Adipocyte-Mononuclear Cell Interaction, Toll-like Receptor 4 Activation, and High Glucose Synergistically Up-regulate Osteopontin Expression via an Interleukin 6-mediated Mechanism*

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Although it has been reported that osteopontin, a matrix glycoprotein and proinflammatory cytokine, mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance, it remains unclear how osteopontin is up-regulated in adipose tissue in obese humans and animals. In this study, we incubated U937 mononuclear cells with adipocytes in a transwell system and studied how cell interaction regulated osteopontin expression. Results showed that coculture of U937 cells with adipocytes led to a marked increase in osteopontin production when compared with that released by independent cultures of U937 cells. Moreover, lipopolysaccharide or palmitic acid-induced TLR4 activation and high glucose further augmented the coculture-stimulated osteopontin secretion. Similar observations were made in the coculture of human primary monocytes and adipocytes. Real time PCR studies showed that coculture of U937 cells and adipocytes increased osteopontin mRNA in U937 cells, but not adipocytes, suggesting that adipocyte-derived soluble factor may stimulate osteopontin expression by U937 cells. In our studies to explore the underlying mechanism, we found that the neutralizing antibodies against interleukin (IL)-6 or IL-6 small interfering RNA transfection in adipocytes effectively inhibited coculture-stimulated osteopontin expression, suggesting that IL-6 released by adipocytes plays an essential role in the coculture-stimulated osteopontin expression by U937 cells. In conclusion, this study has demonstrated that cell interaction, TLR4 activation, and high glucose up-regulate osteopontin expression, and adipocyte-derived IL-6 played a major role in the up-regulation.

A recent study by Kirk et al. (1) showed that MCP-1 (monocyte chemotactic protein-1) deficiency in mice with diet-induced obesity was not associated with reduced macrophage recruitment into adipose tissue and improved insulin sensitivity, suggesting that chemokines other than MCP-1 may play a role in macrophage recruitment into adipose tissue and insulin resistance. Interestingly, Nomiyama et al. (2) reported earlier that osteopontin deficiency in mice with diet-induced obesity was associated with a 50% reduction of macrophage infiltration in adipose tissue and improved insulin sensitivity. It is noteworthy that the genetic backgrounds of the mice (C57BL/6) and the high fat diets (D12492 from Research Diets, Inc.) used in both studies were same. These findings suggest that osteopontin may play a key role in macrophage recruitment in adipose tissue and insulin resistance. In supporting this notion, a very recent clinical study (3) conducted in 52 morbidly obese patients and in mice has provided evidence that elevated expression of osteopontin is related to adipose tissue macrophage accumulation.

Osteopontin is a multifunctional protein secreted by different types of cells, including macrophages, lymphocytes, epithelial cells, vascular smooth muscle cells, and osteoblasts (4). Osteopontin stimulates adhesion molecule expression (5) and osteopontin is associated with the extent of cardiovascular disease independently of traditional risk factors (11). Additionally, osteopontin also plays an important role in bone mineralization, osteoclast differentiation, and bone resorption (13, 14).

Obesity is characterized by increased macrophage infiltration and cytokine production and is associated with insulin resistance and type 2 diabetes (15, 16). Given the crucial role of osteopontin in macrophage infiltration into adipose tissue and insulin resistance, it is important to understand the regulation of osteopontin expression by adipocytes. Although it is known that osteopontin expression in adipose tissue is up-regulated in patients or animal models with obesity (12, 17), the underlying mechanisms have not been well established. In this study, we demonstrated that multiple factors, including interaction

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‡ The abbreviations used are: IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; TNFα, tumor necrosis factor α.
between adipocytes and mononuclear cells, TLR4 activation induced by lipopolysaccharide (LPS) or palmitic acid, and elevated glucose level (high glucose), act in concert to up-regulate osteopontin expression by mononuclear cells through an IL-6-mediated mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture—Human preadipocytes isolated from human adipose tissue in pericardiac fat, preadipocyte growth medium, and adipocyte differentiation medium were purchased from Cell Applications, Inc. (San Diego). For adipocyte differentiation, 2-day postconfluent preadipocytes were treated with adipocyte differentiation medium for 10 days. The medium was changed every 2 days. After differentiation, the conversion of preadipocytes to adipocytes was confirmed by Oil Red O staining. The cells were then incubated in RPMI 1640 medium (Invitrogen) containing normal glucose (5 mM) or high glucose (25 mM), 10% fetal calf serum, 1% minimum Eagle’s medium/nonessential amino acid solution, and 0.6 g/100 ml HEPES for 2 days before being challenged with 100 ng/ml LPS or palmitate. Human monocytes were isolated, as described previously (20), from blood obtained from healthy donors, and monocytes were treated in the medium that was the same as that used for U937 cells. For coculture, adipocytes and U937 cells or human monocytes were grown in 12-well Corning Transwell plates (Fisher), a noncontact coculture system, with two compartments separated by a polycarbonate membrane with 0.4-μm pores. Adipocytes were grown to confluence (about 2×10⁵ cells/well) at the lower compartment, and U937 cells or human monocytes were cultured (1×10⁶/well) in the upper compartment. IL-6, IL-1α, IL-1β, and TNFα were purchased from Sigma. Goat anti-IL-6, IL-1α, IL-1β, and TNFα antibodies were purchased from R&D Systems (Minneapolis, MN). Goat IgG was used as control antibody. Anti-TLR4 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse IgG was used as the control antibody for anti-TLR4 antibody.

Enzyme-linked Immunosorbent Assay (ELISA)—Osteopontin in conditioned medium was quantified using sandwich ELISA kits according to the protocol provided by the manufacturer (R&D Systems). IL-6 in conditioned medium was quantified using ELISA kits from BioLegend (San Diego).

Real Time PCR—Total RNA was isolated from cells using the RNeasy minikit (Qiagen, Santa Clarita, CA). First strand cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad) using 20 μl of reaction mixture containing 0.25 μg of total RNA, 4 μl of 5× iScript reaction mixture, and 1 μl of iScript reverse transcriptase. The complete reaction was cycled for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C using a PTC-
Adipocyte-Mononuclear Cell Interaction Increases Osteopontin

200 DNA Engine (MJ Research, Waltham, MA). The reverse transcription reaction mixture was then diluted 1:10 with nuclease-free water and used for PCR amplification of cDNA in the presence of the primers. The Beacon designer software (PREMIER Biosoft International, Palo Alto, CA) was used for primer designing (osteoponitin, 5′ primer sequence, TCTGAT-GAATCTGATGAAGTG; 3′ primer sequence, GATGTG-CTCGTCTGTAGC). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and real time PCR was performed in duplicate using 25 μl of reaction mixture containing 1.0 μl of reverse transcription mixture, 0.2 μM of both primers, and 12.5 μl of iQ™ SYBR Green Supermix (Bio-Rad). Real time PCR was run in the iCycler™ real time detection system (Bio-Rad) with a two-step method. The hot-start enzyme was

FIGURE 2. Augmentation of osteopontin secretion by LPS and high glucose. A, independent cultures of U937 cells or adipocytes were pre-exposed to normal (5 mM) or high (25 mM) glucose for 2 days and then treated with or without 100 ng/ml LPS for 24 h. Part of U937 cells and adipocytes pre-exposed to normal (5 mM) or high (25 mM) glucose for 2 days were cocultured and treated with or without 100 ng/ml LPS for 24 h. After the treatments, the culture medium was collected for ELISA to quantify osteopontin. Adipo, adipocytes; Coculture, coculture of U937 cells with adipocytes. The data (mean ± S.D.) presented are from one of three independent experiments with similar results. B, similar study as described above was conducted using human primary monocytes. Monocytes were pre-exposed to normal or high glucose for 18 h and then treated with or without LPS for 24 h. Because adipocytes did not release osteopontin as shown in A, no independent culture of adipocytes was included in this experiment. C, time courses of osteopontin secretion and mRNA expression by coculture of adipocytes and U937 cells in response to LPS. High glucose-exposed coculture of U937 cells and adipocytes was treated with 100 ng/ml LPS for 0, 4, 8, 12, 18, 24, or 28 h. After treatment, osteopontin in culture medium and osteopontin mRNA in cells were quantified using ELISA and real time PCR, respectively.
activated (95 °C for 3 min), and cDNA was then amplified for 40 cycles consisting of denaturation at 95 °C for 10 s and annealing/extension at 53 °C for 45 s. A melt-curve assay was then performed (55 °C for 1 min, and the temperature was then increased by 0.5 °C every 10 s) to detect the formation of primer-derived trimers and dimers. Glyceraldehyde-3-phosphate dehydrogenase was used as a control (5’ primer sequence, GAATTTGGCTACAGCAACAGGTG; 3’ primer sequence, TCTCTTCTCTTGTGCTTTGCTG). Data were analyzed with the iCycler iQ™ software (Bio-Rad). The average starting quantity of fluorescence units was used for analysis. Quantification was calculated using the starting quantity of targeted cDNA relative to that of glyceraldehyde-3-phosphate dehydrogenase cDNA in the same sample.

Transfection of Adipocytes with siRNA—Human adipocytes were transfected with 20 nM of stealth siRNA directed against IL-6 (CAGACAGCCAUCCUCUCUUCAGAA) (GenBank™ accession number NM 000600) or control siRNA (CAGACACCAUCCUCUACAGAA) using Lipofectamine RNAi MAX (Invitrogen) as transfection reagent according to the manufacturer’s instruction. After 48 h of transfection, adipocytes were cocultured with U937 cells or human primary monocytes.

Extraction of Nuclear Proteins—Nuclear protein was extracted using NE-PER™ nuclear and cytoplasmic extraction kit (Pierce). The concentration of protein was determined using a protein assay kit (Bio-Rad).

MAPK Phosphorylation—U937 cells were incubated with 50% fresh medium and 50% adipocyte-conditioned medium for 0, 5, 15, 30, and 60 min. Cells were then lysed with lysis buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM Na3VO4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. The cell lysates were cleared by centrifugation, and 50 μg of each sample was electrophoresed in a 10% polyacrylamide gel. After transfer of proteins to a polyvinylidene difluoride membrane, immunoblotting was performed using anti-phosphorylated and total ERK1/2, JNK, and p38 MAPK antibodies (Cell Signaling Technology, Danvers, MA).

Electrophoretic Mobility Shift Assay—Ten μg of nuclear proteins was used for electrophoretic mobility shift assay to determine AP-1 DNA binding activity. DNA–protein binding reactions were performed at room temperature for 20 min in a buffer containing 10 mM Tris-HCl base, pH 7.9, 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 μg of poly(dI-dC), 5% (v/v) glycerol, and ~0.3 pmol of AP-1 oligonucleotide (Promega, Madison, WI) labeled with DIG-ddUTP using terminal deoxynucleotidyltransferase (Roche Applied Science). Protein-DNA complexes were resolved from protein-free DNA in 5% polyacrylamide gels in 50 mM Tris, pH 8.3, 0.38 mM glycine, 2 mM EDTA at room temperature and electroblotted onto positively charged nylon membranes. The chemiluminescence detection of DIG-labeled probes was conducted by following the instructions provided by Roche Applied Science. For competition studies, unlabeled AP-1 oligonucleotides that were 50-fold of the labeled AP-1 oligonucleotides were added to the reaction mixture.

FIGURE 3. Inhibition of LPS-stimulated osteopontin secretion from the coculture of U937 cells and adipocytes by anti-TLR4 antibodies. U937 cells were cocultured with adipocytes in normal glucose-containing medium and treated with 100 ng/ml LPS in the presence or absence of increasing concentrations of neutralizing anti-TLR4 antibodies (Ab) or control antibodies for 24 h. After the treatment, osteopontin in culture medium was quantified using ELISA. The data (mean ± S.D.) presented are from one of two independent experiments with similar results.

PCR Array—The first strand cDNA was synthesized from RNA using the RT² first strand kit (SuperArray Bioscience Corp., Frederick, MD). Human inflammatory cytokines and receptor PCR array (catalog no. PAHS-011, SuperArray Bioscience Corp.) was used to profile the adhesion molecule and cytokine expression by following the instructions from the manufacturer.

Statistical Analysis—Data were presented as mean ± S.D. Student’s t tests were performed to determine the statistical significance of cytokine expression among different experimental groups. A value of p < 0.05 was considered significant.

RESULTS

Coculture of Primary Monocytes or U937 Mononuclear Cells and Adipocytes Augments Osteopontin Secretion—Because obesity is associated with increased monocyte infiltration into adipose tissue and increased interaction between monocytes and adipocytes, we first studied the effects of coculture of primary monocytes or U937 mononuclear cells and adipocytes on osteopontin secretion. Results showed that although osteopontin secreted by monocytes or U937 cells was much more than that secreted by adipocytes, osteopontin released from the coculture of monocytes or U937 cells and adipocytes in the transwell coculture system was markedly increased as compared with total osteopontin released by independent cultures of monocytes or U937 cells and adipocytes (Fig. 1, A and B).

TLR4 Activation and High Glucose Further Increase Coculture-augmented Osteopontin Secretion—Recent studies have shown that the activation of TLR4, a receptor for LPS and some fatty acids such as palmitic acid, is associated with adipose tis-
Sue inflammatory and insulin resistance (21). As it has been shown that osteopontin is also associated with adipose tissue inflammation and insulin resistance (2), TLR4 activation may affect insulin resistance in adipocytes by increasing osteopontin expression. Thus, we examined the effect of TLR4 activation on osteopontin expression by independent culture of U937 cells, adipocytes, or coculture of U937 cells and adipocytes. Results showed that TLR4 activation induced by LPS did not increase osteopontin secretion by adipocytes, but it markedly stimulated osteopontin secretion by independent culture of U937 cells or the coculture of U937 cells and adipocytes (Fig. 2A). Furthermore, considering that hyperglycemia is present in patients with type 2 diabetes, the effect of elevated glucose levels (high glucose) on osteopontin release by independent culture of U937 cells, adipocytes, or the coculture of U937 cells and adipocytes was also examined. Results showed that high glucose increased osteopontin secretion from independent culture of U937 cells and the coculture of U937 cells and adipocytes but not independent culture of adipocytes (Fig. 2A). Strikingly, high glucose in combination with LPS augmented osteopontin secretion by coculture of U937 cells and adipocytes by 10-fold as compared with that released by coculture of U937 cells and adipocytes exposed to normal glucose and LPS (11.02 versus 1.13 ng/ml) (Fig. 2A). These results clearly showed a synergism on osteopontin secretion by coculture of U937 cells and adipocytes, TLR4 activation, and high glucose.

The effect of TLR4 activation and high glucose on osteopontin secretion was also examined in the coculture of human primary monocytes and adipocytes. Results (Fig. 2B) showed that the combination of coculture and LPS increased more osteopontin secretion as compared with the coculture alone. High glucose in combination with LPS increased osteopontin secretion by coculture of monocytes and adipocytes by 4-fold as compared with that released by coculture of monocytes and adipocytes exposed to normal glucose and LPS.
compared with normal glucose in combination with LPS. Thus, our new results show that human primary monocytes and U937 cells have similar response to the coculture, high glucose, and TLR4 activation for osteopontin secretion. The time courses of both osteopontin protein secretion and mRNA expression by coculture of U937 cells with adipocytes in response to LPS were studied, and the results are presented in Fig. 2C. The results showed that osteopontin protein secretion and mRNA expression are nearly parallel. The plateaus of osteopontin protein secretion and mRNA expression were observed at 18 and 24 h, respectively. Thus, we selected 24 h as the time point in the experiments to determine both osteopontin secretion and mRNA expression.

To confirm that LPS stimulates osteopontin secretion by engaging TLR4, the coculture of U937 cells and adipocytes was treated with LPS in the presence of anti-TLR4 antibodies or control antibodies. Results showed that anti-TLR4 antibodies inhibited LPS-stimulated osteopontin secretion in a concentration-dependent manner, although the control antibodies had no blocking effect (Fig. 3). Because it is known that palmitic acid, which is released by adipocytes (22), is also a ligand for TLR4 (23), we examined the effect of palmitic acid on osteopontin secretion by the coculture of U937 cells and adipocytes. Results showed that palmitic acid at 100 and 250 μM stimulated osteopontin secretion, and high glucose further increased osteopontin secretion (Fig. 4). Palmitic acid at 250 μM stimulated osteopontin secretion by 2- and 3-fold from U937 cells pre-exposed to normal and high glucose, respectively, and had no stimulation at 500 μM. Our study further confirmed that the stimulation of osteopontin secretion by palmitic acid was also TLR4-dependent (data not shown).

U937 Cells, but Not Adipocytes, Have Increased Osteopontin mRNA in the Coculture—To determine whether the increased osteopontin secretion by coculture of adipocytes and U937 cells is due to increased osteopontin mRNA expression, we performed real time PCR to quantify osteopontin mRNA. Results showed that U937 cells, but not adipocytes, in the coculture had a markedly increased level of osteopontin mRNA when compared with that in the independent culture of adipocytes without LPS treatment, which was designated as 1.0. The numbers in the parentheses are threshold cycles (Ct) obtained from PCR array analysis.

To provide more evidence that the soluble factor(s) released from adipocytes was involved in the augmentation of osteopontin expression by U937 cells, we collected the conditioned medium from adipocytes after 18 h of incubation and added it to the culture medium of U937 cells. Results showed that exposure of U937 cells with medium that contained 25 or 50% of the adipocyte-conditioned medium led to a significant increase in osteopontin secretion (Fig. 6). When the adipocyte-conditioned medium accounted for 75% of total medium for U937 cells, the augmentation on osteopontin secretion declined, probably due to insufficient fresh medium (25%) that may limit protein biosynthesis.
IL-6 Released by Adipocytes Is Essential for Coculture-increased Osteopontin Secretion—In our attempt to search the soluble factor(s) released by adipocytes and responsible for the stimulation of osteopontin expression in U937 cells, the inflammatory cytokines were considered first because it has been shown that IL-1α stimulates osteopontin expression in pulmonary fibroblasts (24). Also, our gene expression profiling showed that coculture of U937 cells in the presence or absence of LPS and high glucose for 24 h, IL-6 in culture medium was then quantified using ELISA. Results showed that antibody against IL-6, but not those against IL-1α, IL-1β, or TNFα, significantly inhibited the basal and LPS-stimulated osteopontin secretion (Fig. 7). As control, goat IgG had no effect on the basal and LPS-stimulated osteopontin secretion (data not shown). To provide further evidence that IL-6 plays an essential role in coculture-increased osteopontin expression, we took the RNA interference approach to inhibit IL-6 expression in adipocytes. Results showed that transfection of adipocytes with IL-6 siRNA resulted in 60 or 80% decreases in IL-6 release from coculture of adipocytes and U937 cells treated with or without LPS stimulation, respectively, as compared with those released by the cocultures without IL-6 siRNA transfection (Fig. 8A). With the effective inhibition of IL-6 expression, the transfection of adipocytes

FIGURE 8. Inhibition of osteopontin expression in U937 cells or human monocytes by transfection of adipocytes with IL-6 siRNA. A, adipocytes were first transfected with control siRNA (ctr siRNA) or IL-6 siRNA or had no transfection (Non-transfection). Forty eight hours after transfection, adipocytes were cocultured with U937 cells in the presence or absence of 100 ng/ml LPS and high glucose for 24 h. IL-6 in culture medium was then quantified using ELISA. B, osteopontin in the above culture medium was quantified using ELISA. For control, U937 cells were also cultured independently in the presence or absence of LPS for 24 h, and osteopontin in the culture medium was quantified. Non-trans, no transfection. C, similar study as described above was conducted with the coculture of adipocytes and human primary monocytes. IL-6 in culture medium was then quantified using ELISA. D, osteopontin in the above culture medium was quantified using ELISA. The data (mean ± S.D.) presented are from one of two independent experiments with similar results.
with IL-6 siRNA led to 50 or 60% reduction of osteopontin secretion from coculture of adipocytes and U937 cells treated with or without LPS stimulation, respectively (Fig. 8B). Similar experiments were also conducted in the coculture of human primary monocytes and adipocytes. Results showed that transfection of IL-6 siRNA inhibited IL-6 secretion from cocultures with or without LPS by 60 or 84%, respectively, suggesting a significant inhibition of IL-6 expression by IL-6 siRNA. Furthermore, the transfection of IL-6 siRNA resulted in the reduction of osteopontin release by cocultures with or without LPS by 54 or 50%, respectively (Fig. 8, C and D). Taken together, these data indicate that IL-6 released by adipocytes plays an essential role in the coculture- and LPS-stimulated osteopontin secretion by mononuclear cells.

**IL-6 Release Is Also Increased by Coculture and LPS**—To provide further evidence that IL-6 is involved in the coculture-induced osteopontin expression by U937 cells, we investigated the effect of coculture, LPS, and high glucose on IL-6 secretion.

**FIGURE 9. Effect of coculture, LPS, and high glucose on IL-6 secretion.** U937 cells and adipocytes pre-exposed to normal (5 mM) or high (25 mM) glucose were cultured independently or together and treated with or without 100 ng/ml LPS for 24 h. After the treatment, IL-6 in culture medium was quantified using ELISA. The data (mean ± S.D.) presented are from one of three independent experiments with similar results.

**FIGURE 10. Stimulatory effect of IL-6 on osteopontin secretion by U937 cells.** A, U937 cells exposed to normal glucose were treated with 0, 1, 5, or 10 ng/ml IL-6, IL-1α, IL-1β, or TNF-α for 24 h. After the treatment, osteopontin in culture medium was quantified using ELISA. B, U937 cells exposed to normal or high glucose were treated with 10 ng/ml IL-6, 100 ng/ml LPS, or both for 24 h. After the treatment, osteopontin in culture medium was quantified using ELISA. The data (mean ± S.D.) presented are from one of two independent experiments with similar results. Ctr, control.
Results showed that in the absence of LPS, adipocytes released much more IL-6 than U937 cells, and coculture of adipocytes and U937 cells further increased IL-6 secretion (Fig. 9A). High glucose increased IL-6 secretion by 40% in adipocytes but had no effect on U937 cells. For coculture of U937 cells and adipocytes, high glucose only increased IL-6 secretion by 20%. LPS stimulated IL-6 secretion by independent cultures of adipocytes or U937 cells and by the coculture of adipocytes and U937 cells (Fig. 9B). In the presence of LPS, the effect of high glucose on IL-6 was insignificant, and coculture of U937 cells and adipocytes increased IL-6 secretion by 40% as compared with that released by independent culture of adipocytes (Fig. 9B).

The effect of recombinant IL-6 on osteopontin secretion by U937 cells was examined. Results showed that IL-6 at 10 ng/ml significantly increased osteopontin secretion, although IL-1α, IL-1β, and TNFα had no stimulation (Fig. 10A). Additionally, our results (Fig. 10B) showed that under the normal glucose condition, the effects of the combination of IL-6 and LPS on osteopontin secretion were additive. However, when cells were exposed to high glucose, the effect of the combination of IL-6 and LPS on osteopontin secretion was synergistic.

MAPK (ERK, JNK, and p38) Pathways Are Involved in Coculture-increased Osteopontin Secretion—It is known that JAK/STAT3 and MAPK pathways, including the ERK and JNK pathways, mediate IL-6-regulated gene expression (25). Because the above studies indicate that IL-6 plays a major role in the coculture-increased osteopontin expression, we determined which pathway, JAK/STAT3 or MAPK, is involved in coculture-stimulated osteopontin expression using specific inhibitors of these pathways. Results showed that SB203580, PD98059, and SP600125, which inhibit the p38, ERK, and JNK pathways, respectively, not only completely abolished adipocyte-condi-
tioned medium-stimulated osteopontin secretion but also inhibited the basal level of osteopontin secretion (Fig. 11A). In contrast, AG490, an inhibitor for both JAK/STAT1 and JAK/STAT3 pathways (26), and piceatannol, a specific JAK/STAT3 pathway inhibitor (27), had no significant effect on the baseline level and adipocyte-conditioned medium-stimulated osteopontin secretion, suggesting that MAPK pathways, including p38, ERK, and JNK cascades, but not the JAK/STAT3 pathway, mediate the stimulation of osteopontin expression by the coculture. To ensure that the exposure of U937 cells to adipocyte-conditioned medium activates MAPKs, we performed immunoblotting to demonstrate that the adipocyte-conditioned medium increased ERK1/2, JNK, and p38 MAPK phosphorylation (Fig. 11B). Furthermore, because it is known that AP-1 is a key transcription factor involved in osteopontin expression (28) and the activation of MAPK pathways leads to an increase in AP-1 activity (29), the effect of coculture and TLR4 activation on AP-1 activity was determined using electrophoretic mobility shift assay. Results showed that although coculture of adipocytes with U937 cells and LPS moderately stimulated AP-1 activity, the combination of the coculture and LPS markedly increased AP-1 activity (Fig. 12). Similarly, the combination of the coculture and palmitic acid also markedly increased AP-1 cell surface receptors of monocytes/macrophages and adipocytes may trigger signaling activation and subsequent gene expression. In the noncontact interaction, the cross-talking between monocytes/macrophages and adipocytes is mediated by soluble factors released by either monocytes/macrophages or adipocytes. This study, we demonstrated that IL-6 released by adipocytes played an essential role in the stimulation of osteopontin expression by U937 cells in the coculture system. Furthermore, IL-6, LPS or palmitic acid, and high glucose synergistically stimulate osteopontin expression by U937 cells.

**DISCUSSION**

It has been well documented that obesity is associated with increased monocyte/macrophage infiltration into adipose tissue (15, 16). Therefore, it is likely that, with increased content of monocytes/macrophages in the adipose tissue, more interaction between monocyte/macrophage and adipocyte would take place. In this study, we demonstrated that noncontact interaction between U937 mononuclear cells or human primary monocytes and adipocytes markedly increased osteopontin expression. Given the crucial role of osteopontin in the adipose tissue inflammation and insulin resistance (2, 3), this finding has delineated a novel molecular mechanism potentially involved in inflammation and insulin resistance in obesity.

Monocytes/macrophages infiltrated into adipose tissue interact with adipocytes by direct contact and noncontact. In the contact interaction, the binding between

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**FIGURE 13. Illustration for the mechanism involved in the stimulation of osteopontin expression by U937 cell-adipocyte interaction, TLR4 activation by LPS, and high glucose.**

A, in the absence of LPS and high glucose, adipocyte-derived IL-6 in combination with IL-6 released by U937 mononuclear cells stimulates osteopontin expression in U937 cells and thus increases osteopontin secretion in coculture of U937 cells and adipocytes. B, in the presence of LPS or palmitic acid and high glucose, LPS or palmitic acid stimulates IL-6 secretion by adipocytes and U937 cells through TLR4 activation, leading to a marked increase in IL-6 production that contributes to osteopontin expression in coculture system. Furthermore, IL-6, LPS or palmitic acid, and high glucose synergistically stimulate osteopontin expression by U937 cells.
Adipocyte-Mononuclear Cell Interaction Increases Osteopontin

Adipocyte-secreted products (IL-6) up-regulated expression of osteopontin, also a proinflammatory gene, by mononuclear cells. Therefore, it appears that a two-way talk between monocytes/macrophages and adipocytes is present and may affect proinflammatory gene expression by both types of cells, which contributes to adipose tissue inflammation and insulin resistance.

In addition to the interaction between monocytes/macrophages and adipocytes that markedly augments osteopontin expression, our present study showed that TLR4 activation is another powerful factor up-regulating osteopontin expression. Using TLR4 knock-out mice, several studies have demonstrated consistently that TLR4 plays a pivotal role in the inflammation and insulin resistance in adipose tissue (21, 23, 32). Considering the essential role of osteopontin in inflammation and insulin resistance in adipose tissue, our finding in this study that TLR4 activation led to increased osteopontin expression revealed a novel mechanism by which TLR4 promotes inflammation and insulin resistance in adipose tissue.

Our study indicates that TLR4 activation stimulates osteopontin expression by U937 cells in the coculture of U937 cells and adipocytes through two mechanisms. First, we showed that treatment of U937 cells with LPS or palmitic acid increased osteopontin secretion, indicating that TLR4 activation in U937 cells directly stimulates osteopontin expression. Second, we showed that LPS stimulated IL-6 secretion by adipocytes, and IL-6 is capable of stimulating osteopontin expression by U937 cells, suggesting that Toll-like receptor activation in adipocytes may also increase osteopontin expression by U937 cells via adipocyte-derived IL-6. Moreover, we showed that LPS and IL-6 had synergistic effect on osteopontin secretion, suggesting that TLR4 signaling and IL-6 signaling act in concert in the activation of osteopontin transcription.

Type 2 diabetes is associated with obesity and a chronic low-grade inflammation (33). Because hyperglycemia is a major metabolic disorder in type 2 diabetic patients, we examined the effect of high glucose on osteopontin expression by coculture of adipocytes and U937 cells. Results suggest that high glucose increased osteopontin expression by U937 cells through two potential mechanisms. First, high glucose directly increased osteopontin expression, although the augmentation was relatively small (about 2-fold). Second, high glucose augmented LPS-stimulated osteopontin expression by U937 cells, which was more than 10-fold. These findings indicate that when obesity is accompanied with hyperglycemia, the extent of monocyte/macrophage infiltration into adipose tissue may be further increased as a result of up-regulation of osteopontin expression, which may exacerbate adipose tissue inflammation and insulin resistance.

Fig. 9B showed that high glucose could not further enhance IL-6 production in adipocytes, U937 cells, and coculture of adipocytes and U937 cells. However, as shown in Fig. 2A, high glucose and LPS induced osteopontin release by coculture of adipocytes and U937 cells as much as 10-fold of that in the coculture treated with normal glucose and LPS. These observations suggest that factors other than IL-6 released in response to high glucose may contribute to further increased osteopontin production by coculture of adipocytes and U937 cells treated with LPS and high glucose. Indeed, our study showed that neutralizing anti-IL-6 antibody and IL-6 siRNA only blocked about 50% of osteopontin secretion by the coculture exposed to high glucose and LPS (Fig. 7 and Fig. 8). Clearly, although IL-6 plays an essential role in the augmentation of osteopontin production by coculture of adipocytes and U937 cells in response to LPS and high glucose, it is not the only player for the augmentation.

As illustrated in Fig. 13, our present study has demonstrated that the interaction between U937 cells and adipocytes, TLR4 activation, and high glucose synergistically stimulates osteopontin expression by U937 cells. To further explore the underlying mechanisms, we have shown that IL-6 released by adipocytes plays an essential role in the augmentation of osteopontin expression by U937 cell and adipocyte interaction.

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