Impact of Pioglitazone on Macrophage Dynamics in Adipose Tissues of Cecal Ligation and Puncture-Treated Mice

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Pioglitazone improves sepsis-induced organ injury accompanied with anti-inflammatory effects on visceral adipose tissue. However, its action in adipose immune cells remains to be ascertained. We investigated the effects of pioglitazone on visceral adipocyte macrophage population and polarization in cecal ligation and puncture (CLP)-induced sepsis mice. Eight-week-old male mice were assigned to 3 groups: 1) sham-operated group, 2) CLP group, or 3) pioglitazone-treated CLP group. Pioglitazone (10 mg/kg) was injected intraperitoneally for 7 d and CLP surgery was performed. Visceral adipose tissues were collected 24 h after the surgery. mRNA expression of several macrophage markers (inducible nitric oxide synthase (iNOS) for M1, arginase1 (Arg1) and interleukin (IL)-10 for M2, CD163 and F4/80 for mature macrophages) and inflammatory adipokines (IL-6, monocyte chemotactic protein-1: MCP-1) was quantified by real-time RT-PCR. Tissue sections were subjected to the immunohistochemical analysis and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. CLP significantly enhanced Arg1, IL-10 and iNOS mRNA expressions as compared with the sham group, and pioglitazone significantly increased the mRNA level of CD163 and F4/80 in CLP mice. Expression of IL-6 and MCP-1 stimulated by CLP was reduced by pioglitazone treatment. Increased CD11b/c- and CD163-positive cells as well as apoptotic cells were observed in the CLP group and the pioglitazone-treated group. The data indicate that M1/M2 macrophage activation from visceral adipose tissues is induced in CLP-induced mice, and the function of macrophages recruited from surrounding organs may be modulated by pioglitazone treatment.

Key words pioglitazone; sepsis; visceral adipose tissue; macrophage

Severe sepsis is a complex acute inflammatory and systemic disease, which contributes substantially to morbidity and mortality after gastrointestinal surgery.1–3 This inflammatory response is characterized by the systemic circulation of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6). Although these inflammatory cytokines are considered to act as basic mediators in the pathogenesis of sepsis, the mechanism of severe sepsis remains incompletely understood. Furthermore, the incidence of sepsis has increased over the last 20 years despite recent improvements in surgical techniques and intensive care.2 Therapeutic drugs that effectively reduce mortality from severe sepsis are also lacking.

Recently, the concept that adipose tissue is an endocrine/immune-modulating organ has been broadly recognized.4,5 Adipocytes secrete various adipokines including adiponectin, TNF-α, and IL-6. Adipose tissue includes pre-adipocytes, vascular cells, and hemocytes. Adipose regulatory T cells and macrophages have crucial roles in adipose tissue inflammation. Pioglitazone, a peroxisome proliferator-activated receptor (PPAR) γ activator, improved the survival rate in association with increased serum adiponectin levels in cecal ligation and puncture (CLP)-treated mice.6–12 This compound might ameliorate CLP-induced sepsis in part by reducing excessive levels of inflammatory cytokines in adipose tissues.13–15

Infiltration of macrophages to adipose tissue contributes to low-grade adipocyte inflammation, which is closely associated with obesity-related insulin resistance.8,9,13,14 Hence, given our previous and progressing findings, we considered that cross-talk between adipocytes and macrophages in visceral adipose tissue might play an important role in the development of several diseases. Adipose tissue macrophages are divided into three different phenotypes: classically activated M1 macrophages, alternatively activated M2 macrophages, and deactivated macrophages.12 M1 macrophages in obese adipose tissue produce pro-inflammatory and secrete chemokines such as CXCL10 and CXCL11. The cells are involved in promoting inflammation and eliminating the pathogen. M2 macrophages generate anti-inflammatory cytokines such as IL-10, and are characterized by the upregulation of CCR2 and CXCR1/2. The cells produce ornithine and polyamines through the arginase pathway. M2 macrophages, which are the resident macrophages in lean adipose tissue, play a protective role in adipose tissue homeostasis involved in the repair or remodeling of tissues. The ratio of adipose tissue M1/M2 macrophages might have an impact on systemic immune performance.

Both adipocyte and macrophages express high levels of PPARγ and are the targets for pioglitazone.10 However, pioglitazone-associated changes in macrophage populations and polarization in CLP mice remain poorly understood. In the present study, we investigated whether alterations of adipose M1/M2 macrophage balance occur in a mouse model of CLP-induced sepsis and assessed whether pioglitazone treatment before CLP influences the dynamic of macrophages in visceral
adipose tissues.

MATERIALS AND METHODS

Reagents  Pioglitazone (Takeda Pharmaceutical Co., Tokyo, Japan) was dissolved in 0.5% methylcellulose (50cps, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and injected intraperitoneally (10 mg/kg/d) for 7 d before CLP surgery. Antibodies against CD68 (ab125212) and CD163 (ab119996) were purchased from Abcam (Cambridge, U.K.). Antibodies against CD11b/c (ab87) and Alexa Fluor 488-labeled goat anti-rabbit immunoglobulin G (IgG) were obtained from Novus Biologicals (Cambridge, U.K.) and Life Technologies Corporation (Carlsbad, CA, U.S.A.), respectively.

Animals and Experimental Design  Eight-week-old male C57BL/6J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained in an air-controlled room (temperature 23 ± 1°C, humidity 55 ± 5%) under controlled lighting (12-h light/dark cycle) with free access to standard food and water. All animal handling protocols and surgical procedures were reviewed and approved by the Institutional Animal Care Committees at Tokyo University of Pharmacy and Life Sciences Corporation (Carlsbad, CA, U.S.A.). In this study, ca. four hours after surgery, the mice were anesthetized, and each stump was subjected to a midline incision via a 21-ga needle. The needle was then returned to the peritoneal cavity. In this model, approximately 90% animals died within 4 d. Sham-operated mice that received anesthesia and underwent a laparotomy via a midline incision without CLP served as the control for CLP surgery. Twenty-four hours after surgery, the mice were anesthetized, and ca. 100 mg visceral adipose tissues located in the upper parts of the epididymis were removed to prepare paraffin-embedded sections or were homogenized with Isogen II (Wako Pure Chemical Industries, Ltd.) for RNA analysis.

Operative Procedures  CLP was performed to induce polymicrobial sepsis in mice, as described previously. In brief, the cecum was ligated immediately below the ileocecal valve with a silk suture, and each stump was subjected to a single puncture with a 21-ga needle. The cecum was then returned to the peritoneal cavity. In this model, approximately 90% animals die within 4 d. Sham-operated mice that received anesthesia and underwent a laparotomy via a midline incision without CLP served as the control for CLP surgery. Twenty-four hours after surgery, the mice were anesthetized, and ca. 100 mg visceral adipose tissues located in the upper parts of the epididymis were removed to prepare paraffin-embedded sections or were homogenized with Isogen II (Wako Pure Chemical Industries, Ltd.) for RNA analysis.

Quantitative RT-PCR Analysis  Visceral adipose tissues were homogenized with RNA extraction buffer (Isogen II, Wako Pure Chemical Industries, Ltd.), and RNA was extracted. The mRNA levels of CD63, F4/80, arginase1 (Arg1), inducible nitric oxide synthase (iNOS), IL-10, and pro-inflammatory cytokines (IL-6, MCP-1) were determined by quantitative real-time RT-PCR using an iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCRs were run on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), and iQ5 Optical system software was used to collect the data and calculate the threshold cycle (Ct). Quantification of gene expression was assessed with the ΔΔCt method corrected with GAPDH levels, and the relative amounts of mRNA were depicted. To determine the corrections between the expression levels of each marker, the statistical significance of the Pearson r values was tested (Fig. 2B).

Immunohistochemistry and Immunofluorescence  The adipose tissues were embedded in paraffin according to standard procedures. Paraffin sections were rehydrated and stained with hematoxylin-eosin solution (Wako Pure Chemical Industries, Ltd.). The sections were treated with 0.1% sodium citrate containing 0.1% Triton-X100 for 10 min for permeabilization, incubated with 10 nm sodium citrate buffer (pH 6) at 100°C for 20 min for antigen revival, and blocked for 2 h with 10% normal goat serum in 20 nm phosphate-buffered saline (PBS, pH 7.45). The slides were incubated with primary anti-CD11b/c antibody for 18 h at 4°C in immunostaining buffer, treated with an amino acid polymer conjugated to anti-rabbit IgG Fab′ labeled with horseradish peroxidase (Histofine Simple Stain Mouse MAX-PO (R), Nichirei, Tokyo, Japan), and then developed with Histofine Simple Stain DAB solution (Nichirei). Nuclear counterstaining was performed using hematoxylin. Omission of primary antibody was used as negative control. CD163-positive cells were visualized with immunofluorescent staining. The slides were incubated with rabbit anti-CD163 antibody for 18 h at 4°C in immunostaining buffer (Can Get Signal, Toyobo, Osaka, Japan) followed by Alexa Fluor 488-labeled goat anti-rabbit IgG (Life Technologies Corporation). Nuclear counterstaining was performed using 300 nm 4',6-diamidino-2-phenylindole (DAPI, Life Technologies Corporation). The sections were mounted with anti-fading reagent (SlowFade; Invitrogen) and examined using a microscope (Axiovert 200M; Zeiss, Jena, Germany).

Effect of Pioglitazone on M2 and M1 Macrophage Marker mRNA Expression in Visceral Adipose Tissues  To evaluate the effect of CLP and pioglitazone treatment on accumulation of macrophages in visceral adipose tissue, the expression of M2 macrophage markers was quantified 24 h after operative procedures (Fig. 1). As for M2 macrophage markers, the Arg1 and IL-10 mRNA levels in the CLP group were significantly higher than those in the sham group (p < 0.05). Although there was no significant difference in Arg1 mRNA levels between the CLP group and the pioglitazone-treated CLP group, the IL-10 mRNA level in the pioglitazone-treated CLP group was approximately 2-fold higher than the value in the CLP group. M1 macrophage marker, iNOS mRNA level in the CLP group was also significantly higher than those in the sham group (p < 0.05). There was no significant difference in iNOS mRNA levels between the CLP group and the pioglitazone-treated CLP group.
mRNA Expression in Visceral Adipose Tissues

The mRNA expression of global macrophage markers was quantified 24 h after operative procedures (Fig. 2A). The CD163 mRNA level in the CLP group was similar to that in the sham group, but increased in the pioglitazone-treated CLP group, compared with the CLP group. Another mature macrophage marker, F4/80, showed a similar trend, with a significant increase in the pioglitazone-treated CLP group compared to the CLP group (Fig. 2B).

**Fig. 1.** Effects of Pioglitazone on the Expression of Interleukin (IL)-10 and Arginase 1 (Arg1: M2 Macrophage Marker), and Inducible Nitric Oxide Synthase (iNOS; M1 Macrophage Marker) in Adipose Tissue of Cecal Ligation and Puncture (CLP) Mice

Pioglitazone (PGZ) was intraperitoneally injected for 7 d, and CLP were performed. Visceral adipose tissues were collected 24 h after CLP and subjected to extraction of total RNA for quantitative real-time RT-PCR analysis. Each longitudinal axis indicates the relative expression levels in each group. Values are the means ± S.E.M. of eleven mice. *p < 0.05 vs. sham.

**Fig. 2.** Effects of Pioglitazone on the Expression of CD163 and F4/80 (Pan-macrophage Markers), and the Correlation among Macrophage Markers in Adipose Tissue of Cecal Ligation and Puncture (CLP) Mice

(A) Total RNA extracted from adipose tissue in each group was subjected to quantitative RT-PCR analysis, as shown in Fig. 1. Each longitudinal axis indicates the relative mRNA expression levels in each group. Values are the means ± S.E.M. of eleven mice. *p < 0.05 vs. sham, †p < 0.05 vs. CLP PGZ: pioglitazone. (B) Correlation among macrophage markers of adipose tissues was compared in sham, CLP, and pioglitazone-treated CLP animals. Relative mRNA expression levels in individual mice are plotted. Results are shown for the sham group (diamonds), CLP group (squares), and pioglitazone-treated CLP group (triangles). The Pearson correlation coefficient value (r) was depicted in upper-left corners. *p < 0.05: significant differences. PGZ/CLP: pioglitazone-treated CLP group.
marker F4/80 mRNA level in the pioglitazone-treated CLP group was also approximately 3-fold higher than the respective values in the sham and CLP groups. Correlations between the mRNA levels of adipose macrophage markers were examined (Fig. 2B). Significant positive correlations were found between CD163 and F4/80, iNOS and IL-10, Arg1 and IL-10, and Arg1 and iNOS. The correlation between iNOS and IL-10 ($r=0.85$) was the strongest, followed by the correlation between Arg1 and IL-10 ($r=0.78$).

Effects of CLP and Pioglitazone on the Distribution of Adipose Tissue Macrophages and Apoptosis in Adipose Tissues To determine whether CLP affects the structure of adipose tissues and whether pioglitazone could modulate the histological changes, hematoxylin-eosin staining was performed 24 h after CLP (Fig. 3A). No distinct morphological changes were observed in the CLP mice, but increased numbers of infiltrated cells were seen in the pioglitazone-treated CLP group. Immunocytochemical staining of CD11b/c-positive

![Fig. 3. Effects of Pioglitazone on Macrophage Distribution and Apoptosis in Cecal Ligation and Puncture (CLP) Mice](image)
macrophage and CD163-positive macrophages revealed that the cortical area of adipose tissues were characterized by increased monocytes and macrophage influx including M1 and M2 macrophages in the CLP group (Fig. 3B). Infiltration of CD163-positive macrophages into the tissue was also observed in the pioglitazone-treated CLP group. Thus, CLP-induced sepsis markedly increases the number of macrophages, and pretreatment with pioglitazone tended to promote macrophage movement into adipose tissues. The TUNEL assay was applied to the sections to determine the degree of apoptotic cells in adipose tissues (Fig. 3D). There were no apoptotic cells in the sham group, but TUNEL-positive cells were markedly detected in adipose tissues of the CLP group and the pioglitazone-treated CLP group. On double staining with CD163, the apoptotic cells were not consistent with the cells stained with anti-CD163 antibody, suggesting that M2 macrophages could be alive and functional (Fig. 3E).

**Effects of CLP and Pioglitazone on the Expression of IL-6 and MCP-1 in Mouse Adipose Tissues** The expression levels of inflammatory cytokines, IL-6 and MCP-1 in adipose tissues were investigated (Fig. 4). As shown in the previous study,10 CLP treatment up-regulated IL-6 and MCP-1 expression, whereas pioglitazone pretreatment prevented the elevation of these cytokines. Taken together with the results in Figs. 2 and 3, pioglitazone treatment before CLP increases both M1 and M2 macrophages, and might affect the function of these macrophages to result in the reduction of IL-6 and MCP-1 expression in adipose tissues.

**DISCUSSION**

Our previous studies demonstrated that pioglitazone increases circulating adiponectin levels and decreases mortality and morbidity (lung injury) associated with CLP-induced sepsis.10,18 Low preoperative circulating levels of adiponectin might be a risk factor for postoperative infection in patients who undergo colorectal cancer surgery.19 The present study addressed the effects of pioglitazone in adipose tissue macrophage dynamics, and inflammatory and apoptotic responses during sepsis. Our data suggested that CLP-induced sepsis may enhance the appearance of M1/M2 macrophages in visceral adipose tissue and that pioglitazone treatment possibly increases numbers of total macrophages. These results suggest that pioglitazone may affect adipose macrophage activation by enhancing mobilization of macrophages into adipose tissue. Although we still need to clarify the mechanisms why the increase in the number of macrophage in pioglitazone-treated animals decreased the cytokine production, we can speculate that pioglitazone increases M2 macrophages within adipose tissue and local shift in macrophage population to M2 macrophages may be responsible for the beneficial effect of pioglitazone on mortality and morbidity in mice after CLP-induced sepsis. IL-10 derived from M2 macrophages might mediate partially pioglitazone-induced suppression of inflammatory cytokines.

Adipokines play roles in insulin resistance and inflammation of various organs including adipose tissue. PPARγ is related to adipocyte differentiation, and PPARγ agonists are commonly used as insulin sensitizers to treat type II diabetic patients with insulin resistance.20 Several studies have suggested that PPARγ agonists which up-regulate adiponectin production via activation of nuclear PPARγ21,22 may have protective effects on the cardiovascular system by ameliorating endothelial dysfunction and vascular inflammation.23-25 Furthermore, specific PPARγ expression may be required for sustaining obese macrophage subtypes in adipose tissues.60 Obesity induces adipose chronic inflammation,24 and sepsis causes acute inflammation in various organs and affects adipokine production with regard to insulin resistance.60 Interestingly, a previous study has suggested that recruitment of macrophages into adipose tissue and the interaction of adipocytes and macrophages might accelerate inflammatory reactions in obese adipose tissue.14 Our in vitro data using cultured human macrophage and adipocytes (unpublished data) also indicate that co-culture of macrophage and adipocytes enhances lipopolysaccharide (LPS)-stimulated IL-6 expression, suggesting that the interaction of both cells may promote cytokine expression. Pioglitazone may inhibit the CLP-induced interaction of macrophages and adipocytes, because pioglitazone significantly decreased the levels of the cytokine expression in the co-culture system. Furthermore, pioglitazone reduced IL-6 and TNF-α in cultured adipocytes which are elevated by peritoneal fluid collected from CLP animals. These results suggest that major mechanism in which pioglitazone reduces the expression of inflammatory cytokines may be due to direct inhibition of macrophages and adipocytes. Thus, the increase in macrophage infiltration seems to be essential for exerting distinguishing effects of this compound.

Multiple organ dysfunction occurs with systemic inflammation during the septic process. No apparent changes were evident in the structure of adipose tissue except for the appearance of infiltrated cells in this study, although lung injury caused by CLP was attenuated by pioglitazone pretreatment.18 Macrophages exhibit distinct functional phenotypes by undergoing different types of phenotypic polarization in response to various environmental stimuli or under different pathophysiological conditions.14,15,23-27 M1 phenotype is induced by microbial products or pro-inflammatory cytokines such as TNF or Toll-like receptor ligands. The typical characteristics of M1 macrophages include high production of nitric oxide (NO) and reactive oxygen species (ROS). Inflammatory M1 macro-
phages produce many pro-inflammatory cytokines which promote inflammation leading to insulin resistance, and promote T helper 1 (Th1) response and possess strong microbicidal and tumoricidal activities, while M2 macrophages are involved in the promotion of T helper 2 (Th2) response, tissue remodeling, immune tolerance, and anti-inflammatory response. Watanabe et al. has more recently reported that sepsis induces an incomplete polarization to the M2 macrophages in peritoneal exudate cells in CLP mice. In the present study, CLP markedly enhanced the levels of Arg1 and IL-10 mRNA expression serving as M2 markers in septic mice. Further, changes in iNOS expression serving as an M1 marker exhibits similar to those of Arg1 and IL-10. Cooperative expression among M1 and M2 markers was detected in individual animals. Severe impact of CLP operation on macrophage dynamics might complicate to detect the pharmacologic effects of pioglitazone on macrophages. TUNEL positive cells as well as macrophages stained with CD163 and CD11b/c were obviously increased in both CLP and pioglitazone-treated CLP groups.

In addition, the cells stained with CD163 were not consistent with those of Arg1 and IL-10. Cooperative expression among M1 and M2 markers was detected in individual animals. Severe impact of CLP operation on macrophage dynamics might complicate to detect the pharmacologic effects of pioglitazone on macrophages. TUNEL positive cells as well as macrophages stained with CD163 and CD11b/c were obviously increased in both CLP and pioglitazone-treated CLP groups. In addition, the cells stained with CD163 were not consistent with the TUNEL positive cells, suggesting that macrophages are functional in adipose tissues. Macrophages promote tissue repair after injury by producing a variety of growth factors and cytokines. However, they produce highly active mediators such as ROS that induce apoptosis and disrupt cell metabolism during the inflammatory process. Taken together, our findings suggest that macrophages might contribute to inhibiting sepsis-triggered excessive inflammation of adipose tissue by reducing necrotic cell death numbers. These results suggest that macrophages infiltrated into visceral adipose tissues during CLP-induced sepsis may be associated with enhanced apoptosis and improved survival after CLP. In the present study, we did not identify the apoptotic cells induced by CLP, and further investigation would be needed to determine whether macrophages directly contributes to the apoptotic response, and the mechanisms by which pioglitazone affects macrophage dynamics. In addition, immunomodulatory effects of sex hormones are critical factors after the development of polymicrobial sepsis and trauma-hemorrhage. Our previous studies have shown that many factors such as sex hormone, difference related to gender, and genetic background influence inflammatory responses associated with tissue injuries. The effects of sex hormones or differences related to gender in M1/M2 macrophages in adipose tissue on immune functions should also be examined in future studies.

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Conflict of Interest The authors declare no conflicts of interest.

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