Paper

Changes of gene expression profiles in macrophages stimulated by angiotensin II – Angiotensin II induces MCP-2 through AT_1-receptor

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Abstract

Introduction. **Macrophages play critical roles in the development of atherosclerosis and diabetic nephropathy as well as many inflammatory diseases. Angiotensin II type 1 receptor antagonists (AIIA) are beneficial for the prevention of atherosclerosis and diabetic nephropathy suggesting that angiotensin II (Ang II) promotes the development of these diseases. It has recently been reported that Ang II exerts proinflammatory actions *in vivo* and *in vitro*. This study was aimed to clarify the direct effects of Ang II on monocytes/macrophages.**

Materials and methods. **PMA-treated THP-1 cells, a human monocytic leukaemia cell line, were treated with Ang II (10^{-6} mol/L) for 24 hours with or without AIIA (CV11974). We evaluated gene expression profiles of these cells using DNA microarray system and quantified them by real-time RT-PCR.**

Results. **DNA microarray revealed that in total 19 genes, including monocyte chemoattractant protein (MCP)-2, were up-regulated by Ang II and down-regulated by AIIA. Real-time RT-PCR showed that up-regulation of MCP-2 with Ang II is blocked by the AIIA (CV11974) but not by an AT_2-receptor antagonist.**

Conclusions. **These results suggest that Ang II directly stimulates MCP-2 expression through AT_1-receptors in activated macrophages. Ang II may contribute to the persistence or amplification of microinflammation in vessel walls, heart and kidney. Vasculoprotective or renoprotective effects of AIIA might partly depend on direct anti-inflammatory effects on macrophages.**

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Introduction

It is well known that macrophages contribute to the formation and development of atherosclerotic lesions.[1] We have reported that low grade inflammation is also involved in the pathogenesis of diabetic nephropathy in a series of studies in human diabetic patients and animal models of diabetic nephropathy.[2-5] On the other hand, angiotensin II (Ang II) plays important roles in the pathogenesis of atherosclerosis[6] and chronic kidney diseases including diabetic nephropathy. Ang II acts on endothelial cells, vascular smooth muscle cells and immune cells resulting in acceleration of atherosclerosis at least partly through low grade inflammation.[7] Ang II stimulates vascular smooth muscle cells to produce monocyte chemoattractant protein (MCP)-1, one of the major chemokines for macrophage infiltration.[8] Macrophages, which accumulate in atherosclerotic lesions or cardiac tissues, also produce angiotensin-converting enzyme (ACE), renin and Ang II.[9,10]

Concerning the direct effect of Ang II on macrophages, it is reported that the renin-angiotensin system, including angiotensin II type 1 (AT_1) and type 2 (AT_2) receptors, is activated during differentiation of monocytes to macrophages.[11] Yanagitani *et al.*[12] also reported that Ang II directly stimulated up-regulation of peroxide production in macrophages through AT_1-receptors. On the other hand, Kato *et al.*[13] reported that treatment with an ACE inhibitor (ACE-I) and an angiotensin II type 1 receptor blocker (ARB) down-regulated MCP-1 and transforming growth factor (TGF)-β, expression in the kidney, resulting in amelioration of renal damage in experimental diabetic rats. Mezzano *et al.*[14] also reported that activation of the renin-angiotensin system including locally-generated Ang II promoted interstitial inflammation in diabetic nephropathy through up-regulation of MCP-1 and Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES). This evidence suggests that the renin-angiotensin system and macrophages synergistically promote inflammation in atherosclerotic lesions and diabetic kidney.

In the present study, intended to clarify the direct effects of Ang II on macrophages, we evaluated the expression profiles of proinflammatory genes in cultured human monocytes/macrophages after stimulation with Ang II using DNA microarray followed by quantitative real-time reverse transcription (RT)-PCR.

Material and methods

Materials

THP-1 cells, a human monocytic leukaemia cell line, were obtained from Dainippon Co. (Osaka, Japan). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen Corp

(Carlsbad, CA USA). Phorbol 12-myristate, 13-acetate (PMA) and Ang II were purchased from Sigma. CV 11974, an Ang II type 1 (AT_1) receptor antagonist (AIIA), was donated by Takeda Chemical Industries, Ltd. PD123319, an AT_2-receptor antagonist, was purchased from Sigma.

Cell culture

THP-1 cells were cultured in RPMI 1640 medium, containing 4% FBS, 100 U/ml penicillin G and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cells were centrifuged, washed in RPMI 1640, centrifuged, and resuspended in RPMI 1640 medium containing 0.5% FBS. The cells, adjusted to the cell density of 10^{-6} cells/ml in the culture medium, were placed in a 75 cm^2 flask with a filter cap. To differentiate monocytic cells to macrophages, 50 ng/ml PMA was added for 24 hours. After PMA stimulation, non-adherent cells and PMA were washed off 3 times with phosphate-buffered saline without calcium and magnesium [PBS (-)]. Ang II (10^{-6} mol/L) and corresponding vehicle were added six times hourly, after a wash with PBS (-). CV11974 (10^{-6} mol/L) and PD12331 (10^{-6} mol/L) were also added at the same time as Ang II. In flasks without Ang II stimulation, vehicle was exchanged in the same manner as those with Ang II stimulation. Total RNA was isolated from THP-1 cells 24 hours after initial stimulation with Ang II.

Isolation of RNA and cDNA synthesis

Total RNA was extracted from THP-1 cells using RNeasy Midi kit according to the manufacturer's instructions (QIAGEN, Valencia, CA, USA). Single-strand complementary DNA (cDNA) was synthesised from 1.5 µg of the extracted RNA using a reverse transcription (RT)-PCR kit (Perkin Elmer, Foster City, CA, USA) according to the manufacturer's instructions.

Expression of AT_1- and AT_2-receptors in THP-1 cells

For amplification of the cDNA, the following oligonucleotide primers were used: AT_1-receptor, sense 5´-GATGATTGTCCCAAAGCTGG -3´, antisense 5´-TAGGTAATTGCCAAAGGGCC-3´; AT_2-receptor, sense 5´-TTCCCTTCCATGTTCTGACC-3´, antisense 5´-AAACACACTGCGGAGCTTCT-3´. The PCR reactions were carried out in a GeneAmp 97OO machine (Applied Biosystems) and 45 cycles of denaturation (94°C for 30 s), annealing (56°C for 1 minute) and extension (72°C for 1 minute) were performed. Amplified PCR products were run on an ethidium bromide-stained 1% agarose gel. The relative intensity of the autoradiogram was determined using Scion Image analysis software to evaluate semi-quantitatively by densitometry. The messenger ribonucleic acid (mRNA) level of AT_1- and AT_2-receptors was normalised in relation to a house-keeping gene (human GAPDH mRNA) in each sample calculating the relative expression ratio. Four samples were used in each group.

Gene expression analysis by DNA microarrays

The 9,589 gene expression profiles in PMA-treated THP-1 cells were examined using UniSet Human I Bioarray chip (CodeLink™ system, Amersham Bioscience, USA) as described previously.[15] The CodeLink™ platform consists of a glass slide. In brief, double-strand cDNA was synthesised from 5 µg of total RNA using T7-$(dT)_{24}$ oligonucleotide primer. From the double-stranded cDNA complementary RNA (cRNA) was synthesised by an *in vitro* transcription reaction, together with biotin-labelled ribonucleotide. Then, the biotin-labelled cRNA was denatured and hybridised to slides of CodeLink array for 18 hours at 37°C. After hybridisation, slides were washed in TNT buffer at 42°C for 60 minutes and fluorescence-labelled nucleotide was added to generate storeptavidin-Cy5-labelled RNA. Slides were scanned using CodeLink™ Expression Scanning Software (Amersham Bioscience), and images for each slide were analysed using CodeLink™ Expression Analysis Software (Amersham Bioscience). All oligonucleotide probes are 30 bases long.

Gene expression profiles in cultured macrophages were compared between control, Ang II stimulation and treatment with AIIA.

Quantitative analysis by real-time RT-PCR

To evaluate gene expression of cytokines and chemokines in PMA-treated THP-1 cells stimulated by AII, quantitative real-time RT-PCR was performed using a Light Cycler and LightCycler-FastStart SYBR Green 1 (Roche Diagnostics, Tokyo, Japan). After the addition of primers (final concentration: 0.3 µM), $MgCl_2$ (4 mM) and template DNA to the master mix, the following real-time RT-PCR protocol was performed. For monocyte chemoattractant protein (MCP)-2 and tissue growth factor (TGF)-β3, 50 cycles of denaturation (95°C for 10 seconds), annealing (62°C for 10 seconds) and extension (72°C for 7 seconds) were performed, and for MCP1, TGF-β1 and 50 cycles of denaturation (95°C for 10 seconds), annealing (68ºC for 10 seconds) and extension (72°C for 16 seconds) were done. To determine the specificity of each primer set, melting curve analysis was performed after the completion of PCR amplification.

Accumulated levels of fluorescence were analysed by a fit-point method after the melting curve analysis. The mRNA level of cytokines and chemokines was normalised to a house-keeping gene (human GAPDH mRNA) in each sample calculating the relative expression ratio. For amplification of the cDNA, the following oligonucleotide primers were used: MCP-2, sense

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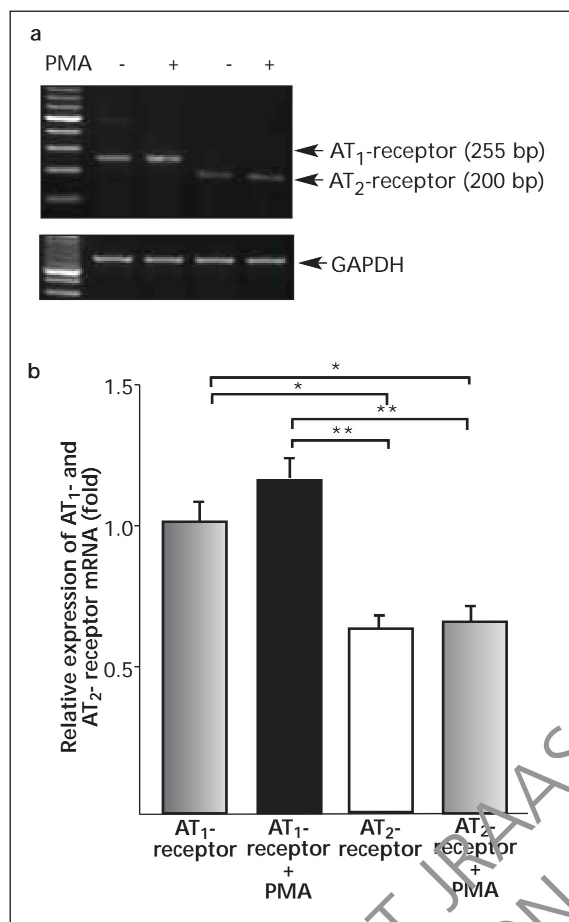

Figure 1
Gene expression of AT_1- and AT_2- receptors in THP-1 cells; **a:** and semi-quantitative evaluation normalised to a house-keeping gene; **b:** Values are expressed as mean±SEM. *p<0.05, **p<0.005.

5´-CTGGAGAGCTACACAAGAA-3´, antisense 5´-TCTGACTCTCAGTCCATGT-3´; TGF-β3, sense 5´-CCATGAACCTAAGGGCTACT-3´, antisense 5´-GGTCCTCCCAACATAGTACA-3´; GAPDH, sense 5´-TGAACGGGAAGCTCACTGG-3´, antisense 5´-TCCACCACCCTGTTGCTGTA-3´. The primers for MCP-1, TGF-β3 and RANTES were purchased from Roche Diagnostics (Tokyo, Japan). Three samples were used in each group and each experiment was performed twice.

Statistical analysis

All data are expressed as the mean±SEM. Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Scheffe's test. A p value less than 0.05 was considered to denote the presence of a statistically significant difference.

Results

Gene expression of AT_1-/AT_2-receptors in THP-1 cells

RT-PCR was performed to confirm mRNA expression of AT_1-/AT_2-receptors in monocytes/ macrophages. Expression of AT_1-/AT_2-receptors in THP-1 cells with or without PMA stimulation was shown in figure 1a.

RT-PCR was also performed semi-quantitatively by densitometry. Both AT_1-/AT_2-receptors expressed in THP-1 cells with or without PMA stimulation and AT_1-receptors were more strongly expressed than AT_2-receptors. Expression of AT_1-/AT_2-receptors seemed to be up-regulated through differentiation to macrophages by PMA stimulation, though differences were not statistically significant (figure 1b).

Comparison of gene expression profiles in a macrophage stimulated by Ang II

Genes up-regulated (1.5-fold and more) by Ang II stimulation compared with control and down-regulated (0.67-fold and less) by AIIA compared with Ang II were listed in table 1. In total, 19 genes were listed. In particular, MCP-2, an important chemokine, was up-regulated by Ang II (1.65-fold) and significantly down-regulated by AIIA (13.80-fold). TGF-β3, a cytokine, was also up-regulated by Ang II (1.76-fold) and down-regulated by AIIA (2.68-fold).

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Quantitative gene expression analysis of chemokines and cytokines

We focused on chemokines and cytokines including MCP-2 and TGF-β3, from the results of the DNA arrays, so quantitative real-time RT-PCR was performed to quantify the gene expression level of chemokines and cytokines in cultured macrophages. Expression of mRNA was normalised to levels of GAPDH mRNA in each sample calculating the relative expression ratio. Ang II stimulation up-regulated MCP-2 mRNA expression by 1.8-fold compared with control (p<0.01). AIIA reduced the increase of MCP-2 expression by 24.9% (p<0.05). The expression of MCP-2 mRNA was remarkably up-regulated by the treatment with AT_2-receptor antagonist (p<0.005 *vs.* control and Ang II+AIIA, n.s. *vs.* AII) (figure 2b). Although MCP-1 mRNA expression was increased by AIIA stimulation and decreased by AIIA, there was no statistical significance (figure 2a). There was no significant difference between four groups in TGF-β1, TGF-β3 and RANTES (figure 2 c,d, and e).

Melting curve analysis confirmed the specificity of the PCR products (data not shown). Values are expressed as mean±SEM.

Discussion

We found the 19 genes were up-regulated by Ang II and down-regulated by AIIA in cultured macrophages using DNA microarray studies. Quantitative analysis with real-time RT-PCR showed that mRNA expression of MCP-2 was significantly increased by Ang II stimulation and markedly reduced by AIIA. We found similar changes of the expression of MCP-1 without significant difference.