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Regulation of Plasminogen Activator Inhibitor-1 in Adipocytes by Macrophages Activated by Low-dose Lipopolysaccharide

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Abstract. *Background/Aim: Increased expression of inflammatory cytokine genes through cell interactions in tissues may cause chronic inflammation, leading to the development of lifestyle-related diseases. Since the activation of inflammatory cytokine genes in monocytes/macrophages by co-culturing with cancer cells or adipocytes was suppressed by pre-treatment with low-dose lipopolysaccharide (LPS), we hypothesized that low-dose LPS-activated macrophages may regulate the expression of immune response-related genes in other cells. Materials and Methods: Phorbol myristate acetate-treated human monocytes (THP-1) were activated by LPS. The conditioned medium of LPS-activated THP-1 cells was added to human adipocytes. After 5 days, the expression of genes encoding interleukin (IL)-6 (IL6), IL-8 (IL8), monocyte chemotactic protein (MCP)-1 (CCL2), adiponectin (ADIPOQ), and plasminogen activator inhibitor (PAI)-1 (SERPINE1) was analyzed using quantitative real-time PCR. Results: The increased expression of inflammation-related genes and SERPINE1 in adipocytes was suppressed by the conditioned medium of THP-1 cells activated by low-dose LPS, whereas the expression of ADIPOQ was significantly increased. Conclusion: Low-dose LPS-activated macrophages convert adipocytes to anti-inflammatory phenotypes.*

Macrophages maintain homeostasis by eliminating foreign substances and pathogens (1, 2). They also change their functions according to the tissue environment. Macrophages have been suggested to be an essential for inducing inflammatory changes in tissues (3). The expression of inflammatory cytokine genes is increased through cell interactions with the macrophages accumulated in tumor tissues or adipose tissues of obese patients, thereby inducing inflammatory changes in these tissues and causing chronic inflammation (4-7). Chronic inflammation leads to the development of cancer, lifestyle-related diseases, and autoimmune diseases, and affects the severity of these diseases (8-11).

In adipose tissues, adipocytes not only accumulate fat but also produce various biologically active substances, such as plasminogen activator inhibitor (PAI)-1. The increased expression of PAI-1 is thought to cause thrombosis. The expression of PAI-1 has been reported to be promoted significantly by inflammatory cytokines (12). The expression of inflammatory cytokine genes in adipocytes is increased through interactions with macrophages (3). Therefore, the expression of *SERPINE1*, which encodes PAI-1, in adipocytes can be increased through adipocytes' interaction with macrophages, leading to the development of lifestyle-related diseases such as arteriosclerosis (13, 14).

Meanwhile, macrophages are activated by lipopolysaccharide (LPS), an extracellular membrane component of gram-negative bacteria and the cause of septic shock; the expression of inflammatory cytokine genes is increased with high-dose LPS (15). On the other hand, the increased expression of inflammatory cytokine genes in monocytes/macrophages by co-culturing with cancer cells or adipocytes using a transwell system was suppressed by pre-treatment with low-dose LPS (16-19). Also, the expression of inflammatory cytokine genes in mouse peritoneal macrophages was not increased with low-dose LPS treatment (20). Thus, it has been suggested that low-dose LPS-activated macrophages have anti-inflammatory effects and regulate the expression of immune response-related genes in other cells (19). Increased expression of

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inflammatory cytokine genes in adipocytes may induce inflammatory changes in adipose tissues and cause chronic inflammation that leads to the development of lifestyle-related disease. Here, we investigated whether low-dose LPS-activated macrophages suppressed the expression of inflammatory cytokine genes in adipocytes. We analyzed the changes in the expression of inflammation-related genes and arteriosclerosis-related genes in adipocytes after treatment with the conditioned medium from LPS-activated macrophages.

Materials and Methods

Cells. Human monocytes (THP-1) were obtained from the American Type Culture Collection. THP-1 cells were cultured in RPMI 1640 medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% fetal calf serum supplemented with 100 units/ml each of penicillin and streptomycin (FUJIFILM Wako Pure Chemical Corporation) in 5% CO_2 atmosphere at 37˚C. Human preadipocytes (HWP-c) were obtained from PromoCell (PromoCell GmbH, Heidelberg, Germany). HWP-c in Preadipocyte Growth Medium (PromoCell GmbH) were incubated in 5% CO_2 atmosphere at 37˚C. After reaching confluency, cells were cultured for 72 h in Preadipocyte Differentiation Medium (PromoCell GmbH). Next, cells were cultured in Adipocyte Nutrition Medium (PromoCell GmbH) until they matured into adipocytes.

Addition of conditioned medium from LPS-activated THP-1 cells. THP-1 cells were seeded at a density of 5×10^5 cells/ml in 100 mm dishes. THP-1 cells were treated with 5 ng/ml phorbol myristate acetate (FUJIFILM Wako Pure Chemical Corporation) for 48 h and activated with ultra-pure *Escherichia coli* LPS (0.1 ng/ml, 10 ng/ml, or 1,000 ng/ml) (InvivoGen Corporation, San Diego, CA, USA). THP-1 culture medium was collected at 24 h after activation with LPS. Adipocyte culture medium was removed, and conditioned medium from LPS-activated THP-1 cells and Adipocyte culture Nutrition Medium (PromoCell GmbH) were added in equal amounts to adipocytes. Then, the cells were incubated in 5% CO_2 atmosphere at 37˚C for 5 days.

RNA extraction. The total RNA from THP-1 cells and adipocytes was extracted using TRIzol® Reagent (Invitrogen Corporation, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. RNA was quantified by measuring the absorbance at 260 nm. cDNA was synthesized using reverse transcriptase with Oligo(dT)20 (TOYOBO Co., Ltd., Osaka, Japan).

Quantitative real-time PCR. Real-time PCR was performed using Thunderbird® SYBR® qPCR Mix (TOYOBO Co, Ltd.). PCR conditions were set at 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 s, 60˚C for 30 s. Relative quantification was performed by normalizing the target expression to that of the housekeeping gene, *ACTB* encoding β-actin.

Statistical analysis. Data were analyzed with Student's *t*-test (Excel 2013). Differences between stimulation with and without LPS were considered statistically significant at $p<0.05$.

Results

Expression of inflammation-related genes in adipocytes following treatment with conditioned medium from low-dose LPS-activated macrophages. The increased expression of inflammatory cytokine genes in adipocytes may lead to the development of lifestyle-related diseases. We investigated the changes in the expression of inflammation-related genes, *interleukin (IL)6* encoding IL-6, *IL8* encoding IL-8, and *CCL2* encoding monocyte chemotactic protein (MCP)-1, in adipocytes after treatment with conditioned medium from LPS-activated THP-1 cells. The expression of *IL-6* tended to be suppressed by the conditioned medium of THP-1 cells activated by 0.1 ng/ml of LPS (p=0.077). The expression of *IL-8* tended to be repressed by the conditioned medium of THP-1 cells activated by 10 ng/ml of LPS (p=0.085). In addition, the expression of *MCP-1* was reduced by the conditioned medium of THP-1 cells activated by 0.1 ng/ml of LPS. In contrast, the expression of *IL6, IL8,* and *CCL2* was increased by the conditioned medium of THP-1 cells activated by 1,000 ng/ml of LPS (Figure 1). These results demonstrated that the increased expression of inflammation-related genes in adipocytes was suppressed by the conditioned medium of THP-1 cells activated by low-dose LPS.

Expression of the adiponectin gene in adipocytes following treatment with conditioned medium from low-dose LPS-activated macrophages. Adiponectin is known as a biomarker of metabolic syndromes (21). It has been shown that the increased expression of *ADIPOQ* encoding adiponectin in adipocytes prevents lifestyle-related diseases (13). We investigated the change in expression of *ADIPOQ* in adipocytes after treatment with conditioned medium from LPS-activated THP-1 cells. The expression of *ADIPOQ* was significantly increased by the conditioned medium of THP-1 cells activated by 0.1 ng/ml of LPS (p=0.016), but decreased by the conditioned medium of THP-1 cells activated by 1,000 ng/ml of LPS (Figure 2). These results demonstrated that the expression of *ADIPOQ* in adipocytes was regulated by the conditioned medium of THP-1 cells activated by low-dose LPS.

Expression of the PAI-1 gene in adipocytes following treatment with conditioned medium from low-dose LPS-activated macrophages. PAI-1 is known to inactivate tissue-type plasminogen activators that regulate the production of plasmin (22). Thus, the increased expression of *SERPINE1* encoding PAI-1 causes thrombosis and leads to the development of lifestyle-related diseases such as arteriosclerosis. We investigated the change in expression of *SERPINE1* in adipocytes after treatment with conditioned medium from LPS-activated THP-1 cells. *SERPINE1* expression was significantly suppressed by the conditioned

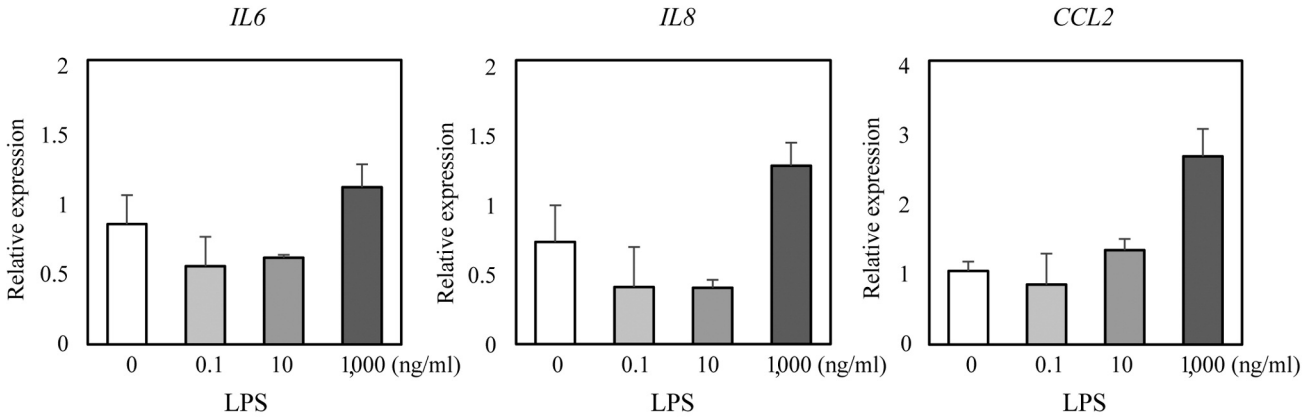

Figure 1. *IL6, IL8, and CCL2 gene expression in adipocytes after treatment with the conditioned medium from LPS-activated macrophages. The mRNA expression of IL6, IL8, and CCL2 in adipocytes was analyzed using quantitative real-time PCR. Relative quantification was performed by normalizing the expression of the target gene to that of ACTB.*

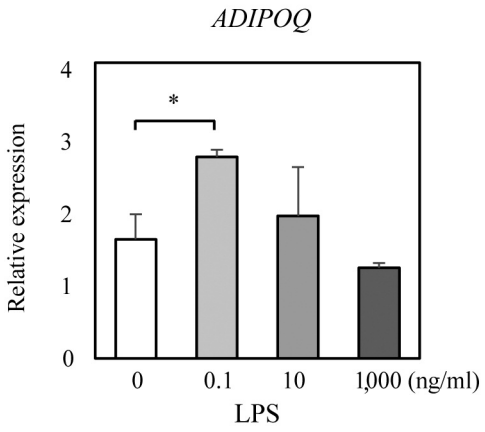

Figure 2. *ADIPOQ gene expression in adipocytes after treatment with the conditioned medium from LPS-activated macrophages. The mRNA expression of ADIPOQ in adipocytes was analyzed using quantitative real-time PCR. Relative quantification was performed by normalizing the expression of the target gene to that of ACTB. *p<0.05.*

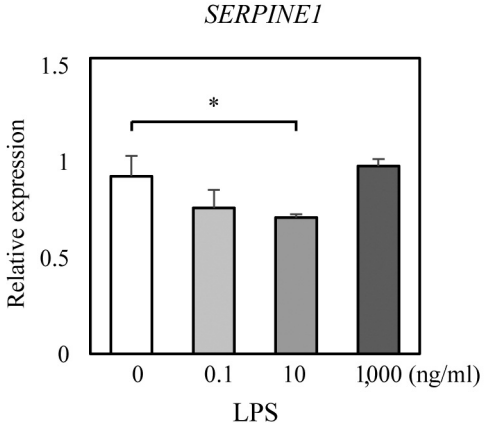

Figure 3. *SERPINE1 gene expression in adipocytes after treatment with the conditioned medium from LPS-activated macrophages. The mRNA expression of SERPINE1 in adipocytes was analyzed using quantitative real-time PCR. Relative quantification was performed by normalizing the expression of the target gene to that of ACTB. *p<0.05.*

medium of THP-1 cells activated by 0.1 ng/ml or 10 ng/ml of LPS (p=0.056 and p=0.036, respectively). On the other hand, *SERPINE1* expression was not suppressed by the conditioned medium of THP-1 cells activated by 1,000 ng/ml of LPS (Figure 3). These results demonstrated that the increased expression of *SERPINE1* in adipocytes was suppressed by the conditioned medium of THP-1 cells activated by low-dose LPS.

Expression of inflammation-related genes in low-dose LPS-activated macrophages. It has been shown that the increased expression of inflammatory cytokine genes in monocytes/macrophages by co-culturing with cancer cells or adipocytes was suppressed by pre-treatment with low-dose LPS (16-19). We confirmed the anti-inflammatory effects of the conditioned medium of low-dose LPS-activated THP-1 cells by investigating the changes in the expression of inflammatory cytokine genes, *IL8* and *CCL2*, and anti-inflammatory cytokine genes, *IL10* and *TGFB1*. The expression of *IL8* and *CCL2* in THP-1 cells activated by 0.1 ng/ml of LPS was unchanged compared to the control THP-1. However, the expression of *IL8* in THP-1 cells activated

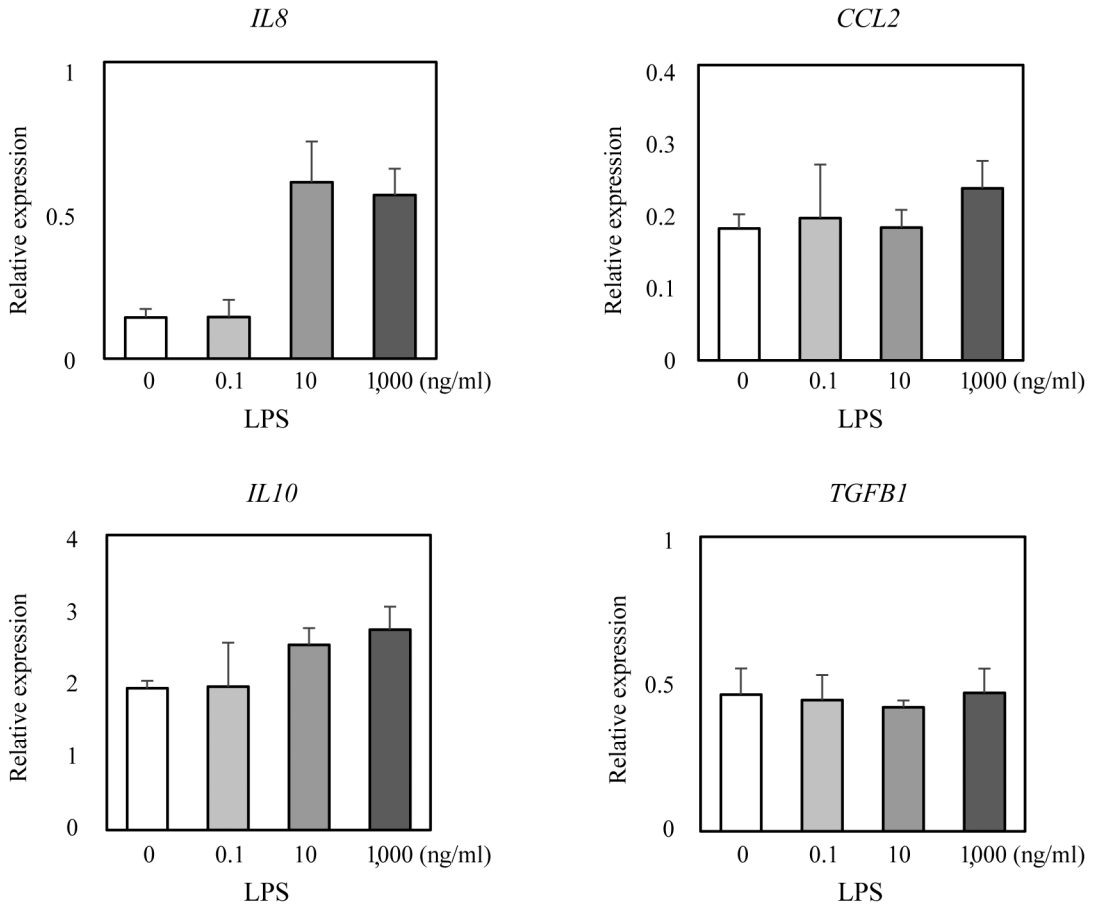

Figure 4. *IL8, CCL2, IL10, and TGFB1 gene expression in LPS-activated THP-1 cells. The mRNA expression of IL8, CCL2, IL10 and TGFB1 in THP-1 cells was analyzed using quantitative real-time PCR. Relative quantification was performed by normalizing the expression of the target gene to that of ACTB.*

by 10 ng/ml or 1,000 ng/ml of LPS was increased. On the other hand, the expression of *IL10* and *TGFB1* in THP-1 cells activated by 0.1 ng/ml of LPS was unchanged compared to the control THP-1, but the expression of *IL10* in THP-1 cells activated by 10 ng/ml or 1,000 ng/ml of LPS was increased (Figure 4). The increased expression of the inflammatory and anti-inflammatory genes was not observed in THP-1 cells activated by 0.1 ng/ml of LPS. These results demonstrated that low-dose LPS had anti-inflammatory effects in THP-1 cells and might regulate the expression of inflammatory cytokine genes.

Discussion

Macrophages are known to change their functions through cell interactions in tissues (1, 2). Previous studies have demonstrated an increased expression of inflammatory cytokine genes in monocytes/macrophages by co-culturing them with cancer cells or adipocytes using a transwell system. Moreover, it has been revealed that the increased expression of inflammatory cytokine genes in monocytes/macrophages was suppressed by pre-treatment with low-dose LPS (16-19). Increased expression of inflammatory cytokine genes in macrophages is thought to cause chronic inflammation in adipose tissues, leading to the development of lifestyle-related diseases. Macrophages have been shown to respond differently to high-dose and low-dose LPS (16-20). It has been reported that LPS might have the characteristic of an exogenous hormone than those of a toxin (23). Low-dose LPS-activated macrophages may have anti-inflammatory effects through cell interactions (19). In this study, we investigated whether low-dose LPS-activated macrophages suppressed the increased expression of inflammatory cytokine genes in adipocytes by studying the expression of the corresponding genes in adipocytes after treatment with conditioned medium from LPS-activated macrophages. It was confirmed that low-dose LPS-activated macrophages did not

increase the expression of inflammatory cytokine genes (Figure 4). Moreover, the increased expression of inflammation-related genes, including *IL6*, *IL8,* and *CCL2*, in adipocytes was negated after treatment with conditioned medium from low-dose LPS-activated macrophages (Figure 1). It was demonstrated that low-dose LPS-activated macrophages may be converting adipocytes to anti-inflammatory phenotypes. Therefore, low-dose LPS-activated macrophages may help prevent the development of lifestyle-related diseases by inducing inflammatory changes in tissues and causing chronic inflammation through cell interactions.

MCP-1 is known to be a chemotaxis-related factor as well as an inflammation-related factor. It was demonstrated that the increased expression of *CCL2* in adipocytes was suppressed after treatment with conditioned medium from low-dose LPS-activated macrophages but the expression of *CCL2* was increased after treatment with conditioned medium from high-dose LPS-activated macrophages (Figure 1). Consequently, low-dose LPS-activated macrophages may help suppress macrophage migration into adipose tissues and the subsequent inflammatory changes in the tissues through cell interactions.

In recent years, the number of patients with lifestyle-related diseases, such as heart diseases and cerebrovascular diseases, has increased. These diseases are thought to be caused by arteriosclerosis associated with visceral fat accumulation, diabetes, and hypertension. PAI-1 likely plays a crucial role in developing lifestyle-related diseases such as arteriosclerosis (24). Increased *SERPINE1* expression is associated with the insulin-resistant syndrome (25). Moreover, plasma PAI-1 levels have been closely correlated with intraperitoneal fat accumulation in humans (26). It has been pointed out that PAI-1 may not only regulate thrombus formation but also suppress the onset of obesity and insulin resistance (27).

This study demonstrated that the increased expression of *SERPINE1* in adipocytes was significantly suppressed after treatment with conditioned medium from low-dose LPS-activated macrophages (Figure 3). *SERPINE1* expression has been reported to be significantly promoted by inflammatory cytokines (12). The increased expression of *SERPINE1* in adipocytes may be suppressed by the anti-inflammatory effects of low-dose LPS-activated macrophages. Therefore, low-dose LPS-activated macrophages may help prevent thrombosis, the onset of obesity, and insulin resistance caused by inflammatory changes in adipose tissues.

Conflicts of Interest

The Authors have no conflicts of interest in relation to this study.

Authors' Contributions

All Authors have contributed to data collection and interpretation. TH performed experiments and drafted the manuscript. HI contributed to critical revision of the manuscript.

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