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Angiotensin II up-regulates CX3CR1 expression in THP-1 monocytes: impact on vascular inflammation and atherogenesis

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Abstract The potential regulatory effect of angiotensins on circulating mononuclear cell activation and migration has not yet been thoroughly evaluated. Using flow cytometry we assessed the possible effect of angiotensin I and II on the expression of CX3CR1 and a single representative of each major chemokine family (CCR5 and CXCR4) in THP-1 monocytes, Jurcat T lymphocytes and primary monocytes—isolated from healthy donors. Fluorescence intensity and the rate of chemokine-positive cells was measured in naïve cells and cells treated with angiotensin I and II. Neither angiotensin I nor angiotensin II exhibited any effect on fluorescence intensity and the rate of CX3CR1-, CCR5- and CXCR4-positive cells in primary peripheral blood mononuclear cells and Jurkat T cells. However, angiotensin II significantly increased the rate of CX3CR1-positive THP-1 cells. This effect was not attenuated by the pre-incubation of THP-1 cells with the AT-1 receptor blocker losartan, suggesting that this was not an AT-1-mediated effect. Angiotensin I and II had no effect on fluorescence intensity and the rate of CCR5- and CXCR4-positive THP-1 cells. In conclusion, angiotensin II increases the rate of CX3CR1-positive THP-1 cells. By extrapolating this in vitro observation to disease mechanisms, we speculate that angiotensin II induces up-regulation of CX3CR1 and promotes firm adhesion of circulation CX3CR1-positive monocytes on CX3CL1 expressing endothelial cells inducing vascular inflammation and atherogenesis.

Keywords Chemokine receptors · CX3CR1 · Angiotensin II · Monocytes

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Introduction

Atherosclerosis is a chronic inflammatory process [1]. It involves interaction of circulating inflammatory cells, with cellular components of the vascular wall. Several chemokine ligands and receptors have been extensively investigated and have been shown to be involved in a more disease-specific way in atherosclerosis [2–4].

As a result of impaired endothelial function, activation of the renin-angiotensin system plays an important role in the initiation and progression of atherosclerosis [5]. Renin is a proteolytic enzyme produced and stored in the granules of the juxtaglomerular cells in the kidney. Renin acts on a circulating A2 globulin, angiotensinogen, to form the decapeptide angiotensin I (Ang I). Angiotensin I is transformed by the angiotensin-converting enzyme (ACE) to angiotensin II (Ang II) [5]. Angiotensin II, the most active component of the renin–angiotensin system, has significant pre-inflammatory functions in the vascular wall, including production of reactive oxygen species, inflammatory cytokines and adhesion molecules [6–12]. However, the potential regulatory effect of angiotensins on circulating monocyte activation and migration has yet to be thoroughly assessed. We previously demonstrated that interferon-γ, a pro-inflammatory cytokine and a critical mediator of

atherosclerosis, enhances the rate and fluorescence intensity of CX3CR1 + THP-1 cells but not that of primary monocytes [7]. We propose that the pre-inflammatory properties of angiotensins and particularly Ang II are not limited to vascular endothelium but are further expanded in circulating mononuclear cells. To support our hypothesis, the expression of CX3CR1 along with that of a representative receptor of each significant chemokine family (CCR5 and CXCR4) was assessed by flow cytometry in human monocytes before and after treatment with Ang I and II.

Materials and methods

Cell cultures

THP-1 is a myelomonocytic cell line. THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% decomplemented FBS and 2 mM glutamine, 25 mM HEPES, penicillin (50 U/ml) and streptomycin (50 U/ml). Jurkat is an HTLV-1-negative acute T-cell leukaemia human cell line. Jurkat T lymphocytes were cultured in RPMI-1640 supplemented with 10% FBS, 4 mM glutamine, penicillin (50 U/ml) and streptomycin (50 U/ml). The two cell lines were cultured at a density of up to 500,000 cells/ml, at 37°C in a humidified 50 ml/l CO2 atmosphere. Using density gradient centrifugation, human peripheral blood mononuclear cells were isolated from buffy-coats drawn from three volunteers, with no prior history of atherosclerotic complication and no cardiovascular risk factors. The chemokine receptor phenotype of the monocyte subpopulation was assessed by re-evaluating the mean fluorescence intensity (MFI) and rate of chemokine receptor-positive cells in the monocyte gates of flow cytometer density plots.

Cells were pre-treated with Ang I and Ang II (Sigma–Aldrich, St. Louis, USA). Three time points of 0, 24 and 48 h, and three concentrations of 0.1, 1 and 10 μM were initially evaluated for the two substances.

Flow cytometry

The expression of chemokine receptors CCR5 and CXCR4 was evaluated by flow cytometry using anti-CCR5 and anti-CXCR4 phycoerythrin (PE) conjugated antibodies (BD Bioscience, New Jersey, USA). The surface expression of CX3CR1 was evaluated by initially staining cells with anti-CX3CR1 rabbit antihuman IgG monoclonal antibody (e-Bioscience, California, USA) and further subjecting them to incubation with mouse anti-rabbit IgG fluoresceine isothiocyanate (FITC) or PE-conjugated secondary antibody (Santa Cruz Biotechnology, California, USA) before analysis on a FACScan flow cytometer (Becton–Dickinson, New Jersey, USA). In all cases, the intra-assay coefficient of variation (CV) was less than 5% while the inter-assay CV was less than 10%.

Statistical analysis

Rates of chemokine receptor-positive cells and the mean fluorescence intensity of positively stained cells are presented as mean values ± standard error of the mean (SEM). The paired sample *t*-test was applied to evaluate the differences between the means. *P* values less than 0.05 were considered statistically significant.

Results

The surface expression of CCR5, CXCR1 and CX3CR1 was measured on THP-1 monocytes, primary monocytes and Jurkat-T lymphocytes and presented as arbitrary units of MFI and mean rates of positive cells. Values obtained from naïve cells and cells treated with Ang I and Ang II are shown in Tables 1,2 and 3.

Baseline phenotype of chemokine receptors

In accordance with previous observations, CX3CR1 is highly expressed in THP-1 monocytes (Fig. 1). The mean

Table 1 Chemokine receptor expression of THP-1 monocytes

	Cells		Angiotensin I*		Angiotensin II*	
	Baseline MFI	Baseline % positive cells	Mean difference of MFI	Mean difference of % positive cells	Mean difference of MFI	Mean difference of % positive cells
CCR5	110.3 ± 18	7.9 ± 1.6	17 ± 7	1.2 ± 0.8	−12 ± 3	−20.8 ± 6
CX3CR1	213.5 ± 25.1	47 ± 4.1**	11 ± 3.5	20.8 ± 2.8	48.7 ± 62.1	40.7 ± 9.2**
CXCR4	125 ± 4.2	93 ± 5.7	−3.3 ± 9.5	1.3 ± 2	1.7 ± 1.5	−2.9 ± 8.3

MFI mean fluorescence intensity

* Results refer to 24-h treatment with either 1 μM of angiotensin I or 1 μM angiotensin II. ** p value for paired sample *t* test = 0.048

Table 2 Chemokine receptor expression of primary monocytes

	Cells		Angiotensin I*		Angiotensin II*	
	Baseline MFI	Baseline % positive cells	Mean difference of MFI	Mean difference of % positive cells	Mean difference of MFI	Mean difference of % positive cells
CCR5	31 ± 8.1	2.3 ± 0.9	1.7 ± 0.4	−0.1 ± 0.15	3.3 ± 2.7	0.3 ± 0.4
CX3CR1	40.2 ± 24.4	6.3 ± 3.3	−4.8 ± 1.9	−2.3 ± 1.9	−8 ± 6.7	−1.9 ± 1.2
CXCR4	75.6 ± 5	22 ± 5	1 ± 3.5	−0.7 ± 1.6	4.6 ± 5.3	3.2 ± 6

MFI mean fluorescence intensity

* Results refer to 24-h treatment with either 1 μM of angiotensin I or 1 μM angiotensin II

Table 3 Chemokine receptor expression of Jurkat cells

	Cells		Angiotensin I*		Angiotensin II*	
	Baseline MFI	Baseline % positive cells	Mean difference of MFI	Mean difference of % positive cells	Mean difference of MFI	Mean difference of % positive cells
CCR5	23.2 ± 5.6	9.2 ± 2.9	1.5 ± 2.9	−0.2 ± 1	−2.6 ± 2.6	−2.1 ± 2.7
CX3CR1	47.2 ± 4.2	13.9 ± 7.2	9.7 ± 3.2	2.2 ± 1.8	6.2 ± 3.3	4.71 ± 1.3
CXCR4	1039 ± 40.3	95.2 ± 1.2	61 ± 32.7	0.8 ± 2.4	63 ± 100	−0.1 ± 0.3

MFI mean fluorescence intensity

* Results refer to 24-h treatment with either 1 μM of angiotensin I or 1 μM angiotensin II

rate and MFI of CX3CR1-positive THP-1 cells were 47 ± 4.1 and 213.5 ± 25.1, respectively. Primary monocytes gated from PBMCs isolated from healthy donors expressed less CX3CR1; the MFI and mean rate of CX3CR1-positive cells were 40.2 ± 24.4 and 6.3 ± 3.3, respectively. CX3CR1 was scarcely detected in the T-lymphocytic Jurkat-cells (Table 3). Similarly, CCR5 was barely detected in the three types of cells in the naïve state, while a higher intensity was detected in THP-1 monocytes (7.9 ± 1.6). Finally, CXCR4 was highly expressed in the two monocytic populations but predominately detected in Jurkat T-lymphocytes.

Effect of angiotensins on chemokine receptor phenotype

Treatment with Ang I and II at the given concentrations and time points did not alter the chemokine receptor phenotype of primary monocytes and Jurkat T cells (Tables 2 and 3). This was reflected in MFI and the rate of positive cells. Despite an impressive tendency Ang I altered neither MFI nor the number of THP-1 positive cells significantly (Fig. 2). However, Ang II altered the rate of CX3CR1-positive THP-1 cells producing a borderline statistical significance (47 ± 4.1 vs. 87.7 ± 5.9, $P = 0.048$). Maximum effect was observed 24 h after treatment with 1 μM of Ang II (Fig. 3), but was neither dose- nor time-dependent. The effect of Ang II on the CX3CR1 receptor phenotype was not attenuated by the pre-treatment of THP-1 cells with the AT-1 receptor blocker (ARB) losartan (Fig. 4), suggesting a non-AT 1-mediated pathway.

Discussion

Monocytes are pivotal mediators in the process of vascular inflammation [13]. Peripheral blood monocytes respond to stressed endothelium and migrate in the intima of the injured vessel. Pre-inflammatory circulating cytokines promote the activation and migration of monocytes by increasing the expression of chemokines and adhesion molecules [1, 2 13]. Angiotensin II exerts pre-inflammatory properties acting on vascular endothelial cells, vascular smooth muscle cells and circulating monocytes [6–12, 14, 15]. Previous studies demonstrated that Ang II up-regulates peroxide production and the expression of chemokines, such as MCP-1, MCP-2 and RANTES, in monocytes/macrophages predominantly through AT1 receptor activity [16–18].

In order to assess a potential effect of angiotensins on monocyte chemokine-receptor phenotypes, we evaluated the surface expression of CX3CR1 along with a single representative of the major chemokine family using flow cytometry. CX3CR1 interaction with its ligand, CX3CL1 (or fractalkine), is a recently discovered pathway strongly implicated in monocyte migration and accumulation during atherogenesis. Fractalkine acts as a chemotactic cytokine and as an adhesion molecule. Although CX3CR1 is our

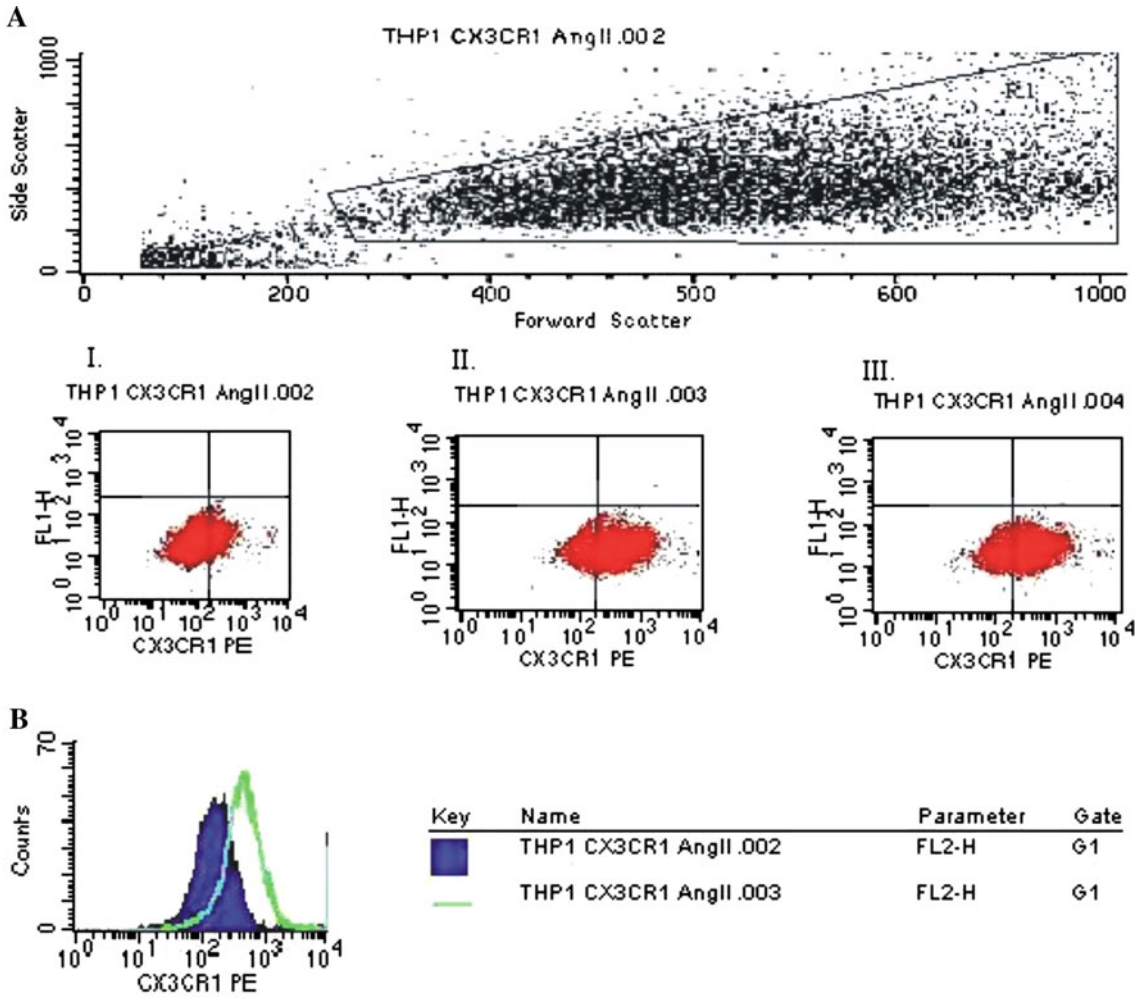

Fig. 1 Representative *dot* plots (**a**) and histogram plot (**b**) presenting effects of angiotensin II on rate and mean fluorescence intensity of CX3CR1-positive THP-1 cells. Rate of CX3CR1 *Positive* cells are presented in naive cells (I), cells treated with 1 μM of angiotensin II (II) and 10 μM of angiotensin II (III). The change of fluorescence intensity of CX3CR1 *Positive* cells after treatment with 1 μM of angiotensin II is presented in panel B. Despite a detectable increase of mean fluorescence intensity the mean difference observed in three experiments did not the exceeded the cut-off point of statistical significance

main field of interest, we deemed it necessary to evaluate the expression of two more chemokine receptors in order to distinguish a possible angiotensin-induced chemokine-specific mechanism from a non-specific up- or down-regulating effect. We demonstrated a direct effect of Ang II on chemokine receptor phenotypes of THP-1 monocytes regarding CX3CR1. We further showed that since the blocking of AT-1 receptors of THP-1 cells did not attenuate the phenomenon this direct effect of Ang II is non–AT 1-mediated effect. The latter hypothesis is also supported by the fact that Ang I -an oligopeptide without known biological activity on AT-1 receptor- partially reproduced the up-regulation of CX3CR1, without, however, exceeding the cut-off point of statistical significance.

Ang II acts as an endocrine, autocrine/paracrine, intracrine hormone, as well as a pro-inflammatory molecule. There is ample evidence in the literature implicating Ang II in the biochemical pathways leading to almost every aspect of cardiovascular disease especially atherosclerosis and heart failure [8–12].

Predisposing factors of cardiovascular disease have also been associated with increased RAS activity. Hypertension has been described as both a cause and a consequence of RAS activation [19, 20]. Similarly, higher levels of rennin, Ang II and aldosterone have been demonstrated in obese, dyslipidemic patients and patients with metabolic syndrome [21, 22], while increased ACE activity has been noted in smokers [23]. Thus, it appears that the activation of RAS is part of the biochemical mechanisms that are triggered by predisposing factors, leading to endothelial injury and overt cardiovascular disease. Activation of FKN/CX3CR1 appears to be part of the same pathways by taking circulating monocytes to a higher level of activation and provoking monocyte adhesion to dysfunctional

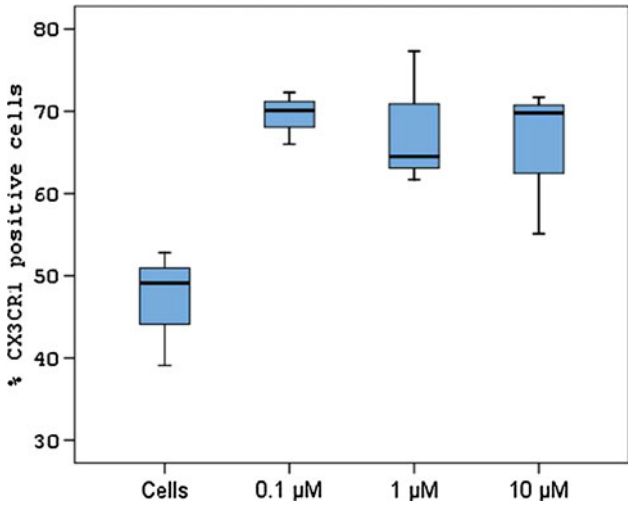

Fig. 2 Rate of CX3CR1-positive THP-1 cells after 24-h treatment with various concentrations of angiotensin I

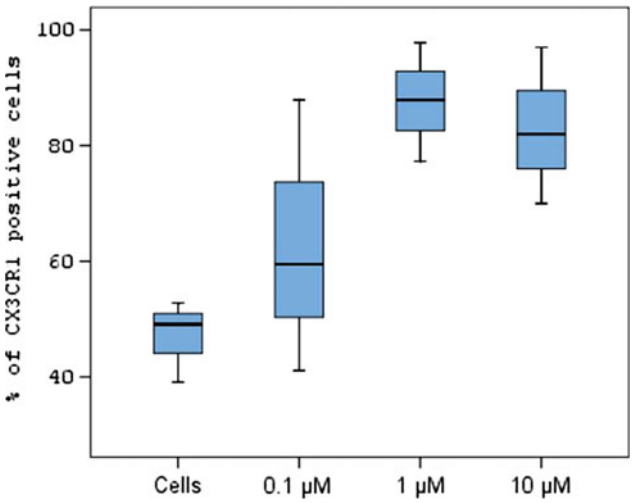

Fig. 3 Rate of CX3CR1-positive THP-1 cells after 24-h treatment with various concentrations of angiotensin II. The strongest statistical significance was obtained for treatment with 1 µM of angiotensin II

endothelium. Our results indicate a possible interaction between RAS- and FKN/CX3CR1-mediated pathways; however, such an association needs to be further confirmed in vivo.

The THP-1 cell line is a well-established model in the study of monocyte behavior since it shares many common characteristics with the normal human monocytes including morphology, and the expression of plasma membrane receptors and cytokines [24]. However, in order to avoid bias related to the particular phenotype of THP-1 cells, we further evaluated our hypothesis in primary monocytes of healthy donors. However, our observation in THP-1 cells was not reproduced in the primary monocytes. No significant effect of Ang I or II was observed in any expression of the tested chemokine receptors in monocytes isolated from

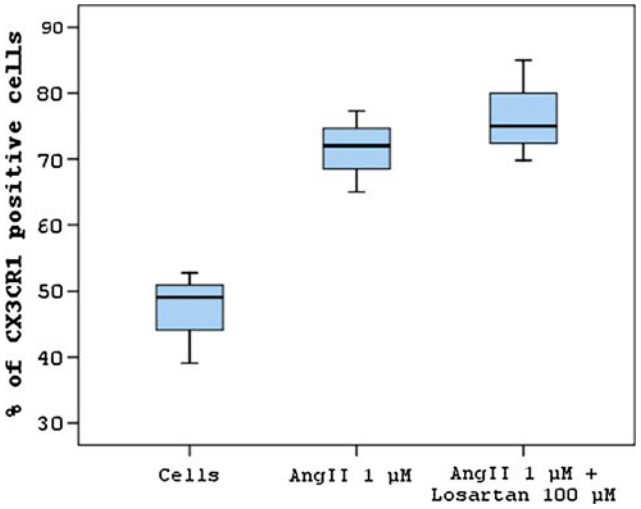

Fig. 4 Rate of CX3CR1-positive THP-1 cells after 24-h treatment with 1 µM of Angiotensin II and 1 µM of angiotensin II plus 100 µM losartan

healthy donors. The latter inconsistency was attributed to many confounding factors related to the isolation of PBMCs or to cell–cell interaction due to the impurity of the cellular population during treatment with angiotensins. Similar inconsistencies were observed in a previous work of ours when treatment with interferon-γ increased the rate of CX3CR1-positive THP-1 cells but not primary monocytes [7].

Finally, we evaluated the effect of Ang I and II on chemokine receptor phenotypes of Jurkat cells. Jurkat cells are an immortalized line of T lymphocytes and have been shown to be an excellent model for the study of T-cell signaling [25, 26]. Under certain conditions, T cells strongly express the three tested chemokine receptors. AT-1 and AT-2 receptor expression has also been demonstrated in T-cells and natural killer (NK) cells. However, no effect of angiotensins was detected on the expression of CCR5, CXCR4 or CX3CR1 by Jurkat cells [27].

In conclusion, it appears that Ang II enhances chemokine receptor expression by THP-1 monocytes. However, the phenomenon could not be reproduced in primary monocytes isolated from healthy donors. We believe that this is due to technical limitations of in vitro systems with mixed cells. In order to be categorical regarding the impact of angiotensins on the chemokine receptor profile of human monocytes, an in vivo experimental evaluation should be conducted.

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