High Glucose-treated Macrophages Augment E-Selectin Expression in Endothelial Cells

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E-selectin expression by endothelial cells (ECs) is crucial for leukocyte recruitment during the inflammatory response. Macrophage accumulation and serum E-selectin elevation are features of type 2 diabetes mellitus. However, the interactions between macrophages and ECs in regulating vascular endothelial function are not clearly understood. We investigated the mechanisms underlying the modulation of EC E-selectin expression by high glucose (HG)-treated macrophages. Macrophage-conditioned media (MCM) were prepared from HG-treated macrophages. EC stimulation with HG-MCM induces increases the expression and secretion of E-selectin. By using specific inhibitors and small interfering RNAs, we demonstrate an important role in atherogenesis in the HG condition and suggest a new mechanism by which arterial disease is accelerated in diabetes.

It is well established that diabetes mellitus (DM) is associated with atherosclerotic and inflammatory disease (1, 2).

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alter their gene expression and that the soluble mediators released from HG-treated macrophages may up-regulate EC E-selectin expression. To gain insights into the mechanisms by which factors released by macrophages after HG treatment may up-regulate EC E-selectin expression, macrophage-conditioned medium (MCM) from patients or from HG and NG treatments were subjected to cytokine protein array analysis to determine the proinflammatory factors produced by macrophages after differentiation from monocytes under these conditions. We found that the chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β produced by HG-treated macrophages exert paracrine effects on ECs to enhance the E-selectin expression and secretion. The E-selectin up-regulation induced by MIP-1α and MIP-1β released from HG-treated macrophages is mediated through the intracellular signaling cascades JNK and p38 MAPK, and the transcription factors NF-κB and activated protein 1 (AP-1). Therefore, our current findings provide a molecular basis for the mechanisms by which HG-treated macrophages enhance E-selectin expression and secretion in ECs.

EXPERIMENTAL PROCEDURES

Materials—All culture materials were purchased from Invitrogen. PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) were purchased from Calbiochem. Mouse mAB against JNK1 and phospho-JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against p38 and mouse monoclonal phospho-p38 antibody were purchased from Cell Signaling Technology (Beverly, MA). sE-selectin ELISA kits and mAB against E-selectin were obtained from R&D Systems (Minneapolis, MN). The ERK siRNA, JNK siRNA, p38 siRNA, NF-κB p65 siRNA, c-Jun siRNA, and control siRNA (scrambled negative control containing random DNA sequences) were purchased from Invitrogen. All other chemicals of reagent grade were obtained from Sigma.

Human Monocyte Isolation—Human monocytes from the buffy coat were isolated as described previously (9). Peripheral blood mononuclear cells were isolated by Histopaque 1077 density-gradient centrifugation. Monocytes were purified from peripheral blood mononuclear cells by negative selection using the magnetic-activated cell sorting monocyte isolation kit (Miltenyi Biotech, Auburn, CA).

Preparation of Human MCM—Monocytes were cultured in fresh RPMI 1640 medium containing 5.5 (NG) or 25 (HG) mmol/liter glucose or 5.5 mmol/liter glucose plus 19.5 mmol/liter mannitol (M) and supplemented with 10% FBS. After 4 days in culture, the macrophages were incubated for another 48 h in fresh serum-free RPMI medium. The conditioned media were then collected and defined as NG-MCM, M-MCM, or HG-MCM (Fig. 1). The cell viability was quantified by using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (16). As shown in supplemental Fig. S1, the viability of macrophages was over 75.6% when cultured in HG-MCM. In addition, the cell viability was similar when macrophages were cultured in NG-, M-, and HG-MCM (supplemental Fig. S1). The final concentrations of glucose in MCM are summarized in supplemental Table S1.

EC Culture—ECs were isolated from human umbilical cords as described previously (10). Briefly, human umbilical cords were washed, filled with 0.1% collagenase, and incubated at 37 °C for 10 min. ECs were resuspended in M199 supplemented with penicillin/streptomycin and 20% FBS. Cultures were maintained at 37 °C in a humidified atmosphere containing 5%
CO2. Cells were kept static, and the culture medium was changed every 3 days. HUVECs of passage 2 were used for the experiments. To investigate the effect of MCM on ECs, the M199 media was removed, and fresh RPMI medium (control), NG-MCM, and HG-MCM containing 2% FBS were added according to different treatments of cells.

Real-time Quantitative PCR—Total RNA preparation and the RT reaction were carried out as described previously (10). PCRs were performed using an ABI Prism 7900HT according to the manufacturer’s instructions. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). The designed primers in this study were as follows: E-selectin forward, 5′-CTGAA AGATG GAGGC TCAAT-3′; and reverse, 5′-CGTTT CAGAG GCCG AAGAG-3′. 18 S rRNA forward, 5′-CGGCG ACGAC CCATT CGAAC-3′; and reverse, 5′-GAATC GAACC CTGAT TCCCC. GTC-3′. Quantification was performed using the 2ΔΔCt method (17).

Western Blot Analysis—ECs were lysed with a buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The total cell lysate (50 μg of protein) was separated by SDS-PAGE (12% running, 4% stacking) and analyzed by using the designated antibodies and the Western-Light chemiluminescent detection system (Bio-Rad), as previously described (16).

ELISA for Cell Surface E-Selectin Expression—E-selectin expression on the EC surface was measured by cell surface ELISA as described previously (18). Briefly, HUVECs cultured in 96-well plates were fixed by 4% paraformaldehyde. Cell surface E-selectin expression was assessed by using the mouse anti-human E-selectin mAb followed by a horseradish-peroxidase-conjugated secondary antibody. The absorbance of each well was measured at 490 nm after the reactions were stopped. ELISA for sE-Selectin—The level of sE-selectin in EC culture medium was determined by using sandwich ELISA (sensitivity 1 pg/ml) (R&D Systems) according to the manufacturer’s protocols, as described previously (9).

Reporter Gene Constructs, siRNA, Transfection, and Luciferase Assays—The plasmid pGL2-E-selectin p540 promoter construct was kindly provided by Dr. Jeng-Jiann Chiu (National Health Research Institutes, Taiwan). Deleted E-selectin promoter constructs (-310/+1, -130/+1, and -40/+1) were subcloned into the pGL-2 basic vector (Promega, Madison, WI) by PCR as described previously (10). DNA plasmids at a concentration of 1 mg/ml were transfected into HUVECs by using Lipofectamine (Invitrogen). The pSV-β-galactosidase plasmid was cotransfected to normalize the transfection efficiency.

For siRNA transfection, HUVECs were transfected with the designated siRNA by using a RNAiMAX transfection kit (Invitrogen).

NF-κB p65 and AP-1 Transcription Factor Assays (TF ELISA)—Nuclear extracts of cells were prepared as described previously (10). Equal amounts of nuclear extracts were used for quantitaive measurements of NF-κB p65 and AP-1 activation using commercially available ELISA kits (Panomics, Redwood City, CA) that measure NF-κB p65 and AP-1 DNA binding activities.

ChIP—After cross-linking with 1% formaldehyde, the cells were centrifuged and then resuspended in a lysis buffer for sonication three times at 15 s each. Supernatants were recovered by centrifugation. Aliquots of the precleared sheared chromatin were then immunoprecipitated using 2 μg of antibodies against IgG, NF-κB p65, or c-Jun. The resulting DNA was used for PCR analysis, and the amplified DNA fragments were visualized on an agarose gel. PCR was performed with primers that amplify the part of the human E-selectin promoters that contain the NF-κB binding sites (5′-GGGAA AGTTT TTGGA TGCCA TT-3′ and 5′-TGTCC ACATC CAGTA AAGAG GAAAT-3′) and AP-1 binding sites (5′-ATCTA CCTTG TGAGT CATTCC-3′ and 5′-TAGTT GTGGT AGTAA TTAGA AT-3′).

Monocytic Cell Adherence Experiment—The human monocytic cell line THP-1 was obtained from the American Type Culture Collection and maintained in RPMI 1640 culture medium supplemented with 10% FBS. Before the adhesion experiments, THP-1 cells were suspended in RPMI 1640 containing 0.1% FBS and labeled with 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine (Molecular Probes, Eugene, OR) for 20 min. The labeled THP-1 cells (1 × 10⁵ cells/ml) were added to NG-MCM- or HG-MCM-treated ECs and incubated for 30 min. In parallel experiments, ECs were treated with isotype-matched IgG or neutralizing antibody against E-selectin (20 μg/ml), or HG-MCM were treated with neutralizing antibodies against MIP-1α or MIP-1β during HG-MCM stimulation. Non-adherent cells were removed by washing with RPMI. The adherent THP-1 cells on the EC surface were identified and counted in 10 randomly selected microscopic fields (1.37 × 1.07 mm) under a Nikon Ti-E inverted epifluorescence microscope with 10× objective, and the adhesion was expressed as fold compared with controls.

Statistical Analysis—The results are expressed as mean ± S.E. Statistical analysis was determined by using an independent Student t test for two groups of data and analysis of variance followed by Scheffe’s test for multiple comparisons. p values less than 0.05 were considered significant.

RESULTS

HG-MCM Enhanced E-Selectin Expression in ECs—The effects of macrophages from a high-glucose concentration on the expression and secretion of E-selectin in ECs were studied using HG-MCM (versus NG- and M-MCM). The time courses determined for the E-selectin mRNA levels (Fig. 2A) revealed an increase after 1 h of HG-MCM stimulation and a peak expression at 4 h, followed by a gradual reduction thereafter. At the 4-h time point, the culturing of the ECs in HG-MCM induced increases in the E-selectin mRNA levels by 5.3-fold compared with NG-MCM-treated ECs (Fig. 2A). The exposure of ECs to HG-MCM also caused significant increases in the surface E-selectin expression at 6 h after treatment (Fig. 2B) and the sE-selectin secretion at 12 h (C). Fig. 2D shows the dosedependent induction of E-selectin mRNA expression by HG-MCM. In addition, HG-MCM also induced slight increases in the EC ICAM-1 and VCAM-1 transcript levels (by 1.5- and 1.43-fold, respectively) compared with NG-MCM-treated ECs (Fig. 3A). NG-4D medium, HG-4D medium, and NG-MCM had marginal effects only on EC E-selectin expression, whereas
HG-MCM augmented this expression significantly in ECs (Fig. 3B). The effect of glucose and insulin on EC E-selectin expression is shown in Fig. 3C. The increases in E-selectin mRNA expression were 2.12-fold in HUVECs stimulated with 25 mM glucose for 4 h. HUVECs stimulated with 50 microunits insulin had no effect on E-selectin expression.

HG-MCM-induced E-Selectin Expression in ECs Is Mediated by the JNK and p38 Pathways—Members of the MAPK superfamily (i.e. ERK, JNK, and p38) are known to regulate gene expression and cellular functions (19). To determine whether HG-MCM-induced E-selectin expression is mediated through the MAPK-dependent pathways, ECs were incubated with specific inhibitors for ERK (PD98059, 30 μM), JNK (SP600125, 20 μM), or p38 (SB203580, 10 μM) for 1 h before and during stimulation with HG-MCM. The HG-MCM-induced mRNA expression (Fig. 4A), EC surface protein expression (B), and sE-selectin secretion (C) were found to be significantly inhibited by SP600125 and SB203580 but not by PD98059. Treatment of ECs with SP600125 and SB203580 resulted in the additive inhibition of HG-MCM-induced E-selectin expression and secretion (Fig. 4, A–C). To further confirm the involvement of JNK and p38, but not ERK, in the modulation of E-selectin expression by HG-MCM stimulation, we examined the effects of expressing specific siRNAs that target these signaling pathways upon HG-MCM-induced E-selectin expression in ECs. HG-MCM-induced E-selectin mRNA (Fig. 4A) and protein expression (B) and sE-selectin secretion (C) were inhibited by transfection with JNK- and p38-specific siRNAs but not by transfection with ERK-specific or control siRNAs (100 μmol/ml for each). The effectiveness of the silencing was validated because ERK siRNA, JNK siRNA, and p38 siRNA (compared with control siRNA) caused an 85% reduction in ERK, JNK, and p38 protein expressions, respectively (supplemental Fig. S2, 100 μmol/ml for each).

The phosphorylation of JNK and p38 in ECs increased rapidly after HG-MCM stimulation, reaching maximal levels at 10 min (Fig. 4D). After such transient increases, the levels of phosphorylation decreased to nearly basal levels by 1 h. In addition, HG-MCM significantly caused JNK and p38 phosphorylation after 10 min of treatment, whereas NG-MCM had a minor effect on JNK and p38 phosphorylation in HUVECs (Fig. 4E).

NF-κB and AP-1 Binding Sites Are Essential for the HG-MCM Induction of Human E-Selectin Promoter Activity—To identify the cis-acting elements in the E-selectin gene promoter that mediate HG-MCM-induced E-selectin transcription, luciferase assays were conducted with the p540-Luc plasmid and several deletion promoter constructs (Fig. 5A). In HUVECs, the −540/+1 region of E-selectin was found to direct maximum luciferase activity. A sequence deletion from −310 to −130 caused this reporter activity to decrease to 40–45% of control levels, whereas a further deletion to the −40 position almost completely abolished the activity (Fig. 5A).

To investigate whether NF-κB and AP-1 binds the E-selectin promoter region in HUVECs, we performed quantitative analysis of the NF-κB p65 and AP-1 binding activities in vitro using TF ELISA kits from Panomics. The treatment of ECs with HG-MCM caused both NF-κB p65- and AP-1 DNA binding activities to increase after 1 h and remain elevated for at least 2 h (Fig. 5B). These results were confirmed by ChIP analysis. Immunoprecipitated chromosomal DNA with a p65 antibody was sub-

**FIGURE 2. Induction of E-selectin expression in HUVECs by MCM stimulation.** Data are mean ± S.E. from three independent experiments. *p < 0.05 versus CL ECs. #, p < 0.05 versus NG-MCM-stimulated ECs. A, ECs were maintained as controls (CL) or stimulated with NG-, M-, or HG-MCM (A–C). A, RNA samples were isolated at the indicated time points and subjected to real-time PCR analysis. The mRNA data are presented as fold changes in fluorescent density from CL ECs normalized to the 18 S rRNA levels. B, the dose-dependent induction of E-selectin mRNA expression by HG-MCM. ECs were grown as CL or were stimulated with different concentrations of NG- or HG-MCM for 4 h. The mRNA data are presented as fold changes in fluorescent density from CL ECs normalized to the 18 S rRNA levels.
jected to PCR using primers designed to amplify the E-selectin promoter region harboring the NF-κB binding site. NF-κB was indeed found to bind the E-selectin promoter region containing the NF-κB sites (Fig. 5C, left panel). Similarly, the region containing the AP-1 sites was specifically immunoprecipitated with c-Jun antibodies (Fig. 5C, right panel).

**FIGURE 3.** Induction of ICAM-1 and VCAM-1 expression in HUVECs by MCM stimulation (A). ECs were maintained as untreated controls (CL) or stimulated with NG- or HG-MCM for 4 h. RNA samples were then isolated and subjected to real-time PCR analysis. The mRNA data are presented as the fold changes in the fluorescent signals compared with CL ECs normalized to the 18 S RNA level. Data are mean ± S.E. from three independent experiments. *, p < 0.05 versus CL ICAM-1. #, p < 0.05 versus CL VCAM-1.

**FIGURE 4.** JNK and p38 pathways are required for HG-MCM-induced E-selectin expression in ECs. ECs were untreated (CL) or stimulated with NG- or HG-MCM prepared from different concentrations of macrophages for the periods (t) indicated. The results shown are the mean ± S.E. from three to four independent experiments. A–C, effects of HG-MCM on E-selectin mRNA levels (t = 4 h), protein levels (t = 6 h), and sE-selectin concentration (t = 10 h), respectively. Prior to culturing as control or HG-MCM-stimulated cells, ECs were pretreated with PD98059 (PD, 30 μM), SP600125 (SP, 20 μM), or SB203580 (SB, 10 μM) individually for 1 h or transfected with control siRNA (si-CL) or siRNAs targeting ERK (si-ERK), JNK (si-JNK), or p38 (si-p38) (100 μmol/ml for each). *, p < 0.05 versus CL. #, p < 0.05 versus dimethyl sulfoxide-treated or control siRNA (si-CL)-transfected ECs under HG-MCM stimulation. &, p < 0.05 versus SP- or SB-pretreated ECs under HG-MCM stimulation. D; control or HG-MCM-stimulated ECs were maintained for the times indicated, and the phosphorylation of JNK and p38 in these cells was determined using Western blotting. E, the phosphorylation of JNK and p38 in HUVECs after 10 min of NG-MCM (NG) or HG-MCM (HG) stimulation was determined using a Western blot analysis. The results shown are representative of three independent experiments that gave similar results (D and E).
We further tested whether NF-κB and AP-1 activation are involved in the signal transduction pathway leading to the HG-MCM induction of E-selectin gene expression. HUVECs were transfected with siRNAs for p65 or c-Jun, followed by stimulation with HG-MCM for 4 h. The HG-MCM-induced E-selectin mRNA and E-selectin p540-Luc promoter activity levels (Fig. 5D) were significantly down-regulated by the inhibition of p65 or c-Jun by siRNA, indicating that NF-κB and AP-1 are involved in the regulation of E-selectin gene expression. The p65- and c-Jun-specific siRNAs (compared with control siRNA) caused an 80% reduction in p65 and c-Jun protein expressions, respectively (supplemental Fig. S3).

The JNK and p38 Signaling Pathways Are Involved in HG-MCM-induced E-Selectin Promoter Activity—To evaluate whether the inhibition of E-selectin expression by the JNK and p38 signaling pathways occurs at the transcriptional level, we studied the effects of inhibitors or siRNAs against JNK and p38 upon HG-MCM-induced E-selectin mRNA and E-selectin p540-Luc promoter activity levels (Fig. 5D) were significantly down-regulated by the inhibition of p65 or c-Jun by siRNA, indicating that NF-κB and AP-1 are involved in the regulation of E-selectin gene expression. The p65- and c-Jun-specific siRNAs (compared with control siRNA) caused an 80% reduction in p65 and c-Jun protein expressions, respectively (supplemental Fig. S3).

The JNK and p38 Signaling Pathways Are Involved in HG-MCM-induced E-Selectin Promoter Activity—To evaluate whether the inhibition of E-selectin expression by the JNK and p38 signaling pathways occurs at the transcriptional level, we studied the effects of inhibitors or siRNAs against JNK and p38 upon HG-MCM-induced E-selectin p540-Luc promoter activity. Culturing of the HUVECs in HG-MCM increased the luciferase activity by 4.5-fold compared with unstimulated cells after normalization with a transfection control (Fig. 6A). Pretreatment of the cells with SP600125 and SB203580 or transfection with JNK siRNA and p38 siRNA resulted in a marked inhibition of HG-MCM-induced E-selectin promoter activity (Fig. 6A). The simultaneous treatment of ECs with SP600125 and SB203580 resulted in the additive inhibition of HG-MCM-induced E-selectin promoter activity (Fig. 6A). In HUVECs, transfection with siRNA or promoter constructs at the concentrations used in these experiments was found not to cause cytotoxicity, on the basis of cell numbers and morphology (data not shown).

To explore whether JNK and p38 activate the promoter responsible for E-selectin transcription via NF-κB and AP-1, HUVECs were pretreated with MAPK inhibitors followed by HG-MCM stimulation. NF-κB p65 and AP-1 activation was then assessed by TF ELISA. Pretreatment of the cells with SP600125 and SB203580 significantly inhibited the HG-MCM-induced p65 DNA and AP-1 DNA binding activity (Fig. 6B). Pretreatment with these inhibitors simultaneously caused an additive inhibition of HG-MCM-induced p65 DNA and AP-1 DNA binding activity (Fig. 6B). In addition, ChIP analysis revealed that the combined pretreatment of ECs with SP600125 and SB203580 inhibited the HG-MCM induction of the p65 (Fig. 6C) and c-Jun promoter (D) binding activities.

HG-MCM-induced E-Selectin Expression Is Mediated by MIP-1α and MIP-1β—The increases in E-selectin expression in ECs by HG-MCM suggested that macrophages under high glucose condition release soluble mediators to exert paracrine effects on ECs and thereby induce the E-selectin expression. We examined the expression levels of cytokines in NG-MCM and HG-MCM using a human cytokine array system. Using this array, we identified that the MIP-1α and MIP-1β proteins are released at significantly higher levels in HG-MCM cultured cells compared with those exposed to NG-MCM (Fig. 7A). To confirm these results, the protein levels of MIP-1α and MIP-1β in cells grown in NG-, M-, and HG-MCM were analyzed by
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FIGURE 6. JNK and p38 signaling pathways are involved in HG-MCM-induced E-selectin promoter activity. All bar graphs represent the fold increases over the control EC levels (CL), calculated as the mean ± S.E. from three independent experiments. *p < 0.05 versus CL. A, p < 0.05 versus dimethyl sulfoxide-treated or si-CL-transfected ECs under HG-MCM stimulation. &p < 0.05 versus SP- or SB-pre-treated ECs under HG-MCM stimulation. A, E-selectin p540-Luc activity determined in HUVECs pretreated with PD98059 (PD), SP600125 (SP), or SB203580 (SB) individually or with SP and SB in combination (SP+SB) for 1 h, or transfected with si-CL, si-ERK, si-JNK, or si-p38 and then stimulated with HG-MCM for 4 h. B, NF-κB p65 and AP-1 activation levels determined by TF ELISA in HUVECs pretreated with DMSO, PD, SP, or SB individually or with SP and SB in combination, and then stimulated with HG-MCM for 2 h. C and D, ChIP assays were performed for NF-κB (C, using p65 antibodies) and AP-1 (D, using c-Jun antibodies) in HUVECs pretreated with dimethyl sulfoxide or simultaneously with SP and SB.

ELISA. Culturing of monocyte-derived macrophages for 4 days in NG-4D, M-4D, or HG-4D conditions caused only slight increases in MIP-1α and MIP-1β in the culture medium, but significant increases of MIP-1α and MIP-1β were observed in HG-MCM (Fig. 7B).

The incubation of ECs with neutralizing antibodies against MIP-1α or MIP-1β significantly inhibited HG-MCM-induced E-selectin mRNA expression and promoter activity (Fig. 7C) in addition to HG-MCM-induced NF-κB p65 and AP-1 activation (D). The simultaneous treatment of ECs with neutralizing antibodies against MIP-1α and MIP-1β caused further inhibition of E-selectin promoter activity and mRNA expression (Fig. 7C) and NF-κB p65 and AP-1 activation (D). In addition, ECs directly stimulated with MIP-1α or MIP-1β (5 ng/ml each) had no effect on the expression of E-selectin (Fig. 7E). However, the costimulation of ECs with NG-MCM and MIP-1α or MIP-1β had effects upon E-selectin expression that were similar to HG-MCM (Fig. 7E).

HG-MCM-induced THP-1 Cell Adhesiveness Was Inhibited by Neutralizing Antibodies Against E-Selectin, MIP-1α, and MIP-1β—Because the up-regulation of E-selectin in ECs induces monocyte adherence, we examined the alterations in adhesiveness of the HG-MCM-treated ECs for monocytic THP-1 cells. ECs treated with HG-MCM for 4 h increased the adhesion of THP-1 cells about 6.6- and 4.1-fold in comparison to that of controls and NG-MCM, respectively (Fig. 8A). Pretreatment of ECs with neutralizing antibody against E-selectin (Fig. 8B) or pretreatment of HG-MCM with neutralizing antibodies against MIP-1α or MIP-1β (C) significantly inhibited the adhesion of THP-1 cells to the HG-MCM-treated ECs. The simultaneous treatment of ECs with MIP-1α and MIP-1β neutralizing antibodies resulted in the additive suppression of HG-MCM-induced THP-1 adhesiveness (Fig. 8C).

DISCUSSION

Diabetic patients have an increased susceptibility to atherosclerosis (20, 21). E-selectin, which plays significant roles in atherosclerosis and type 2 DM, is a major adhesion molecule expressed by vascular ECs. In this study, we cultured primary human macrophages under HG and NG conditions and elucidated the molecular mechanisms underlying the roles of HG-treated macrophages in modulating E-selectin expression in ECs. The major findings of our present analyses are 1) that conditioned media from HG-treated macrophages induced slight increases in the EC ICAM-1 or VCAM-1 transcript levels compared with NG-MCM-treated ECs, 2) that HG-MCM significantly increases the expression and secretion of EC E-selectin compared with NG-MCM-treated ECs, 3) that HG-4D medium and NG-MCM only caused moderate increases in E-selectin expression, 4) that this increase in E-selectin expression is attributable to the paracrine effect of the chemokines MIP-1α and MIP-1β released by HG-treated macrophages, and 5) that the HG-MCM-increased expression of E-selectin is mediated by the JNK/p38 and NF-κB/AP-1 pathways.

Endothelial dysfunction or damage can be evaluated by the measurement of biomarkers released from ECs, such as sE-selectin, sICAM-1, sVCAM-1, and von Willebrand factor (15, 22). Several studies have now demonstrated that the plasma level of sE-selectin is also increased in patients with hypertension, type 2 DM, atherosclerosis, and dyslipidemia, all of which are well...
established risk factors for cardiovascular diseases (23–26). In diabetic patients in several earlier studies, sE-selectin levels have also been found to be elevated and to correlate with the onset of glycemia (13, 23, 27). In particular, sE-selectin is a strong independent predictor of the incidence of DM after adjustment for obesity and other clinical and lifestyle parameters in both men and women (28). The results of this study demonstrate that conditioned media from HG-treated macrophages not only promote the secretion of sE-selectin but also induces their gene transcription and expression in human ECs. Our analysis of the E-selectin promoter activity with different plasmid constructs further revealed that the NF-κB and AP-1-binding regions function as the cis-element for HG-MCM responsiveness via JNK and p38 phosphorylation. Previous studies have shown that NF-κB and AP-1 can be activated through the JNK and p38 pathways in ECs (29, 30). The regulation of gene expression through the use of combinations of different transcription factors, including NF-κB and AP-1, has been reported, and JNK and p38 have been shown to be involved in the activation of p65 and c-Jun (31, 32). In our present experiments, we performed luciferase assays and found that NF-κB and AP-1 cooperate to activate the human E-selectin promoter. We employed TF ELISA and ChIP assays to demonstrate that the regulation of E-selectin gene expression in ECs is mediated by increased NF-κB p65 and AP-1-DNA binding activities following JNK and p38 phosphorylation.

Because there is considerable evidence that inflammation plays a major role in atherogenesis, it was important to evaluate the proatherogenic activity of monocytes/macrophages in diabetes. Macrophage accumulation is a feature of type 2 DM and is associated with the development of atherosclerosis (33). Monocytes in a proinflammatory state have also been detected

**FIGURE 7.** MIP-1α and 1β present in HG-MCM are the major factors underlying HG-MCM-induced E-selectin expression in ECs. A, membranes spotted with antibodies against cytokines and chemokines were incubated with MCM diluted 2-fold, incubated with a mixture of biotin-labeled antibodies, and then detected by ECL. The results shown are representative of three independent experiments that gave similar results. B, the expression levels of MIP-1α and MIP-1β in NG-, M-, or HG-MCM determined by sandwich ELISA. *, p < 0.05 versus MIP-1α in NG-MCM. #, p < 0.05 versus MIP-1β in NG-MCM. C and D, ECs were untreated (CL) or stimulated with NG- or HG-MCM for the indicated periods (t). The results are the mean ± S.E. of three to four separate experiments. C, HG-MCM- and ECs were preincubated with isotype-matched IgG or neutralizing antibodies against MIP-1α or MIP-1β (20 ng/ml each) upon HG-MCM-induced E-selectin mRNA expression and p540-Luc promoter activity (t = 4 h) and upon NF-κB- and AP-1 activation (t = 2 h), respectively. Prior to culturing under control conditions or stimulation with HG-MCM, the HG-MCM and ECs were preincubated with isotype-matched IgG or neutralizing antibodies against MIP-1α (Ab-α) or MIP-1β (Ab-β) individually or in combination (Ab-α + β) for 1 h. *, p < 0.05 versus CL. #, p < 0.05 versus IgG-treated ECs under HG-MCM stimulation. &p, p < 0.05 versus Ab-α- or Ab-β-treated ECs under HG-MCM stimulation. E, ECs cultured as unstimulated CL, stimulated with MIP-1α or 1β (5 ng/ml), or costimulated with NG-MCM and MIP-1α and MIP-1β, separately or in combination for 4 h. RNA samples were then isolated and subjected to real-time PCR analysis. The mRNA data are presented as the fold changes in fluorescence intensities from CL ECs normalized to the 18S rRNA levels. *, p < 0.05 versus CL or ECs stimulated with MIP-1α and 1β. #, p < 0.05 versus ECs costimulated with NG-MCM + MIP-1α and MIP-1β.
in severe hyperglycemic type 2 DM patients, indicating that the proinflammatory status of circulating monocytes can contribute to the development of the atherosclerotic plaques when they are recruited to the vascular lesion. It is also becoming evident that chronic inflammation is associated with type 2 DM conditions and is involved in all stages of the atherosclerotic process. The macrophage effector chemokines MIP-1α and MIP-1β, produced and secreted by activated macrophages to attract other proinflammatory cells, are crucial in recruiting macrophage themselves to sites of inflammation. By using protein arrays to perform a systematic analysis and ELISA assays of macrophage themselves to sites of inflammation. The modulatory effects of a high-glucose environment on monocyte/macrophage function have been documented previously. Glucose has been shown to increase the production of cytokines by human monocytes. HG treatments of THP-1 monocytes have also been found to induce changes in the expression levels of several cytokines, chemokines, and related molecules. In this study, we observed for the first time that MIP-1α and MIP-1β are significantly increased only in macrophages after differentiation in an HG environment. It has been shown previously that glycated human serum albumin enhances MIP-1β mRNA expression in macrophage-like differentiated U937 cells. Our present data also demonstrate that the protein levels of MIP-1α and MIP-1β are similar when monocytes are cultured in an HG, NG, or mannitol environment for 4 days. HG-4D medium and NG-MCM only caused moderate increases in E-selectin expression. These results suggest that the gene expression of macrophages may be altered after transformation from monocytes under hyperglycemic conditions. Hence, we speculate that activated monocytes under high-glucose conditions may bind to ECs and, following transmigration, may differentiate into macrophages in the subendothelial space. The accumulation of macrophages in the arterial wall thereby increases the MIP-1α and MIP-1β levels, and contributes to proinflammatory responses. We have also demonstrated the functional consequence of the MIP-1α and MIP-1β modulation of the HG-MCM-induced EC gene expression by measuring the adhesiveness of the monocytic THP-1 cells to HG-MCM-treated ECs. HG-MCM
treated with neutralizing antibodies against MIP-1α and MIP-1β blocked the HG-MCM-induced THP-1 cell adhesion. On the basis of our results, we propose a possible signal transduction pathway in ECs in which HG-treated macrophages release MIP-1α and MIP-1β, which induce NF-κB/AP-1 activation, thus resulting in E-selectin transcriptional activation, expression and secretion.

In summary, our results characterize the mechanisms by which HG-treated macrophages induce E-selectin expression in ECs. HG-treated macrophages induce the activation of JNK/p38 and NF-κB/AP-1 signaling pathways and ultimately enhance E-selectin expression in ECs. These findings provide insights into the mechanisms underlying the interplay between hyperglycemic macrophages with ECs in modulating EC signaling and gene expression, which may well be involved in the development of vascular complications in patients with diabetes.

REFERENCES

1. Kim, J. A., Montagnani, M., Koh, K. K., and Quon, M. J. (2006) Circulation 113, 1888–1904
2. Orasanu, G., and Plutzky, J. (2009) J. Am. Coll. Cardiol. 53, S35–42
3. Monnier, L., Mas, E., Ginet, C., Michel, F., Villon, L., Cristol, J. P., and Colette, C. (2006) JAMA 295, 1681–1687
4. Wright, R. J., and Frier, B. M. (2008) Diabetes-Metab. Res. Rev. 24, 353–363
5. Ross, R. (1999) N. Engl. J. Med. 340, 115–126
6. Libby, P. (2002) Nature 420, 868–874
7. Shankugam, N., Reddy, M. A., Gulla, M., and Natarajan, R. (2003) Diabetes 52, 1256–1264
8. Tedgui, A., and Mallat, Z. (2001) Circ. Res. 88, 877–887
9. Chen, C. N., Chang, S. F., Lee, P. L., Chang, K., Chen, L. J., Usami, S., Chien, S., and Chiu, J. J. (2006) Blood 107, 1933–1942
10. Sung, M. L., Wu, C. C., Chang, H. I., Yen, C. K., Chen, H. I., Cheng, J. C., Chien, S., and Chen, C. N. (2009) Circ. Res. 105, 755–763
11. Chiu, J. J., Lee, P. L., Chen, C. N., Lee, C. I., Chang, S. F., Chen, L. J., Lien, S. C., Ko, Y. C., Usami, S., and Chien, S. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 73–79
12. Tesfamariam, B., and DeFelice, A. F. (2007) Vascul. Pharmacol. 46, 229–237
13. Matsumoto, K., Nakamura, H., Ueki, Y., Tominaga, T., and Miyake, S. (2001) Diabet. Med. 18, 224–228
14. Matsumoto, K., Fujishima, K., Moriuchi, A., Saishoji, H., and Ueki, Y. (2010) Metabolism 59, 320–324
15. Meigs, J. B., Hu, F. B., Rifai, N., and Manson, J. E. (2004) JAMA 291, 1978–1986
16. Chou, M. T., Chang, S. N., Ke, C., Chang, H. I., Sung, M. L., Kuo, H. C., and Chen, C. N. (2010) Biomaterials 31, 4367–4375
17. Yeh, C. C., Chang, H. I., Chiang, I. K., Tsai, W. T., Chen, L. M., Wu, C. P., Chien, S., and Chen, C. N. (2009) Arthritis Rheum. 60, 2350–2361
18. Sands, W. A., Martin, A. F., Strong, E. W., and Palmer, T. M. (2004) Mol. Pharmacol. 66, 1147–1159
19. Johnson, G. L., and Lapadat, R. (2002) Science 298, 1911–1912
20. Renard, C., and Van Obberghen, E. (2006) Diabetes Metab. 32, 15–29
21. Kreutz, A. J., Clough, G., and Byrne, C. D. (2007) Diabetes Obes. Metab. 9, 781–791
22. Verma, S., and Anderson, T. J. (2002) Circulation 105, 546–549
23. Boulbou, M. S., Koukoulis, G. N., Makri, E. D., Petinaki, E. A., Gourgoulianis, K. I. L., and Germinis, A. E. (2005) Int. J. Cardiol. 98, 39–44
24. Hackman, A., Abe, Y., Insull, W., Jr., Pownall, H., Smith, L., Dun, K., Gotto, A. M., Jr., and Ballantyne, C. M. (1996) Circulation 93, 1334–1338
25. Hwang, S. J., Ballantyne, C. M., Sharrett, A. R., Smith, L. D., Davis, C. E., Goto, A. M., Jr., and Boerwinkle, E. (1997) Circulation 96, 4219–4225
26. DeCaterina, R., Ghiaidoni, L., Taddei, S., Virdis, A., Almerigogna, F., Basta, G., Lazzzerini, G., Bernini, W., and Salvetti, A. (2001) Am. J. Hypertens. 14, 259–266
27. Matsumoto, K., Sera, Y., Nakamura, H., Ueki, Y., and Miyake, S. (2002) Diabetes Res. Clin. Pract. 55, 131–138
28. Thorand, B., Baumert, J., Chambless, L., Meisinger, C., Kolb, H., Döiring, A., Löwel, H., Koenig, W., and MONICA/KORA Study Group. (2006) Arterioscler. Thromb. Vasc. Biol. 26, 398–405
29. Chiu, J. J., Chen, L. J., Lee, C. I., Lee, P. L., Lee, D. Y., Tsai, M. C., Lin, C. W., Usami, S., and Chien, S. (2007) Blood 110, 519–528
30. Zhu, W., Chandrasekharan, U. M., Bandyopadhyay, S., Morris, S. M., Jr., DiCorleto, P. E., and Kashyap, V. S. (2010) Am. J. Physiol. Cell Physiol. 298, C952–960
31. Read, M. A., Whiteley, M. Z., Gupta, S., Pierce, J. W., Best, J., Davis, R. J., and Collins, T. (1997) J. Biol. Chem. 272, 2753–2761
32. Lin, S. J., Shyue, S. K., Hung, Y. Y., Chen, Y. H., Ku, H. H., Chen, J. W., Tam, K. B., and Chen, Y. L. (2005) Arterioscler. Thromb. Vasc. Biol. 25, 334–340
33. Marx, N., Walcher, D., Raiche, C., Alexekis, M., Bach, G., Gröh, M., Hombach, V., Libby, P., Zieske, A., Homma, S., and Strong, J. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 540–545
34. Dandona, P., Aljada, A., and Bandyopadhyay, A. (2004) Trends Immunol. 25, 4–7
35. Liang, C. P., Han, S., Senokuchi, T., and Tall, A. R. (2007) Circ. Res. 100, 1546–1555
36. Dasu, M. R., Devaraj, S., and Jialal, I. (2007) Am. J. Physiol. Endocrinol. Metab. 293, E337–346