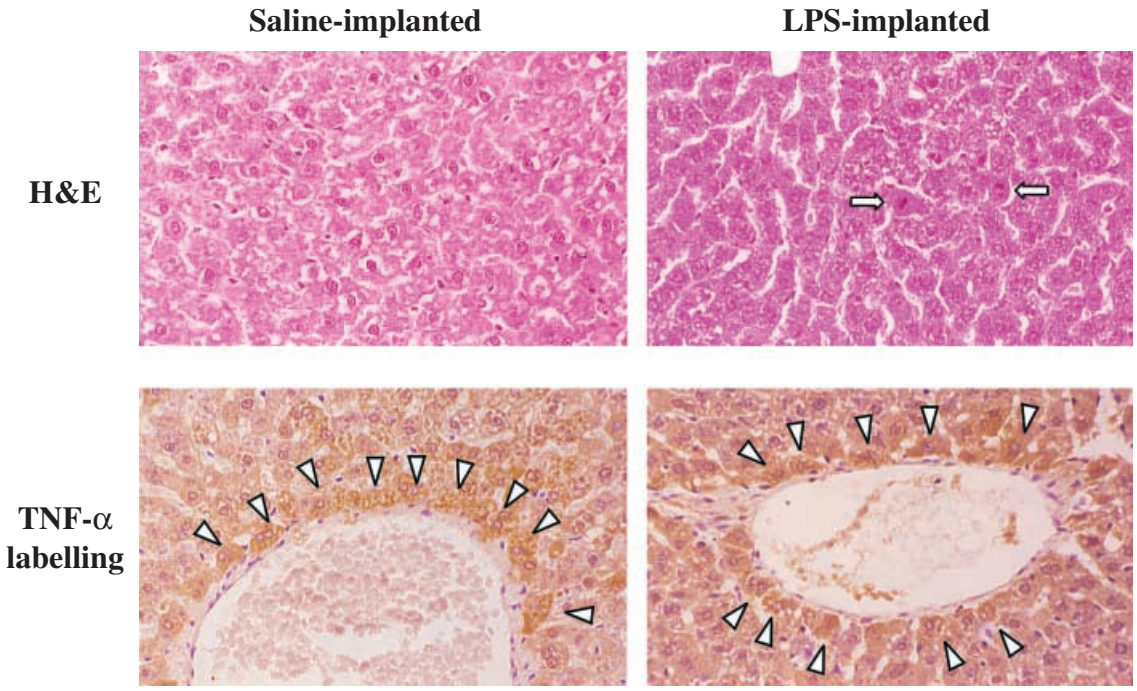

Fig. 4. Histological and immunohistochemical assessments of lipopolysaccharide (LPS)-implanted rats. Livers were collected from some of the animals on day 10 following LPS implantation. Sections were stained with hematoxylin and eosin (H&E) and/or immunoperoxidase for tumor necrosis factor-α (TNF-α). (A) H&E staining; ×600, arrowheads indicate hepatocytes with nuclear morphological changes. (B) TNF-α labeling; ×600, arrows indicate TNF-α-positive hepatocytes.

increased to reach a maximum on day 14, whereas no increase of serum TNF-α levels could be detected from days 1–15. In addition, immunohistochemical observations showed that the number of TNF-α-positive cells in the livers of LPS-implanted rats was the same as that of saline-implanted rats on day 10 after implantation. Therefore, when using a continuous administration of LPS as a model of chronic infection, the increase of serum TG levels induced by LPS may not be mediated by TNF-α.

A pivotal role for chemokines such as MCP-1 in the pathogenesis of atherosclerosis has been postulated. Recently, the MCP-1/MCP-1 receptor (CCR2) pathway has been speculated to be involved in the inflammatory aspect of atherogenesis, because atheroma-forming cells express MCP-1 and CCR2, and the MCP-1/CCR2 pathway is activated in atherosclerotic lesions (24). In addition, activation of this pathway induces the expression of adhesion molecules (25), proinflammatory cytokines (25, 26), chemokines and matrix metalloproteinases (27), thus acerbating atherosclerosis in hypercholesterolemic animals (28, 29). These findings indicate that MCP-1 may contribute not only to vascular inflammation but also to plaque destabilization and thrombosis. Thus, we finally investigated the effects of subcutaneous and continuous or transient administration of LPS on serum MCP-1 levels. Interestingly, after both implantation and injection of LPS, increased serum MCP-1 levels were maintained throughout each experimental period at significantly higher levels, as compared to the controls. These results suggest that chronic infection causes an increase in serum MCP-1 levels, which participates with the induction of atherosclerosis.

In summary, subcutaneous and continuous administration of LPS induced hypertriglyceridemia, as it increased serum TG levels significantly and FFA levels slightly. This increase of serum TG levels induced by the continuous administration of LPS was not considered to be mediated by TNF-α, because the increase of serum TNF-α levels induced by LPS rapidly declined to the baseline. Conversely, the increase of serum MCP-1 levels induced by continuous administration of LPS was maintained throughout the experimental period. These findings suggest that chronic infection such as periodontal disease induces hypertriglyceridemia and increases serum MCP-1 levels in a manner that increases the risk of atherosclerotic cardiovascular disease.

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