

## Gene expression of monocyte chemoattractant protein-1 in human monocytes by exposure to advanced glycosylation end products

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**KEY WORDS** advanced glycosylation end products; monocytes; monocyte chemoattractant protein-1; reverse transcriptase polymerase chain reaction; atherosclerosis; human

### ABSTRACT

**AIM:** To explore the effects of advanced glycosylation end products (AGEP) on monocyte chemoattractant protein-1 (MCP-1) gene expression in human peripheral blood monocytes/macrophages (PBMC). **METHODS:** Expression of MCP-1 mRNA in PBMC incubated with AGEP-bovine serum albumin (AGEP-BSA) was examined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) with  $\beta$ -actin as an internal standard. Sequencing of RT-PCR products was performed to confirm the specificity of amplification for MCP-1 gene.

**RESULTS:** AGEP-BSA stimulated monocytes to express MCP-1 mRNA in a glucose-concentration-related fashion. The levels of MCP-1 mRNA were increased slightly when monocytes were exposed to AGEP-BSA 200 mg/L (glycosylated with glucose 20 mmol/L), and increased markedly when exposed to AGEP-BSA 200 mg/L (glycosylated with glucose 50 mmol/L), but decreased slightly when exposed to AGEP-BSA 200 mg/L (glycosylated with glucose 80 mmol/L). Expression of MCP-1 mRNA was undetectable in freshly isolated monocytes, but was induced at 12 h and reached a maximal level at 24 h and was almost undetectable at 36 h after the monocytes were incubated with AGEP-BSA 200 mg/L ( $P < 0.01$ ). **CONCLUSION:** AGEP enhanced MCP-1 mRNA expression in human PBMC.

### INTRODUCTION

The principal cause of morbidity and mortality in

diabetes is the vascular complications. Accelerated atherosclerosis is a major vascular complication of this disorder. Advanced glycosylation end products (AGEP) are ultimately formed as the result of proteins exposed to aldoses and play an important role in the pathogenesis of diabetic complications<sup>[1]</sup>. It was demonstrated that AGEP was accumulated and deposited in vascular wall expressing its specific receptor, especially in atherosclerotic lesions<sup>[2]</sup>. The intimal recruitment of peripheral blood monocytes (PBMC) to the lesion-prone areas of the arterial wall is one of the earliest events in atherogenesis<sup>[3]</sup>. It was reported that AGEP accumulated in the vessel wall played a role in attracting blood monocytes into the intima through the endothelium<sup>[4]</sup>. Monocyte chemoattractant protein-1 (MCP-1) is a recently characterized protein of 76 amino acids with high specificity for monocytes. MCP-1 is an important mediator which plays a role in host responses and in human diseases, such as atherosclerosis<sup>[5]</sup>. It has been found that many types of cells such as endothelial cells, smooth muscle cells, monocytes/macrophages could produce MCP-1 at mRNA and protein levels, and the production of MCP-1 in monocytes could be increased by oxidized lipoproteins<sup>[6]</sup>. The present study was designed to demonstrate whether the expression of monocyte MCP-1 gene was stimulated by AGEP.

### MATERIALS AND METHODS

**Reagents** AGEP-BSA was prepared by incubating BSA in phosphate-buffered saline (PBS) with glucose 20, 50, and 80 mmol/L at 37 °C for 6 weeks. AGEP-BSA was dialyzed extensively against PBS 10 mmol/L pH 7.4 before experiments. Non-glycosylated BSA was treated identically as AGEP-BSA except that no glucose was present in the reaction mixture. AGEP-BSA exhibited characteristic yellow-brown pigment and fluorescence.

**Isolation and culture of monocytes** Monocytes were isolated from heparinized blood samples, taken

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from young healthy volunteers as described before<sup>(7)</sup>. Following incubation with hypertonic NaCl thrice, Ficoll 1.077 kg/L gradient centrifugation and adherence, the cells were incubated in RPMI-1640 culture medium at a concentration of  $5 \times 10^9 \text{ L}^{-1}$  with the groups AGEP-BSA or BSA for the indicated period of time.

#### Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from the cultured cells using the method of guanidinium isothiocyanate<sup>(8)</sup>. After purity and integrity of total RNA were confirmed by measuring the absorbency ratio of  $A_{260}/A_{280}$  (1.75 - 1.96) and electrophoresis, total RNA was stored in DEPC-treated water at  $-30 \text{ }^\circ\text{C}$ . The first-strand cDNA was synthesized from the total RNA using Avian myelo-blastosis virus (AMV) reverse transcriptase and random Oligo (dT) 15 primers. The reverse transcription reaction was performed at  $42 \text{ }^\circ\text{C}$  for 55 min. At the end of reverse transcription, the mixture was heated at  $92 \text{ }^\circ\text{C}$  for 5 min and immediately cooled on ice. The second-strand DNA synthesis and 30-cycle amplification were performed using DNA thermal cycler (Bio-Rad). Direct and reverse oligo primers for MCP-1 (5' TCA AAC TGA AGC TCG CAC TCT CG 3' for sense and 5' AGC TGC AGA TTC TTG GGT TGT GG 3' for antisense) and  $\beta$ -actin (5' GGT CAG AAG GAA TCC TAT GTG 3' for sense and 5' ATT GCC AAT GGT GAT GAC CTG 3' for antisense) were constructed to amplify the full coding sequences according to the sequences downloaded from the Gene Bank. A 50- $\mu\text{L}$  reaction mixture containing the first-strand cDNA as a template corresponding to 0.5  $\mu\text{g}$  total RNA, Tris-HCl 10 mmol/L, KCl 50 mmol/L, Gelatin 0.01 %, Triton-X 100 0.1 %,  $\text{MgCl}_2$  2.5 mmol/L, dNTP 0.2 mmol/L, each of direct and reverse primer 50 pmol, and Taq polymerase 2.5 U (in a 50  $\mu\text{L}$  reaction mixture) was applied to the thermal cycler for amplification. Amplification was performed for 30 cycles of denaturation (45 s at  $94 \text{ }^\circ\text{C}$ ), annealing (120 s at  $55 \text{ }^\circ\text{C}$ ) and elongation (120 s at  $72 \text{ }^\circ\text{C}$ ). To ensure that the amounts of PCR products obtained were linear in respect to input RNA, a kinetic analysis was performed by varying numbers of amplification cycles as well as by varying the amounts of input RNA. Then suitable cycle numbers of amplification and amounts of input RNA located at the linear area were selected. In all experiments, a template-free control tube was amplified at the same time to monitor the accuracy of the PCR method. PCR products were electrophoresed through a 2 % agar gel. The amounts of MCP-1 mRNA were standardized relative to the amount

of  $\beta$ -actin mRNA via densitometric analysis. The results were expressed as AU ( $D_{\text{area}} \cdot D_{\text{density}}$ ).

#### Nucleotide sequences of RT-PCR products

The sequencing of RT-PCR products was performed by Takara Biotechnology Co, Ltd, Dalian.

**Data analysis** Data were expressed as  $x \pm s$  and statistically compared by ANOVA.

## RESULTS

**Optimization of RT-PCR parameter** According to kinetic analysis of RT-PCR, suitable amplification cycle was selected as 30 cycles and amount of input RNA as 0.5  $\mu\text{g}$ .

**Effects of AGEP-BSA on MCP-1 mRNA expression** AGEP-BSA enhanced expression of MCP-1 mRNA in human PBMC (Fig 1,2). AGEP-BSA stimulated monocytes to express MCP-1 mRNA in a glucose-concentration-dependent fashion compared with BSA group (Tab 1). The level of MCP-1 mRNA in PBMC was slightly increased when PBMC were exposed to AGEP-BSA 200 mg/L (glycosylated with glucose 20 mmol/L), markedly increased in glucose 50 mmol/L, and slightly decreased in glucose 80 mmol/L. MCP-1 mRNA was undetectable in freshly isolated monocytes, but was induced at 12 h and reached a maximal level at 24 h and was undetectable at 36 h during incubating monocytes with AGEP-BSA 200 mg/L (glucose 50 mmol/L, Tab 2).

Tab 1. Different effects of glucose-concentration-dependent AGEP-BSA on expression of MCP-1 in cultured monocytes for 24 h.  $n=3$ .  $x \pm s$ .  $^*P < 0.01$  vs control.

Glucose/ $\text{mmol} \cdot \text{L}^{-1}$	Ratio of MCP-1/ $\beta$ -actin mRNA
0	1.04 $\pm$ 0.06
20	3.18 $\pm$ 0.25 <sup>*</sup>
50	10.54 $\pm$ 0.83 <sup>*</sup>
80	7.9 $\pm$ 0.5 <sup>*</sup>

The monocytes were incubated with AGEP-BSA 200 mg/L for 0 h, 12 h, 24 h, or 36 h, and quantitated via UVP densitometry.

**Sequence of RT-PCR products** It was found that the first six base sequence of PCR products was different from the sequence of MCP-1 mRNA from Gene Bank, all other coding were identical to the reported sequence which may be due to machine error reading, and

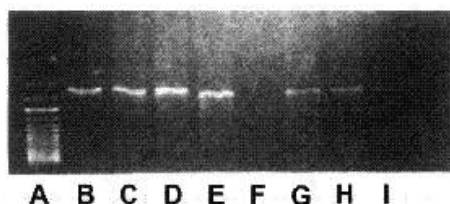


Fig 1. Expression of MCP-1 mRNA by monocytes incubated with BSA 200 mg/L or AGEP-BSA. A: Molecular marker; B: non-glycosylated-BSA; C: AGEP-BSA (glucose 20 mmol/L); D: AGEP-BSA (glucose 50 mmol/L); E: AGEP-BSA (glucose 80 mmol/L); F: AGEP-BSA (glucose 50 mmol/L), 0 h; G: AGEP-BSA (glucose 50 mmol/L), 12 h; H: AGEP-BSA (glucose 50 mmol/L), 24 h; I: AGEP-BSA (glucose 50 mmol/L), 36 h.

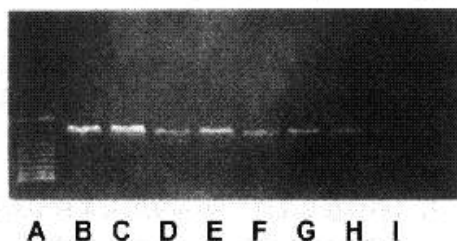


Fig 2.  $\beta$ -Actin mRNA expression by monocytes incubated with BSA 200 mg/L or AGEP-BSA. A: Molecular marker; B: nonglycosylated-BSA, 24 h; C: AGEP-BSA (glucose 20 mmol/L), 24 h; D: AGEP-BSA (glucose 50 mmol/L), 24 h; E: AGEP-BSA (glucose 80 mmol/L), 24 h; F: AGEP-BSA (glucose 50 mmol/L), 0 h; G: AGEP-BSA (glucose 50 mmol/L), 12 h; H: AGEP-BSA (glucose 50 mmol/L), 24 h; I: AGEP-BSA (glucose 50 mmol/L), 36 h.

Tab 2. Expression of MCP-1 gene in monocytes induced by AGEP-BSA for various incubation periods.  $n=3$ .  $x \pm s$ .  $^*P < 0.05$ ,  $^*P < 0.01$  vs control.

Incubation time/h	Ratio of MCP-1/ $\beta$ -actin mRNA
0	0.13 $\pm$ 0.08
12	4.9 $\pm$ 0.3 <sup>*</sup>
24	10.5 $\pm$ 0.9 <sup>*</sup>
36	0.70 $\pm$ 0.10 <sup>#</sup>

not due to the non-specific amplification. When the machine reads the base sequence of PCR products, it usually cannot clearly read the first 10 - 40 bases after the primers. The error bases were at the end of PCR products with the non-amplification for the gradually decreas-

ing capacity of the enzyme.

## DISCUSSION

The movement of specific populations of monocytes into the tissue is mediated by MCP-1, which is a recently described cytokine specifically chemotactic for monocytes, and which is also a potent activator of monocytes/macrophages, inducing a respiratory burst in monocytes/macrophages and the release of lysosomal enzymes. MCP-1 is expressed in macrophages-rich areas of human atherosclerotic lesions but not in normal blood vessels. The monocyte/macrophage plays an important role throughout all stages of atherogenesis, from the initial events (increased vascular permeability, increased monocyte adherence, and intimal recruitment) to the advanced lesions (monocyte-macrophage-derived foam cells, cell necrosis, and formation of a necrotic lipid core). MCP-1 may mediate its chemotactic effects on PBMC through specific high-affinity receptors on cell surface, including further intimal monocyte recruitment and the release of hydrolytic enzymes, ROS, and other mediators of tissue damage.

It has been suggested that AGEP is involved directly in the early development of atherosclerosis in diabetic patients as well as in non-diabetic disorders. The vascular cell alterations induced by AGEP or AGEP bearing blood cells may result in various vascular dysfunctions. We demonstrated previously that AGEP-BSA could enhance SMC proliferation associated with elevated cytosolic free calcium<sup>[9]</sup>. It is reported that MCP-1 is a mitogen for cultured rat vascular smooth muscle cells<sup>[10]</sup>. Expression of MCP-1 in vascular smooth muscle cells could result in SMC differentiation to synthetic phenotype in the process of atherogenesis<sup>[11]</sup>. Moreover, MCP-1 has an autoinductive effect on its own production by monocytes after being exposed to initial stimulation, and this process could be regulated by IL-10<sup>[12]</sup>. Therefore, our results that monocytes/macrophages expressing MCP-1 mRNA induced by AGEP are of great significance for pathogenesis of atherosclerosis in diabetic patients. Recently, demonstration that expression of MCP-3 mRNA in rat vascular SMC and in carotid artery after balloon angioplasty suggests a potential role of MCP-3 in the pathogenesis of restenosis and atherosclerosis<sup>[13]</sup>. It is reasonable to suggest that specific, early interventions directed to MCP-1 expression and inhibition of glycosylation might be a promising new approach to atherosclerotic disorders.

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高级糖基化终产物对人单核细胞表达单核细胞趋化蛋白-1基因的影响

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**关键词** 高级糖基化终产物; 单核细胞; 单核细胞趋化蛋白-1; 逆转录聚合酶链反应; 动脉粥样硬化; 人类

**目的:** 探讨高级糖基化终产物(AGEP)对单核细胞趋化蛋白-1 (MCP-1) 基因表达的影响. **方法:** 以  $\beta$ -actin 为内标, 用反转录聚合酶链反应(RT-PCR)半定量检测 AGEP 对人外周血单核细胞 MCP-1 mRNA 表达的影响, 并对 RT-PCR 产物测序. **结果:** AGEP 能增加人血单核巨噬细胞 MCP-1 基因的表达, 在 0-50 mmol/L 葡萄糖修饰浓度范围内, AGEP 的作用呈明显的剂量依赖效应, 80 mmol/L 葡萄糖修饰的 AGEP 作用稍降低. AGEP 增加 MCP-1 的表达呈明显的时间效应. 刚分离出来的新鲜单核细胞并不表达 MCP-1 mRNA, 12 h 已能检测到 MCP-1 的 mRNA, 24 h 达高峰, 36 h MCP-1 的 mRNA 几乎检测不到. **结论:** AGEP 能增加人血单核巨噬细胞 MCP-1 基因的表达.

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