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Molecular Genetic Analysis Of C-X-C Motif Chemokine Ligand 9 (CXCL9) As A Novel Biomarker In Atherosclerosis

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ABSTRACT

For almost a century, many have considered lipids as the sine qua non of atherosclerosis. However, in 1856 Rudolf Virchow introduced a theory that inflammation is the driving force of atherogenesis. 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 STAT1. Our study indeed provides evidence that in HMECs STAT1 coordinates a platform for cross-talk between IFN γ 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 Cxcl9. HMECs may therefore provide a better model for in vitro studies of atherosclerosis.

KEYWORDS

Atherosclerosis, biomarkers, chemokine, Cxcl9, IFN γ , LPS, HMECs

INTRODUCTION

According to the latest statistics published by the American Heart Association cardiovascular

disease (CVD) is the largest cause of death worldwide and accounts for over 750,000

deaths in the USA annually(1,2,3). Atherosclerosis is a multifactorial disease that results from a complex interaction between genetic predisposition and well-recognized cardiovascular risk factors (CVRF), such as diabetes mellitus, arterial hypertension, hypercholesterolemia, smoking, obesity and age (4). Now there is compelling evidence that chronic systemic inflammation also has a major impact on progression of CVD, with accelerated secondary CVD being noted in obese individuals, and as a risk factor for patients with diabetes or autoimmunity (1, 3). 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 (5).

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 (6,7); 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 (8), interferons (IFNs) (9). 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 (10). In support of this hypothesis, our very recent observations have shown, that with the effects of inflammatory factors, elevated level of gene expression of C-X-C Motif Chemokine Ligand 9 (CXCL9), in HMECs (Human microvascular epithelial cells), is associated 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 (11). 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 (12). As a general rule, C chemokines mainly recruit lymphocytes, while CC chemokines recruit monocytes. CX3CL1 is the sole member of CX3C chemokine subgroup, combines the properties of chemoattractant (T cells and monocytes) and adhesion molecules. The last family of chemokines, the CXC chemokines, are further classified according to the presence of the

tripeptide motif glutamic acid-leucine-arginine (ELR) in the Nterminal region. ELR+ CXC chemokines mediate neutrophil attraction and angiogenesis, typically via interaction with chemokine (C-X-C motif) receptor 2 (CXCR2), while ELR- CXC chemokines attract lymphocytes and have angiostatic properties through their common receptor CXCR3 (13, 14).

CXCL9, also known as SCYB9/ MIG gene (located on 4q21.1), is a small cytokine belonging to the ELR- CXC family of chemokines. Under the influence of a pro-inflammatory cytokine milieu, CXCL9 can be produced by a wide variety of cell types, such as endothelial cells, fibroblasts, monocytes, and T lymphocytes, and exerts its biological effects mainly via binding to CXC chemokine receptor 3 (CXCR3 /GPR9/CD183) (15-17). The protein encoded is thought to be involved in T cell trafficking. The encoded protein binds to C-X-C motif chemokine 3 and is a chemoattractant for lymphocytes but not for neutrophils. Number of cytokines may control the cellular expression of the CXCL9 gene (18). Although, CXCL9 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(Lipopolysaccharides), and leads to a significant phosphorylation of STAT1 as compared to both factors alone (19-20). Hence, phosphorylated STAT1 in VSMCs and ECs of human atheromatous plaques correlated with elevated gene and protein expression of the chemokines CXCL9 and CXCL10 (21). To study if a similar mechanism

affected the expression of CXCL9 in HMECs, the following scientific studies have been conducted. Taken together, the aim of this cohort study was to examine the role of chemokine (C-X-C motif) ligand 9 (CXCL9) on experimental atherosclerosis.

MATERIALS AND METHODS

Cell culture experiments

1. Human Microvascular Endothelial Cells (HMECs) (28) 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 μ g/ml streptomycin, 0.01 μ g/ml EGF, 0.05 μ M hydrocortisone and 2 mM L-glutamine.
3. Serum starved-medium (containing 1% of FBS instead of 10%).
4. Recombinant IFN α and IFN γ 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 μ g/ml streptomycin, 0.01 μ g/ml EGF, 0.05 μ M 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 IFN α and murine IFN γ 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 (22), 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.

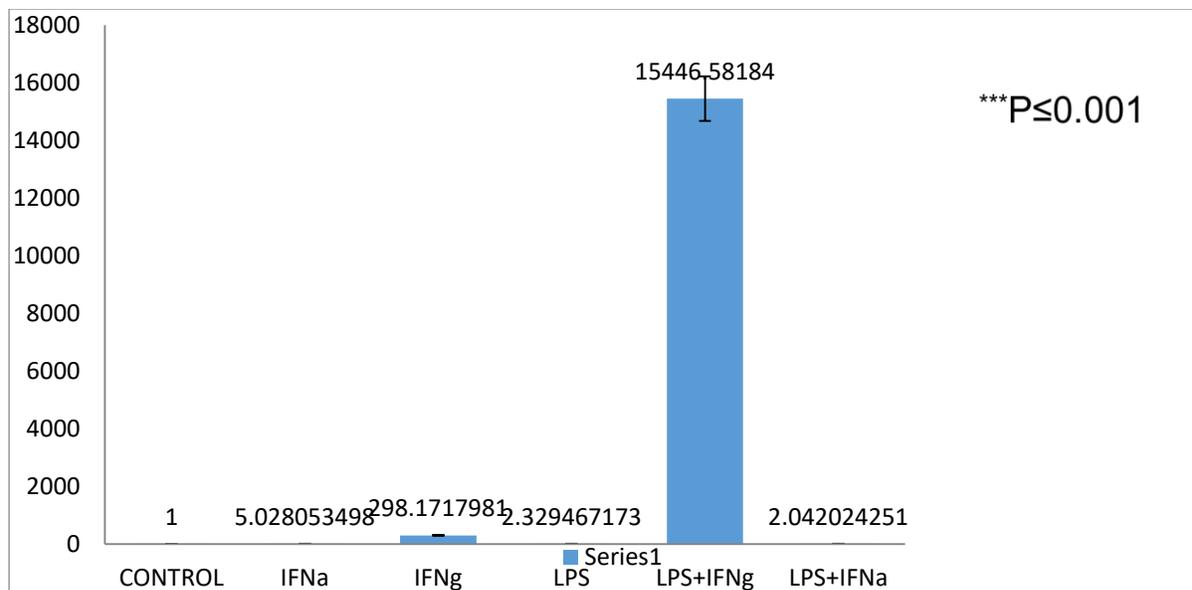
Table 1. Primer sequences used in experimental procedure.

GeneName	Forward	Reverse
CXCL9	TCATCCCTGCGAGCCTATCC	GGAGCCCTTTTAGACCTTTTT

RESULTS

It is already known that, in ECs cross-talk between IFN γ and TLR4 leads to augmented phosphorylation of STAT1 and expression of the chemokine CXCL9(21). In HMECs, CXCL9 gene showed a mild response to interferon treatment such as IFN α and IFN γ alone, 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 dependent CXCL9 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 IFN γ and TLRs. Recently, it is shown that also in VSMCs cross-talk between IFN γ and LPS resulted in augmented STAT1 phosphorylation and increased expression of the chemokine CXCL9 (21). Here, a similar STAT1-dependent mechanism for CXCL9 expression in response to IFN γ and LPS was observed in HMECs at the RNA level. Although CXCL9 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 CXCL9 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 IFN γ 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

CXCL9. HMECs may therefore provide a better model for in vitro studies of atherosclerosis.

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