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CXCL12: A New Player in Coronary Disease Identified through Human Genetics

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Abstract

Genome-wide association studies (GWAS) in over 100,000 people have revealed novel loci associated with coronary artery disease (CAD) and myocardial infarction (MI) which present exciting opportunities to discover novel disease pathways. One such recently identified locus is on chromosome 10q11, near the gene for the chemokine CXCL12 which has been implicated in cardiovascular disease (CVD) in both mouse and human studies. These GWAS demonstrate that CXCL12 may emerge as a potential therapeutic target for atherosclerosis and thrombosis.

Keywords

CXCL12; atherosclerosis; GWAS; cardiovascular disease; inflammation

Introduction

Genome-wide association studies (GWAS) have revealed several novel loci associated with coronary artery disease (CAD) and myocardial infarction (MI) which present opportunities to explore novel proteins and pathways that may be implicated in these disease processes. Human genomics can be utilized as a tool for understanding and discovering novel therapeutic targets. One such recently identified locus is a region near the gene for the chemokine CXCL12 (Samani et al. 2007). Though both human and mouse studies of CXCL12 and cardiovascular disease (CVD) have been conflicting (Damas et al. 2002; Nagasawa et al. 1994; Yamaguchi et al. 2003), CXCL12 may emerge as an important therapeutic target for atherosclerosis and thrombosis. This review will focus on the role of the chemokine CXCL12 in CVD.

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Genome Wide Association Studies of Coronary Artery Disease and Myocardial Infarction Implicate a Novel Locus at *CXCL12*

Thus far, GWAS have identified several novel loci for CAD and MI. The first reported GWAS locus for CAD was a strong signal on chromosome (chr) 9p21 (Helgadottir et al. 2007; McPherson et al. 2007; Schunkert et al. 2008) that has subsequently been replicated in all GWAS for MI and CAD (Assimes et al. 2008; Hinohara et al. 2008; Shen et al. 2008). With the recent publication of data from the CARDIoGRAM (H. Schunkert, et al. 2011) and C4D CAD consortia GWAS (Peden et al. 2011), there are now at least 35 independent genome-wide significant loci associated with CAD/MI: as expected, many are associated with LDL cholesterol while several others are novel and not related to lipids.

One of the earliest and most widely replicated novel GWAS locus for CAD/MI is a region on chr 10q11 3' 80 Kb downstream of *CXCL12*. The Wellcome Trust Case Control Consortium Study (WTCCC) of complex diseases was the first large scale CAD/MI GWAS that consisted of 1,926 case subjects with CAD and 2,938 controls (Consortium 2007). CAD cases had a validated history of either MI or coronary revascularization before age 66. Two SNPs on chr 10q11.21 (rs501120, T allele, frequency = 0.833, per allele odds ratio (OR) 1.24, p-value = 1.31×10^{-3} and rs1746048, C allele, frequency = 0.84, OR 1.21, p-value = 2.96×10^{-3}) had a suggested association with CAD/MI. These SNPs were within a region approximately 80 Kb 3' downstream of a gene for an inflammatory chemokine *CXCL12* and in correlation with each other (linkage disequilibrium r^2 value of 1.0). Both SNPs have been replicated in subsequent GWAS which are described below. The results from the WTCCC study and others are summarized in Table 1. Supplementary Table 1 provides the adjustments used in the statistical analyses of each study.

Following the WTCCC study, an analysis performed in 2008 of 875 German MI case subjects (MI before age 60 and at least one first-degree relative with premature CAD) and 1,644 controls (age and sex matched) (Samani et al. 2007) strengthened the findings on chr10q11 downstream of *CXCL12* (rs501120, OR 1.54, p-value = 5.28×10^{-6} and rs1746048, OR 1.47, p-value = 1.61×10^{-5}). The combined WTCCC-German MI data revealed more statistically significant signals for both SNPs near *CXCL12* (rs501120, OR 1.33, p-value = 9.46×10^{-8} and rs1746048, OR 1.29, p-value = 5.73×10^{-7}) (Consortium 2007; Kathiresan et al. 2009; Samani et al. 2007).

Following these discovery studies, a large GWAS was conducted by the Myocardial Infarction Genetics Consortium in a four-stage design (Kathiresan et al. 2009) and this large-scale effort replicated SNP rs1746048 (OR 1.17, p-value = 7.4×10^{-9}) in the same region 3' 80 Kb downstream of *CXCL12* (Kathiresan et al. 2009) at genome-wide significance. This study provided further supporting evidence of a CAD/MI relationship with the *CXCL12* locus.

A recent meta-analysis performed by a large international consortium, CARDIoGRAM, confirmed the association of the *CXCL12* locus with CAD/MI (Schunkert et al. 2011). The CARDIoGRAM consortium brought together GWA data from fourteen studies of subjects of European ancestry with CAD. Studies were initially done in a discovery sample size of 22,233 cases and 64,762 controls, and then replicated in 42,343 additional subjects. The SNP rs1746048 near *CXCL12* was again found to be a CAD/MI risk locus in the combined analysis (OR 1.09, p-value 2.12×10^{-10}). Interestingly, in a sub-group analysis of MI cases and those with only angiographic CAD, the SNP rs1746048 odds ratio for MI was more significant than that for angiographic CAD (OR (MI) 1.12, p-value = 8.19×10^{-6}) (Schunkert et al. 2011). The distinction of this phenotypic heterogeneity of CAD was recently demonstrated in a study of CAD and MI in approximately 20,000 individuals where

the authors reported differential associations in those with angiographic CAD versus controls (ADAMTS7; p-value = 4.98×10^{-13}) compared to those with angiographic CAD who had myocardial infarction versus those with angiographic CAD but no myocardial infarction (ABO locus; p-value = 7.62×10^{-9}) (Reilly et al. 2011). These results, which complement findings from the CARDIoGRAM study, suggest that specific genetic predispositions may promote the development of coronary atherosclerosis whereas others may lead to myocardial infarction in the presence of coronary atherosclerosis.

Another large recent consortium known as C4D failed to replicate the *CXCL12* locus association with CAD/MI at genome-wide significance (OR 1.06; p-value = 8.52×10^{-3}), but was able to demonstrate consistent directionality in the effect of the risk allele (Peden et al. 2011). This study included European and South Asian subjects, and the weaker findings at the locus near *CXCL12* suggest the possibility of ancestry-specific associations. Though larger studies of more ethnicities will better define the association of the chr 10q11 region with CAD/MI and plasma CXCL12 levels, data in European descent Caucasians strongly suggest an association between the *CXCL12* locus and CVD. Whether this relationship is due to gain/loss of function of the *CXCL12* protein secondary to coding point mutations within the gene or variations in gene transcription is unknown at this point.

Chemokines, Atherosclerosis and Plaque Rupture: An Overview

Chemotactic cytokines, or chemokines, are a large superfamily of low-molecular-weight proteins. Over forty chemokines have been identified in humans that predominantly act on neutrophils, monocytes, lymphocytes, and eosinophils (Koenen et al. 2010).

Chemokines are central to both the development of atherosclerosis and rupture of atherosclerotic plaque (Zernecke et al. 2010) - the leading cause of acute ischemic syndromes within the vascular system. For example, CCL2 (monocyte chemotactic protein/MCP-1) has been shown to play a central role in the recruitment of monocytes into the vascular wall (Han et al. 1998). Another CC chemokine expressed by monocytes, T lymphocytes, and smooth muscle cells (SMCs), CCL5, has been shown to be atherogenic and increase lesion formation (Pattison et al. 1996). The CXC chemokine CX3CL1 has been shown to be present in higher levels in CAD patients when compared to healthy individuals (Apostolakis et al. 2007), and CX3CL1 knockout murine models demonstrate less atherosclerosis (Teupser et al. 2004). Further, knockout murine models of a chemokine receptor CXCR3 have implicated an atherogenic role for its ligands, the chemokines CXCL9, CXCL10, and CXCL11 (Veillard et al. 2005).

As platelets are known to release chemokines, platelet-mediated inflammation may also contribute to the development of atherosclerosis and acute coronary syndromes (Koenen et al. 2010). Activated platelets are known to release CXCL12 (Massberg et al. 2006) and several other chemokines (e.g. CCL5, CXCL1, epithelial neutrophil activating peptide-78 (ENA-78/CXCL5), CXCL4, and CXCL7 (Zernecke, Shagdarsuren et al. 2008)).

Following plaque formation, cells within the plaque continue to secrete cytokines and matrix proteases. These molecules contribute to the thinning of the stability-providing fibrous cap (Hansson 2005). Chemokines destabilize plaques via recruitment of inflammatory cells such as T lymphocytes and monocytes which further secrete proteolytic enzymes contributing to plaque destabilization. Chemokines also trigger oxidative stress and apoptosis in the lesion and tissue factor release in vascular smooth muscle and endothelial cells (Aukrust et al. 2008).

Chemokine Structure, Function, and Molecular Overview of CXCL12

CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is one of 17 members of the CXC chemokine family, the “X” designating the one amino acid that separates the first two cysteine groups. This family is further subdivided into two subgroups depending on whether a specific amino acid motif (glutamic acid – leucine – arginine, or “ELR”) is present before the first cysteine of the CXC sequence (Murdoch et al. 1999). Interestingly, CXC chemokines that are “ELR” positive attract neutrophils. Those that are ELR negative, like CXCL12, are chemotactic for T lymphocytes and natural killer cells (Ali et al. 2007; Fujiwara et al. 2002).

CXCL12 binds to the CXCR4 receptor and has been implicated in a variety of processes ranging from embryonic development, hematopoiesis, angiogenesis, and cancer metastasis to HIV infection (Yu et al. 2006). It is the natural ligand for the CD4 co-receptor CXCR4, a G-protein coupled receptor (GPCR) that mediates the biological effects of its ligand (De La Luz Sierra et al. 2004). This receptor is also used by HIV T-tropic strains to enter cells in advanced stages of HIV, and selectively binds CXCL12 (Coll et al. 2005). CXCR4 is expressed on a variety of tissues including lymphocytes, platelets, hematopoietic cells, and endothelial cells (Wang et al. 1998; Wang et al. 2000).

CXCL12 is ubiquitously expressed in both embryonic and adult life, and mice deficient in CXCR4 have severe defects in the hematopoietic and nervous systems (Ma et al. 1998; Yu et al. 2006). Though it was previously thought that CXCR4 was the only receptor for CXCL12 (Ma et al. 1998), a second receptor, CXCR7, has also been described (Balabanian et al. 2005). CXCR7 is highly expressed in monocytes and mature B cells and has a high affinity for CXCL12. However, binding of the chemokine with CXCR7 does not lead to leukocyte chemotaxis or coupling of the receptor to G-proteins as is seen after CXCL12-CXCR4 interactions. CXCL12-CXCR7 associations have been shown to affect cell growth and survival (e.g. B cells, fibroblasts, endothelial cells). It is currently thought that CXCR7 may act as a scavenger receptor to modulate the CXCL12 gradient, and may also activate some signaling pathways (Thelen et al. 2008).

The six isoforms of CXCL12 that have been identified are derived from alternative splicing of mRNA (Yu et al. 2006). CXCL12- α and CXCL12- β are both expressed throughout the body, with highest expression levels in the liver, pancreas, and spleen (Yu et al. 2006). Though CXCL12- α and CXCL12- β were the first isoforms to be described, four more isoforms with differential expression patterns have more recently been identified. Of all six, CXCL12- α is the most highly expressed in both adult and fetal human tissues (Yu et al. 2006) and also the isoform specifically identified by a commercial ELISA assay for SDF-1/CXCL12- α (R&D Systems) (Personal communication with R&D, Christopher Larson).

Until earlier this decade, CXCL12 was thought of mainly as an oncologic and immunologic mediator of disease in humans (Modi et al. 2005; Mohle et al. 1998; Zou et al. 1998). Further, studies have shown that CXCL12 acts on lymphocytes and monocytes in vitro, and strongly attracts mononuclear cells in vivo. CXCL12 induces intracellular polymerization of actin in lymphocytes which is crucial for cell motility (Bleul et al. 1996). Because CXCL12 is involved in the cell trafficking of monocytes, macrophages, and endothelial progenitor cells (EPCs) (Hansson 2005), key components in the pathogenesis of atherosclerosis, it is not surprising that this chemokine has emerged in the context of CVD.

CXCL12 in Animal Models of Vascular Disease

Vascular Injury Models

Early studies of CXCL12 in vascular biology demonstrated its role in the repair of injured vascular tissue (Yamaguchi et al. 2003), as the chemokine plays a pro-proliferative role in recruitment of bone marrow derived endothelial progenitor cells (EPCs) in acute vascular injury models (Yamaguchi et al. 2003). *In vitro* studies showed a dose-dependent EPC migration towards CXCL12, and CXCL12 induced reduction of EPC apoptosis. *In vivo*, CXCL12 injection increased ischemic neovascularization in ischemic mice muscle by increasing recruitment of EPC to ischemic tissues (Yamaguchi et al. 2003).

In another study, the treatment of apolipoprotein E (apoE) $-/-$ mice with a neutralizing CXCL12- α monoclonal antibody after carotid injury led to a reduced neointimal lesion area and SMC content, suggesting that CXCL12 promotes vascular repair in acute injury to the intima (Schober et al. 2003). In this study, plasma levels of CXCL12- α were shown to increase one day after injury, and blockade of CXCL12- α led to a significant decrease in neointimal SMC content (Schober et al. 2003).

Further, an *in vivo* study conducted in a mouse model showed that after arterial injury, CXCL12 bound to platelets at the site of injury and ultimately promoted vascular remodeling by recruiting a subset of SMC progenitors. The mice were repopulated with CXCR4 knockout bone marrow or were treated with a lentivirus containing a CXCL12- α antagonist. Mice repopulated with CXCR4 $-/-$ bone marrow showed a reduction in neointimal hyperplasia which was linked to a decrease in SMC content (Zernecke et al. 2005).

Together, these animal models suggest a reparative role for CXCL12. Though vascular repair can be beneficial, it is possible that such repair could be pathologic if hyperproliferative. Excessive neointimal hyperplasia, neovascularization, and tissue repair could lead to restenosis of vessels following stent implantation or reparative mechanisms in response to ongoing chronic endothelial damage observed in states of atherosclerotic risk factors (e.g tobacco, hypertension, diabetes). Further studies are needed to better understand the role of CXCL12 in various forms of vascular remodeling observed after injury.

Chronic Atherosclerosis Models

Some animal studies have implicated an anti-atherogenic role for the CXCL12-CXCR4 interaction in models of chronic atherosclerosis. For example, treatment of apoE $-/-$ mice with constant delivery of a CXCR4 antagonist, AMD3465, increased the size of atherosclerotic lesions and necrotic core area size within the plaque. This blockade of the CXCL12 receptor led to an increase of neutrophils from the bone marrow and increased the number of neutrophils in aortic root plaques and adventitia (Zernecke, Bot et al. 2008). The involvement of neutrophils in the pathogenesis of CAD/MI has not yet been completely understood, but neutrophils have been found in areas of plaque erosion and rupture (Sainz et al. 2008). However, this preliminary work has not yet been replicated and it is possible that this increase of neutrophils was due to the administration of the specific antibody. The increase of neutrophils following the CXCL12 blockade suggests a potentially anti-inflammatory role that is opposite of the role that most chemokines play (Charo et al. 2004). However, in the absence of knockout mouse models, it is difficult to interpret these findings. Furthermore, ontologic evidence supports significant differences in cytokine systems between non-human species and humans (Mestas et al. 2004). Thus, there should be cautious interpretation of extrapolation of these data to humans, even though these animal study data suggest a potentially anti-atherogenic role for CXCL12.

CXCL12 in Humans and Relationship to Coronary Artery Disease

Prior to the association of the *CXCL12* locus with CAD and MI discovered through human genetics, there was limited data describing the role of CXCL12 in vascular disease. CXCL12 is highly expressed in SMCs, endothelial cells, and macrophages in human atherosclerotic plaques, but not in normal vessels (Abi-Younes et al. 2000). This finding pointed to the chemokine as a potential regulatory agent in atherosclerosis. In 2002, a study found that plasma CXCL12 levels in patients with stable angina (n = 30) and unstable angina (n=30) were decreased when compared to healthy controls (n=20). In these patients with angina, the gene expression of the CXCL12 receptor CXCR4 was increased in peripheral blood mononuclear cells (PBMCs) when compared to controls. Further, the surface expression of the receptor in these same cells from patients with angina was decreased (Damas et al. 2002). PBMCs from patients in this study with unstable angina were also used to describe the role of CXCL12 treatment on the *ex vivo* production of inflammatory atherogenic signals (MCP-1, interleukin 8 (IL-8), and matrix metalloproteinases (MMPs)). It was found that *ex vivo* CXCL12 treatment reduced production of these inflammatory mediators (Damas et al. 2002). Though this small study has not been replicated, it added support to the thought that CXCL12 may play an anti-atherogenic role in human CAD.

Though the acute versus chronic modulation of plasma CXCL12 levels in human CAD is not yet well defined, a study conducted in 101 patients with acute coronary syndromes (ACS) found that plasma levels of CXCL12 were significantly elevated 24 hours after the ACS. After this elevation, there was a subsequent decrease in the days following the event (Stellos et al. 2009). This study was done in a small sample size and has not yet been replicated, but suggests that there may be differences in the acute versus chronic modulation of CXCL12 plasma levels that depend on the clinical manifestation of atherosclerosis.

In contrast, a subsequent large study in 2009 found that patients with ACS (n = 418, ACS defined as non-ST-segment elevation MI, ST-segment elevation MI, or unstable angina with coexistence of positive troponins) showed significantly enhanced CXCL12 expression on the platelet surface compared to those with stable angina pectoris (n = 486). Platelet-bound CXCL12 correlated with platelet P-selectin expression and the degree of systemic platelet activation (Stellos et al. 2009). CXCL12 was also shown to induce aggregation of platelets collected from healthy volunteers (Abi-Younes et al. 2000). These findings support an atherogenic, pro-thrombotic, and plaque destabilizing role for CXCL12 (Falk et al. 1995). Moreover, this study suggests that CXCL12 is a potential mediator of platelet involvement in atherosclerosis, as activated platelets play a critical role in the rupture and early repair of unstable atherosclerotic plaques (Hansson 2005).

A recent human study in late 2010 reported an association between the genotype T/T at rs501120 discovered in CAD/MI GWAS in the 3' region downstream of *CXCL12*. The authors found that the risk allele (T) increased carotid atherosclerosis. Interestingly, they also reported that this allele was associated with *lower* plasma levels of CXCL12 (Kiechl et al. 2010). However, the sample size was small (n=738), the statistical modeling utilized a recessive model rather than an additive model, and the study was performed in a single sample of patients without replication.

We analyzed the association of the two SNPs discovered by CAD/MI GWAS (rs1746048 and rs501120) with plasma CXCL12 levels (Mehta et al. 2011) by performing analyses in a two-stage study (n = 2939): first (n = 1182) in a Caucasian, angiographic CAD case-control study and second (n = 1757) in a community-based study of CAD risk factors. Both SNPs were associated with *higher* plasma levels of CXCL12 in a dose-response fashion per copy of risk allele in an additive genetic model adjusted for age, gender, CV risk factors, and

CAD-status. Consistent with this relationship, the rs1746048 risk allele tended to have higher transcript levels of CXCL12 in natural killer and liver cells. These data together not only suggest that these loci in the region 80 Kb 3' downstream of *CXCL12* may modulate CXCL12 plasma levels, but also suggest that CXCL12 may be atherogenic because the CAD/MI risk allele SNPs were associated with higher mRNA transcript and plasma levels of CXCL12. The GWAS discovered region 3' 80 Kb from the gene may harbor regulatory elements which modulate plasma CXCL12 levels, or one sample of patients from the study may have been confounded by hospital-based recruitment during an MI which modulated plasma CXCL12 levels. Larger ongoing studies will address these issues in community-based and prospective samples.

Though the precise role of CXCL12 in atherosclerosis remains to be elucidated, both animal models and human studies suggest modulation of plasma CXCL12 levels and genetic predisposition of *CXCL12* in CAD/MI: from initiation to acute coronary syndrome to repair of injured vascular tissue. Further, studies in both conditional knockout and transgenic mouse models are needed to better understand the role of CXCL12 in various tissues. Larger prospective cohort studies are needed to understand whether there is an association between the CXCL12 protein, *CXCL12* common variation, and hard CV endpoints. Studies to analyze the poorly understood modulation of CXCL12 in acute and chronic CVD states such as stable coronary disease, unstable angina, and MI are also necessary. Studies of all CXCL12 isoforms in cells and tissues relevant in atherosclerosis will also be needed to define whether there are tissue specific expression and isoform specific effects. Finally, large re-sequencing efforts should be performed to understand if the 3' 80 Kb downstream of *CXCL12* harbors rare, functional variants that modulate transcription of the *CXCL12* gene, mRNA splicing, or function of the CXCL12 protein. The completion of these studies will allow for a better understanding of the role of CXCL12 in vascular repair, atherosclerosis, and may reveal other unknown functions.

Clinical implications of CXCL12 in CVD

As current therapeutics for atherosclerosis have focused on lipid metabolism and hemostasis, chemokines may emerge as a therapeutic target as they have a role in both atherosclerosis and thrombosis (Rader et al. 2008). Several studies have pointed towards a complex role for CXCL12 in CVD. These studies beget the question of the potential clinical and therapeutic significance of the *CXCL12* gene as well as plasma CXCL12 levels. For example, given that CXCL12 is a strong platelet activator and inducer of platelet aggregation, blockade of either the chemokine or its receptor may be used as adjunct anti-platelet therapy during ACS. However, carefully conducted platelet studies in humans are needed prior to these trials.

Several questions remain within the clinical context: 1) Are higher levels of plasma CXCL12 levels protective or detrimental in humans? 2) What is within the region 3' 80 Kb downstream of *CXCL12* and will genetic variation closer to the gene demonstrate a similar relationship to plasma CXCL12 levels? 3) Will interrogation of the other predominant isoform, CXCL12- β , clarify the role of plasma CXCL12 levels in vascular disease? Future research should focus on understanding how the modulation of plasma levels in response to acute ischemia compared to chronic atherosclerosis phenotypes alters the plasma levels in humans. However, these studies will be difficult and require plasma samples that are drawn *prior* to an acute ischemic event in large numbers of individuals. In addition to the 3' 80 Kb region downstream of the gene, studies focusing on the region closer to *CXCL12* may also inform biology of CXCL12 and demonstrate findings which answer the question of whether common variation within the gene region is associated with modulation of plasma CXCL12 levels. There is tremendous work still needed to better understand how plasma levels relate

to hard CV outcomes, and how genetic variation at this locus and within the gene modulates tissues and cells in atherosclerosis.

Despite the complex relationship between CXCL12 and atherosclerosis, vascular injury, and CAD/MI, animal and human studies done thus far highlight this chemokine as a novel player discovered through human genomics in CVD. Work thus far demonstrates the exciting science which follows up novel discovery in the modern era of high throughput genomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Results from CAD/MI Genome-Wide Association Studies (GWAS) for SNPs within Locus Near *CXCL12* on Chromosome 10q11.

Study	SNP	OR	p-value
WTCCC (2007)	rs501120	1.24	1.31×10^{-3}
WTCCC (2007)	rs1746048	1.21	2.96×10^{-3}
German Family MI Study (2007)	rs501120	1.54	5.28×10^{-6}
German Family MI Study (2007)	rs1746048	1.47	1.61×10^{-5}
MIGen (2009)	rs1746048	1.17	7.4×10^{-9}
CARDIoGRAM (2011)	rs1746048	1.09	2.12×10^{-10}

Results from GWAS analyses are included from the following studies: Wellcome Trust Case Control Consortium (WTCCC), German Family MI, Myocardial Infarction Genetics Consortium (MIGen), and CARDIoGRAM. SNP = single nucleotide polymorphism; OR = per allele odds ratio. Supplementary Table 1 provides the adjustments used in the statistical analyses of each study.