International Journal of Multicultural and Multireligious Understanding

http://ijmmu.com
editor@ijmmu.com
ISSN 2364-5369
Volume 8, Issue 6
June, 2021
Pages: 205-211

The Specific Role of CCL8 Chemokine in Atherosclerosis

Yulduz Yusupbaevna Baltaeva[1]; Moxigul Turdialievna Yuldasheva[2]; Matlyuba Satvaldievna Palvanova[3]; Mansurjon Safaralievich Hoshimov[4]

[1] Research Assistant at the Department of Medical Biology and Chemistry, Fergana Medical Institute of Public Health, Fergana, Uzbekistan

[2] PhD, Fergana Medical Institute of Public Health, Fergana, Uzbekistan

[3] Candidate of Medical Sciences, Fergana Medical Institute of Public Health, Fergana, Uzbekistan

[4] Scientific Researcher, Fergana Medical Institute of Public Health, Fergana, Uzbekistan

http://dx.doi.org/10.18415/ijmmu.v8i6.2779

Abstract

Atherosclerosis is a chronic inflammatory disease that represents the primary cause of heart disease and stroke. C-C motif chemokine ligand 8 (CCL8) has been found in many diseases' pathogenesis. Nevertheless, its molecular mechanism in atherosclerosis (AS) remains to be elucidated. Human microvascular endothelial cells (HMEcs) were stimulated by IFNα, IFNγand LPS, to establish experimental atherosclerosis. Recruitment of blood leukocytes to the injured vascular endothelium characterizes the initiation and progression of atherosclerosis and involves many inflammatory mediators, modulated by cells of both innate and adaptive immunity. The pro-inflammatory cytokine, interferon (IFN)-γ derived from T cells, is vital for both innate and adaptive immunity and is also expressed at high levels in atherosclerotic lesions. As such, IFN-γ plays a crucial role in the pathology of atherosclerosis through activation of signal transducer and activator of transcription (STAT)1. Our study indeed provides evidence that in HMECs STAT1 coordinates a platform for cross-talk between IFNγ and TLR4, and identifies a STAT1-dependent gene signature that reflects a pro-atherogenic state in coronary artery disease (CAD) and carotid atherosclerosis. Taken together, our data indicate that in the presence of appropriate stimuli, HMECs are highly responsive and consistently express CCL8. However, upregulation of CCL8 led to suggest CCL8 could be an atherosclerosis therapeutic target. HMECs may therefore provide a better model for *in vitro* studies of atherosclerosis.

Keywords: *Atherosclerosis; Chemokines; Vulnerable Plaque; Biomarkers; CCL8; IFNγ; LPS; HMECs*

Introduction

For almost a century, many have considered lipids as the *sine qua non* of atherosclerosis. However, atherosclerosis is currently viewed as an inflammatory disease in which the initiation and progression of the atherosclerotic plaque towards a rupture prone, unstable plaque is driven by leukocyte

recruitment mediated by various inflammatory mediators (Kraaijeveld: 2007; Hans: 2012). Over the last few decades, a plausible model linking lipids and inflammation to atherogenesis has emerged, and finally, scientific proof for this was discovered only 30 years ago. This admission has had a significant impact on our understanding of atherosclerosis as more than a disease of lipid accumulation, but rather as a low-grade vascular inflammation disorder. Atherosclerosis, as well as arteriosclerosis are a type of vascular disease, characterized by endothelial dysfunction and contractility alteration of vascular smooth muscle cells (Hans: 2012). Despite the fact that, it was initially believed as a bland lipid storage ailment, substantial advances in basic and experimental science have illuminated the role of inflammation and the underlying cellular and molecular mechanisms that contribute to the atherogenesis (Libby: 2002). Various factors can injure the vascular endothelium, which leads to the release of numerous inflammatory mediators resulting the recruitment, activation and adhesion of various types of leukocytes to inflammatory foci. Thus, cells of both innate and adaptive immunity modulate the chronic inflammatory process initiating and acting in the atherosclerotic plaque development (Hansson: 2006).

The best human data relating inflammation to the prospective development of vascular diseases have come from large-scale, population based studies. To date, upraised levels of several inflammatory mediators among apparently healthy men and women have proven to have predictive value for future vascular events. In particular, prospective epidemiological studies have found increased vascular risk in association with increased basal levels of cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α (Ridker: 2000); cell adhesion molecules such as soluble intercellular adhesion molecule (ICAM)-1, P selectin, and E selectin, downstream acute-phase reactants such as C-reactive protein (CRP), fibrinogen, serum amyloid (Haverkate: 1997), interferons (IFNs) (Borne: 2014). Several traditional cardiovascular risk factors track with these inflammatory biomarkers. These conducted researches have significant importance because inflammatory cytokines can be produced by a wide variety of cell types, and a common underlying disorder of innate immunity (Hans: 2018). In support of this hypothesis, our very recent observations have shown, that elevated levels of Homo sapiens C-C motif chemokine ligand 8 (CCL8), also known as HC14, associate with subsequent development of atherosclerosis.

Chemokines are a large family of small cytokines with a molecular weight between 7 and 15 kDa, soluble and are involved in a wide variety of processes during physiological and pathological conditions (Taub: 1996). These pro-inflammatory mediators and their receptors play crucial roles in recruitment, activation and adhesion of various types of leukocytes to inflammatory foci. Subgroups of chemokines that have been identified are C, CC, CX3C, and CXC, defined based on the configuration of a conserved amino-proximal cysteine-containing motif. With the exception of the C subgroup, all chemokines contain a common four cysteine residue motif linked by disulphide bonds in conserved positions, one between the first and third cysteines and one between the second and fourth cysteines, to form triple stranded b-sheet structures (Fernandez: 2002). As a general rule, C chemokines mainly recruit lymphocytes, while CC chemokines recruit monocytes.

The CC (β) subfamily of chemokines is a group of chemotactic cytokines known as CC motif chemokine ligands (CCL)1–28. Their shared characteristic is the N-terminal CC domain and digits in their symbols depend on the order of discovery (Zlotnik: 2000; Hughes: 2018). The actual number of CC chemokines is 27, as CCL9 and CCL10 denote the same chemokine. All these chemokines are ligands for 10 receptors—CC motif chemokine receptors (CCR)1–10. Just like the rest of chemokines, CC chemokines are crucial for the functioning of the immune system cells (Hughes: 2018). C-C motif chemokine ligand 8, also known as MCP-2 gene is one of several chemokine genes clustered on the q-arm of chromosome 17. Chemokines form a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes. The superfamily is divided into four subfamilies based on the arrangement of N-terminal cysteine residues of the mature peptide. This chemokine is a member of the CC subfamily which is characterized by two adjacent cysteine residues. This cytokine displays chemotactic activity for monocytes, lymphocytes, basophils and eosinophils. By recruiting leukocytes to sites of inflammation this

cytokine may contribute to tumor-associated leukocyte infiltration and to the antiviral state against HIV infection.C-C motif Chemokine ligand 8 (CCL8) has been found in many diseases' pathogenesis. But its molecular mechanism in atherosclerosis (AS) remains to be elucidated (Hughes: 2018; Struyf: 2009; Lin: 2014; Ridiandries: 2016).

CCL8 known as MCP-2. MCP-2 genes were isolated from a YAC contig from human chromosome 17q11.2. The amplified genomic MCP-2 fragment was used to isolate an MCP-2 cosmid from which the gene sequence was determined. The MCP-2 gene shares with the MCP-1 and MCP-3 genes a conserved intron–exon structure and a coding nucleotide sequence homology of 77%. (Luster: 1985; Shehadeh: 2009; Antonelli: 2008; Wang: 2013; Mohty: 2010). Number of cytokines may control the cellular expression of the CCL8 gene (Groom: 2011). Although, CCL8 is strongly induced by IFN-II, also known as IFN-γ(produced by T lymphocytes), type I interferons-IFN-α,β. Furthermore, recent studies have shown evidence to suggest that, also in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) signal transducer and activator of transcription 1 (STAT1) provides a platform for cross-talk between IFN γ and LPS, and leads to a significant phosphorylation of STAT1 as compared to both factors alone (Tedgui: 2006; Hans: 2011). Hence, phosphorylated STAT1 in VSMCs and ECs of human atheromatous plaques correlated with elevated gene and protein expression of the chemokines CXCL9 and CXCL10 (Hans: 2014). To study if a similar mechanism affected the expression of CCL8 in HMECs, the following scientific studies have been conducted.Taken together, the aim of this cohort study was to examine the gene expression of C-C motif chemokine ligand 8 (CCL8)in experimental atherosclerosis.

Materials and Methods

Cell Culture Experiments

1. Human Microvascular Endothelial Cells (HMEC 1) (Ades: 1992) were provided by the Center for Disease Control and Prevention (Atlanta, GA)

2. MCDB-131 medium (IITD PAN, Wroclaw, Poland) containing 10% of fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.01 µg/ml EGF, 0.05 µM hydrocortisone and 2 mM L-glutamine.

3. Serum starved-medium (containing 1% of FBS instead of 10%).

4. Recombinant IFNα and IFNγ were purchased from Merck, and LPS was provided by Sigma-Aldrich.

Microvascular Endothelial Cells were cultivated in MCDB-131 medium containing 10% FBS (PAA), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.01 µg/ml EGF, 0.05 µM hydrocortisone (Sigma), 2 mM L-glutamine (PAA) for 48 hours in 37C. 24 hours before the experiment, full medium was exchanged into medium containing 2% serum. After minimum 12 h-starvation, to detect the effect of inflammatory factors to the STAT1 dependent genes expression, HMECs were treated with murine IFNα and murine IFNγ alone for 8 hours and IFN treatment was followed separately by treatment with LPS for an additional 4 hours and at the end LPS alone for 4 hours to induce signal integration pathway between IFNs and TLRs.

RNA Isolation and Real-Time PCR

Total RNA was isolated from HMECs using GeneMATRIX Universal RNA Purification Kit (EUR x, Gdansk, Poland) according to the manufacture's protocol. Afterwards, isolated RNA was

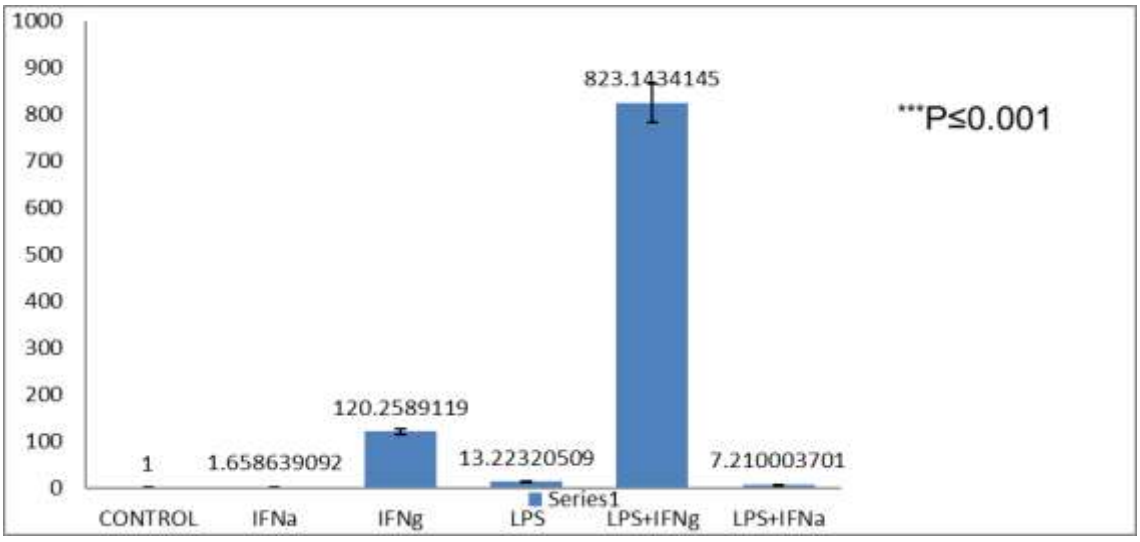
subjected to reverse transcription and PCR amplification was performed in Maxima SYBR Green/ROX qRT-PCR Master Mix (Thermo Fisher Scientific) on the Eco qRT-PCR System (Illumina).Perhaps, β-actin (ACTB) is the most widely used gene for normalization in the experiments of gene expression, and the expression of this gene is believed to remain stable across all experimental conditions, then relate the concentrations of gene(s) of interest to those of this housekeeping gene(26), thus the amount of target gene in each sample was normalized to β-actin gene endogenous control. The 2^{-ddCT} method was applied for quantitative data analysis.

Table 1 Primer sequences used in experimental procedure
Homo sapiens C-C motif chemokine ligand 8 (CCL8), mRNA

Gene Name	Forward	Reverse
CCL8	GTAGTGTTGTGGGGTCCTCC	TACAGGAGCACTGATTGCCA

Results

It is already known that, in ECs cross-talk between IFNγ and TLR4 leads to augmented phosphorylation of STAT1 and expression of the chemokine CCL8 (25).In HMECs, CCL8 gene showed a mild response to interferon treatment such as IFNα and IFNγalone, and the expression levels remarkably increased by the contribution of LPS compound, in comparison to untreated cells. Another important factor is STAT1 as a critical mediator of signal integration pathway. Genes, which are induced by the presence of STAT1 protein, showed high levels of expression with the treatment combination of IFNs and LPS together. STAT1 dependentCCL8 gene showed significant patterns in HMECs.



p<0.05 was considered as significant. Data were tested for significance by one-way ANOVA followed by post-hoc Dunnett's.

Discussion

Many studies have revealed that signal transducer and activator of transcription 1 (STAT1), That mediates cellular responses to interferons (IFNs), cytokine KITLG/SCF and other cytokines and other

growth factors, plays a significant role in cardiovascular disease. It is additionally accepted that in immune cells STAT1 is a unique point of convergence for the antimicrobial and inflammatory synergism between IFNγ and TLRs. Recently, it is shown that also in VSMCs cross-talk between IFNγ and LPS resulted in augmented STAT1 phosphorylation and increased expression of the chemokine CCL8 (Hans: 2014). Here, a similar STAT1-dependent mechanism for CCL8 expression in response to IFNγ and LPS was observed in HMECs at the RNA level. Although CCL8 have been extensively studied in *in vivo* and *in vitro* angiogenesis models, the expression of receptors for these chemokines on HMECs remains controversial. Moreover, we have studied the expression of CCL8 in human microvascular ECs with HUVECs to test the hypothesis that HMECs may be better indicators of the role of chemokines in somatic angiogenesis and to evaluate the prediction that differences in receptor expression are responsible for different functional abilities of various EC types.

Our study indeed provides evidence that in HMECs STAT1 coordinates a platform for cross-talk between IFNγ and TLR4, and identifies a STAT1-dependent gene signature that reflects a pro-atherogenic state in coronary artery disease (CAD) and carotid atherosclerosis. Taken together, our data indicate that in the presence of appropriate stimuli, HMECs are highly responsive and consistently express CCL8. HMECs may therefore provide a better model for *in vitro* studies of atherosclerosis.

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