Methylation status of CpG sites in the MCP-1 promoter is correlated to serum MCP-1 in Type 2 diabetes

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ABSTRACT. Aim: Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine and plays an important role in atherosclerosis of Type 2 diabetes. The aim of this study was to investigate the methylation status of CpG sites in the MCP-1 promoter in Type 2 diabetic patients and its correlation to serum MCP-1 level, and blood glucose level. Methods: The 32 patients with Type 2 diabetes and 15 healthy controls were enrolled into the study. Body mass index, blood pressure, blood lipid, blood glucose, glycosylated hemoglobin (HbA1c), and serum MCP-1 were measured. Genomic DNA was isolated from the peripheral blood mononuclear cells (PBMC). Methylation status of CpG sites in the MCP-1 promoter was determined using methylation specific polymerase chain reaction. Results: The promoter region (2890-3050 bp) was predominantly methylated in PBMC from controls. Methylation of CpG motifs were less methylated in the patients than in the controls (25% vs 80%; p<0.001), while the level of MCP-1 in serum was higher in patients with Type 2 diabetes (193.95±74.96 vs 88.46±55.10; p<0.001). MCP-1 promoter methylation was significantly correlated to serum MCP-1, HbA1c, fasting blood glucose, and triglyceride. Conclusion: These data suggest that hypomethylation of CpG sites in the MCP-1 promoter region may be affected by blood glucose and TG, which then increase the serum MCP-1 level and may play a role in the vascular complications of Type 2 diabetes. (J. Endocrinol. Invest. 35: 585-589, 2012)
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INTRODUCTION

Monocyte chemoattractant protein-1 (MCP-1) is a potent monocyte attractant, which plays an important role in atherosclerosis. The chemokine expression might be an important step in the recruitment and activation of peripheral blood leucocytes in atherosclerotic lesions (1-3). It has been demonstrated that Type 2 diabetes have elevated MCP-1 level (4-6). Emerging evidence suggests a possible contributing factor in the inflammation and complications of Type 2 diabetes remain unclear.

In this study, we intended to investigate 1) the methylation status of CpG sites of MCP-1 gene promoter in patients with Type 2 diabetes and healthy controls by methylation specific polymerase chain reaction (MSP); 2) the serum MCP-1 level and its relationship with the methylation status of promoter; 3) whether blood glucose had an effect on the epigenetic modification of MCP-1.

SUBJECTS AND METHODS

Subjects

This study was reviewed and approved by the local Ethics Review Committee (Union Hospital, Tongji Medical College, Huazhong University of Science and Technology). Written informed consent was obtained from all patients and healthy controls before they entered the study. Diagnosis of Type 2 diabetes was based on the guidelines in the Expert Committee Report of the American Diabetes Association (12). Thirty-two Type 2 diabetic subjects (21 male and 11 female, mean age 51.97±16.57 yr) who had been hospitalized for treatment of poor glucose control were recruited. The mean duration of diabetes was 5.16±7.17 yr. Mean fasting plasma glucose and glycosylated hemoglobin (HbA1c) were 8.83±3.36 mmol/l and 9.5±2.8%, respectively. Twelve patients had macrovascular complications.
including ischemic heart disease, myocardial infarction, stroke or peripheral vascular disease. All Type 2 diabetic patients were free from microangiopathic complications. Health controls (7 male and 8 female, mean age 50.40±5.69 yr) were recruited from the people who took health examinations in the hospital. Subjects were included in the control group according to the following criteria: no past history of diabetes, normal fasting glucose, and free of any previous vascular event. Any subject suspected of having any infectious disease (including a common cold) shortly before or during the admission was excluded from study, as were patients with autoimmune disease. One patient was treated with dietary modification alone, while 25 patients were treated with diet and oral hypoglycemic agents, specifically sulfonylureas (glibenclamide, gliclazide, or glimepiride), and 6 patients were treated with diet and insulin injections. Eleven patients were taking antihypertensive drugs: ACE-inhibitors or angiotensin II receptor blockers, calcium channel blockers or both.

### Study assessments

#### Sample collection

Blood was drawn after an 8-12-h overnight fast for fasting blood glucose (FBG), triglyceride (TG), total cholesterol (TC), HDL-cholesterol, LDL-cholesterol, and HbA1c and analyzed by conventional methods immediately. After centrifugation, serum was obtained for the assessment of MCP-1 and stored at −70 C. Additional 3-4-ml blood from each subject was collected in the presence of anticoagulant and processed immediately for peripheral blood mononuclear cells (PBMC) isolation. PBMC were isolated by Ficoll-Paque density gradient centrifugation. Blood was diluted with equal volumes of phosphate buffered saline. An equal volume of diluted blood was overlaid on Ficoll-Paque (TBD, Tianjin, CHN) in 1:1 ratio and centrifuged at 1200× g for 20 min at 18-20 C. The PBMC were collected from the interface and washed with PBS several times to remove plasma and Ficoll. Then PBMC were stored in liquid nitrogen until assayed.

#### Measurements

Height and weight were measured in light indoor clothing and without shoes. The body mass index (BMI) was calculated in kg/m². Diastolic blood pressure (DBP), and systolic blood pressure (SBP) were measured in all subjects. FBG, TC, HDL-cholesterol and TG were measured by means of standard enzymatic techniques. LDL-cholesterol was calculated using Friedewald’s formula. HbA1c was evaluated by high performance liquid chromatography. Sandwich enzyme-linked immunosorbent assay for MCP-1 was performed according to the manufacturer’s instructions (R&D systems Inc, USA). Intra-assay coefficient of variation (CV) was <7.8% and the detection limit of the MCP-1 assay was 5.0 pg/ml.

#### DNA extraction

Genomic DNA was extracted by using the TiANamp Genomic DNA Kit (Tiangen, Beijing, CHN) according to the manufacturer’s protocol.

### MCP-1 promoter methylated patterns analysed with methylation-specific PCR

To address the fact that the altered expression of atherosclerosis-related genes is associated with their promoter methylated patterns, MSP was performed in CpG-rich regions MCP-1 promoters as previous procedure (13). Bisulfite modification protocols of DNA were completed with EpiTect Bisulfite Kit (Qiagen, Germany). EpiTect control DNA (Qiagen) was used as positive control and negative control for methylation. The MCP-1 unmethylation (U) and methylation (M) primer sequences used were as follows; U-F 5'-TGTTTGGAAGTTAAGTTGACT-3’ and U-R 5'-TAAAAAAAAACAAAAATCAAAAC-3’, M-F 5'-TGTTTGGAAGTTAAGTTGAGC-3’ and M-R 5'-AAAAAAAACAAAAATCAAAACCGAAA-3’ which gave 234 and 228 bp (13) fragments, respectively. The corresponding sequence was located in promoter region 2890-3050 bp. PCR reactions (20 µl) containing 1U FastStart Taq DNA Polymerase (Roche), 5 pmol of each forward and reverse primer, and supplemented with MgCl₂ to 3.5 mmol/l, were subjected to the following cycling conditions: 1 cycle of 95 C for 5 min, 50 cycles of 95 C for 30 sec, 60 C for 30 sec, 72 C for 30 sec followed by a 10-min extension at 72 C. Ten-µl PCR products were run on 1.5% ethidium bromide-stained agarose gel electrophoresis.

#### Statistical analysis

All statistical analyses were performed using SPSS 13.0 software package (SPSS Inc. Chicago, IL, USA). Results are presented as mean±SD. Independent sample t-test was used to compare variables of Type 2 diabetes and controls. Methylation pattern was classified into two categories (Unmethylated 0, Methylated 1). Categorical variables were compared by the chi-square test or continuity correction. Odds ratios (OR) with 95% confidence intervals (CI) were calculated. Correlation analyses were performed using Spearman or Pearson coefficients. p<0.05 was considered statistically significant. Power to detect differences in serum MCP-1 level between Type 2 diabetes patients and controls was calculated to 99.8% (α=0.05).

#### RESULTS

### Characteristics of the subjects

Table 1 shows the characteristics of Type 2 diabetes and age-matched controls. A total of 47 subjects; 32 Type 2

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Type 2 diabetes</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>51.97±16.57</td>
<td>50.40±5.69</td>
<td>0.635</td>
</tr>
<tr>
<td>Gender, male (%)</td>
<td>21 (65.6)</td>
<td>7 (46.7)</td>
<td>0.217</td>
</tr>
<tr>
<td>Duration of diabetes (yr)</td>
<td>5.16±7.17</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.37±3.93</td>
<td>22.33±2.83</td>
<td>0.005</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>136.43±22.49</td>
<td>118.60±9.49</td>
<td>0.005</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80.27±9.81</td>
<td>80.47±9.93</td>
<td>0.941</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>8.83±3.36</td>
<td>4.70±0.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.5±2.8</td>
<td>5.7±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>4.84±1.29</td>
<td>4.49±0.55</td>
<td>0.32</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.51±1.75</td>
<td>0.98±0.35</td>
<td>0.006</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>2.66±1.13</td>
<td>2.30±0.35</td>
<td>0.298</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.24±0.40</td>
<td>1.56±0.30</td>
<td>0.020</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>193.95±74.96</td>
<td>88.46±55.10</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented mean±SD or number (%). Independent sample t-test was used to compare variables of Type 2 diabetes and controls. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin; TC: total cholesterol; TG: triglyceride; MCP-1: monocyte chemotactic protein-1.

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**Table 1 - Characteristic of the subject studies.**
diabetes (51.97±16.57 yr) and 15 healthy controls (50.40±5.69 yr) were included in the study. The mean diabetic duration of Type 2 diabetes group is 5.16±7.17 yr. The patients with Type 2 diabetes had higher BMI, SBP, FBG, HbA1c, and TG, lower HDL-cholesterol compared with healthy controls. Serum MCP-1 level was elevated in Type 2 diabetes group. On the other hand, there were no significant differences in gender, age, DBP, TC, and LDL between the two groups.

Methylation frequency distribution between the Type 2 diabetes and the control groups

MCP-1 promoter methylation was detected in 8 Type 2 diabetic patients and 12 controls, whereas 24 Type 2 diabetic patients and 3 controls lacked MCP-1 promoter methylation (Fig. 1). The frequency of the MCP-1 promoter unmethylation was significantly higher in Type 2 diabetes group than that in the control group (p<0.001). The OR for Type 2 diabetes was 12 (95% CI 2.686-53.616) (Table 2).

Relationship between clinical parameters, MCP-1 levels, and MCP-1 promoter methylation

MCP-1 promoter methylation status was negatively correlated with serum MCP-1, BMI, HbA1c, FBG, and TG. There were no significant correlations between MCP-1 promoter methylation and age, TC, LDL-cholesterol or HDL-cholesterol (Table 3). No significant correlation was found between MCP-1 promoter methylation status and the disease duration in the Type 2 diabetes group (r=0.177, p=0.331).

DISCUSSION

In the present study, we showed that the patients with Type 2 diabetes had higher unmethylation ratio of MCP-1 promoter than healthy controls. The corresponding methylation sequence was located in MCP-1 promoter region 2890-3050 bp. This epigenetic change is associ-
Methylated DNA in promoter regions cause stable heritable transcriptional silencing (22). On the other hand, aberrant methylation in DNA promoter cause sustained gene expression. Given the evidence implicating that the patients with Type 2 diabetes had elevated MCP-1 level and were associated with increased risk of cardiovascular mortality (5), our results suggest that lower DNA methylation in MCP-1 gene promoter may contribute to the pathogenesis of macrovascular complications of Type 2 diabetes. Serum MCP-1 concentrations were also significantly associated with future Type 2 diabetes (23) and incident coronary heart disease events (24–26). Therefore, the identification of epigenetic variant may provide a novel biomarker that could be used to evaluate the risk of Type 2 diabetes and its complications.

The frequency of the unmethylation in MCP-1 promoter increased with blood glucose. Hyperglycemia leads to increased production of reactive oxygen species that is associated with increased DNA damage and chromosomal degradation with alteration of hypomethylation of the DNA (27). Chronic increase of ROS in the cells can also result in lipid peroxidation and generation of a wide range of other reactive products with the potential to damage DNA (28). Such DNA lesions have been shown to interfere with the ability of DNA to function as a substrate for the DNA methyltransferases, resulting in global hypomethylation (29).

Adipocytes can also be induced to secrete a number of inflammatory factors including MCP-1. MCP-1 expression increased in obese mice in the adipose tissue (30). We observed that the hypomethylation of MCP-1 negatively correlated with BMI. Analysis of the changes in MCP-1 regulation together with DNA methylation in adipocytes will be required.

As seen in our study MCP-1 methylation was negatively related to TG. An increase in TG can be a causal factor or it may be a result of methylation. Peroxisome proliferator-activated receptor activation can inhibit hypermethylation of the inducible nitric oxide synthase gene which implies that fatty acids or TG could impose epigenetic modifications on critical genes participating in atherogenesis (31). In yeast, TG accumulation was observed accompanied by down-regulation of S-adenosyl-L-homocysteine hydrolase (SAH1) expression, demonstrating that SAH1-regulated methylation has a major impact on cellular lipid homeostasis (32).

Based on our data, we suggest that blood glucose and lipid may lead to the high frequency of unmethylation of MCP-1 promoter in the Type 2 diabetic patients, and the long-lasting unmethylation of MCP-1 promoter then induced the MCP-1 expression. Persistent increase in MCP-1 expression under hyperglycemic condition maybe plays an important role in diabetic cardiovascular complications.

This is the first study to reveal that the methylation level of MCP-1 promoter region (2890-3050 bp) decreased in the diabetes patients. However, our study has several limitations. The population being studied was small and larger and multiple races studies are needed to confirm our results. Furthermore, the differences identified between Type 2 patients and healthy controls could be a primary phenomenon, implying that lower methylation is related to hyperglycemia; alternatively, the difference could be secondary to the disease process or treatment. Prospective studies in patients with recent onset Type 2 diabetes will be required to elucidate this.

In conclusion, our study implies that DNA methylation in gene promoter may take part in MCP-1 expression regulation in Type 2 diabetes, which provides one possible mechanism for the development of Type 2 diabetes and its complications. The therapy targeting of the epigenome may be a novel therapeutic pathway in the treatment of chronic inflammatory diseases such as Type 2 diabetes and its complications.

Acknowledgments

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Table 3 - Spearman correlation coefficients between methylation pattern of monocyte chemotactic protein-1 (MCP-1) and the clinical parameters in Type 2 diabetes.

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>-0.022</td>
<td>0.882</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.339</td>
<td>0.020</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>-0.490</td>
<td>0.001</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>-0.374</td>
<td>0.011</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>-0.059</td>
<td>0.702</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>-0.394</td>
<td>0.008</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>-0.278</td>
<td>0.078</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.253</td>
<td>0.110</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>-0.317</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Correlation analyses were performed using Spearman coefficients. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; HbA₁c: glycosylated hemoglobin; TC: total cholesterol; TG: triglyceride; MCP-1: monocyte chemotactic protein-1.

REFERENCES


