Chemokines in vascular pathology (Review)

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Abstract. Clinical complications of atherosclerosis are major causes of morbidity and mortality in Western societies. Recent evidence suggests that formation of atherosclerotic lesions is an inflammatory process involving multiple molecular pathways. Chemokine-mediated mechanisms are potent regulators of such processes by orchestrating the interactions of inflammatory cellular components of the peripheral blood with cellular components of the arterial wall. The increasing evidence supporting the role of chemokine-pathways in atherosclerosis renders chemokine ligands and their receptors potential therapeutic targets. In the following review, we intend to highlight the special structural and functional features of each chemokine sub-family in respect to their role in atherosclerosis and discuss to what extent such knowledge could be applied in diagnostic, prognostic or therapeutic practices.

Contents

1. Introduction

Atherosclerosis is a complex, multifactorial disease associated with numerous environmental risk factors interacting with the genetic background of the individual. Elevated plasma cholesterol levels, hypertension, diabetes mellitus and smoking are, among others, recognized as major inducers of endothelial damage. Increasing evidence also suggests that atherosclerosis is an inflammatory disease involving accumulation and activation of inflammatory cells onto the vessel wall (1). In fact, the development of atherosclerotic lesions is the outcome of the interaction of multiple cellular populations of the peripheral blood, mainly monocytes and T lymphocytes, with cell components of the arterial wall, mostly endothelial and smooth muscle cells, in response to multifactorial vascular injury. Several pro-inflammatory factors, chemoattractant cytokines (chemokines) and adhesion molecules are essential in orchestrating this process (2).

Atherosclerosis and chronic inflammation. Despite the fact that the exact nature of endothelial damage is currently not clear, it is well established that the endothelium reacts to injury by increased cell surface expression of adhesion molecules (1,2). Circulating leucocytes that roll on the endothelium are trapped by the increased number of adhesion molecules and migrate between endothelial cells, predominantly at sites of disrupted blood flow patterns and increased shear stress. In the intima of the injured endothelium, monocytes differentiate into macrophages, they absorb modified lipoprotein particles and become foam cells. Accumulation of foam cells in the intima results in the formation of "fatty streaks". Fatty streaks are the first atherosclerotic lesions and can be identified even in infants and young children. If the trigger factor remains, the inflammatory process continues and progresses. The initially protective inflammatory response starts to damage the arterial wall. Dysfunctional endothelial cells and activated leukocytes release cytokines, chemokines and growth factors, and promote the migration of smooth muscle cells (SMCs) into the intima of the arterial wall and give rise to the next stage of lesion formation, the intermediate or fibro-fatty lesion. At this stage, the lesion can contain multiple layers of smooth muscle cells, connective tissue, macrophages and T cells (1,2). Under the effect of pre-disposing factors, remodelling of the vessel wall occurs resulting in advanced lesion formation. Advanced lesions are susceptible to rapture, the pathophysiological background of acute ischemic events.

Chemokine structural and functional characteristics. Chemokines are low molecular weight chemoattractant cytokines

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known to be major regulatory proteins in leukocyte trafficking and activation. They consist of an expanding family of approximately 50 ligands and 20 receptors which are classified into four sub-groups based on the number and structural arrangement of conserved cysteine residues within their amino-terminal polypeptide sequence (C, CC, CXC and CX3C). CXC (or α) chemokines have a single amino acid separating the two amino-terminal cysteine residues of the protein, while CC (or ß) chemokines have no amino acid separating the amino-terminal cysteines. Fractalkine is the single member of the CX3C sub-family having three amino acids separating the two amino-terminal cysteine residues. Finally, the latest-discovered lymphotactine (XCL1) and single C motif chemokine 1-α (SCM1-α or XCL2) are the only currently known members of the C sub-family and lack two of the four conserved cysteines in the mature protein (3,4,5).

Chemokines induce cell activation by binding to specific seven-transmembrane G-protein coupled cell-surface receptors on target cells. Six human CXC chemokine receptors, ten human CC chemokine receptors, and a single receptor for each of the CX3C and C chemokine sub-families have been up to now identified. An unusual characteristic of most chemokine receptors is their high-affinity for multiple ligands. Chemokines interacting with their receptors on the cell surface lead to the generation of an intracellular signal via the G-protein complex, resulting in cell chemotaxis towards the source of the chemokine (3,4,5).

Chemokines are involved in almost every inflammatory response and they play an important role in the pathogenesis of a wide variety of infectious and inflammatory diseases, such as human immunodeficiency virus-1 (HIV-1) infection, respiratory syncytial virus-induced bronchiolitis, asthma, sarcoidosis, rheumatoid arthritis, glomerulonephritis, inflammatory bowel disease, multiple sclerosis, and atherosclerosis (2,3,6,7).

There is increasing interest in recent literature regarding the role of chemokines in atherosclerosis since they are implicated in essential aspects of atherogenesis, such as recruitment of inflammatory cells onto the vessel wall and proliferation of SMCs in atherosclerotic plaques (8). This vital involvement of chemokines in the establishment and progression of athrosclerosis suggests that medically-important chemokines and their receptors could provide novel targets for therapeutic interventions in atherosclerosis-related diseases, such as coronary artery disease, peripheral artery disease and cerebrovascular disease.

The present review attempts to provide recent evidence supporting the role of chemokines in atherosclerosis and discusses how such knowledge could be applied in diagnostic, prognostic and therapeutic practices. We attempt to highlight the special structural and functional features of each chemokine sub-family in relation to their role in atherosclerosis along with *in vitro* and *in vivo* derived supporting evidence. Because of this special focus, we restricted our assessment to selected chemokine aspects. It is quite likely, however, that other facets related to chemokines may well play an important role in atherogenesis. The abbreviations, localization, and main functions of the chemokines implicated in atherosclerosis, are summarized in Table I.

2. The CC chemokines and chemokine receptors

The CC chemokines form the largest chemokine sub-family, containing over 25 currently identified ligands and ten receptors (5).

MCP-1 and CC receptor 2. Monocyte chemoattractant protein-1 (MCP-1 or CCL2) has been the leading target of research in the field of experimental atherosclerosis. It is the prototype molecule of the CC class and a strong chemoattractant for monocytes. The presence of MCP-1 in atherosclerotic lesions was first demonstrated in 1991 by *in situ* hybridisation and has been confirmed since by several studies and different techniques (9). MCP-1 mRNA expression has been detected in endothelial cells, macrophages and vascular SMCs in atherosclerotic arteries (10,11,12). Several experimental models of atherosclerosis, including low density lipoprotein (LDL) receptor and apolipoprotein E knockout (ApoE-/-) mice, have been used to confirm the role of MCP-1 or its receptor, CCR2, in atherosclerotic lesion formation. Gu *et al* reported less lipid deposition and fewer macrophages in the aortic walls of LDL-receptor deficient mice lacking also the MCP-1 encoding gene (13). Similarly, Boring *et al,* using ApoE-/- knockout mice, observed an overall decrease in atherosclerotic lesions in mice also deficient of the MCP-1 receptor (14). In addition to those findings, Aiello *et al* demonstrated that overexpression of MCP-1 in the bone marrow-derived cells of ApoE-/- mice resulted in increased lesion formation as well as increased deposition of oxidized lipid and macrophages (15). Experimental blocking of the MCP-1/CCR2 pathway in atherosclerotic models also resulted in reduction of lesion development. Ni *et al* and Inoue *et al* used a mutant analogue of MCP-1 that was able to bind to its receptor, CCR2, without producing activation. When the mutated gene was transfected into the skeletal muscle cells of ApoE-/- mice, atherosclerosis was attenuated (16,17). MCP-1/CCR2 pathways have also been implicated in the accumulation and migration of SMCs in atherosclerotic lesions. In a study by Roque *et al* mice deficient in CCR2 significantly reduced intimal hyperplasia following injury to the femoral artery. As macrophage infiltration to the injured femoral arteries of both CCR2+/+ and CCR2-/- mice is negligible, this suggests that MCP-1/CCR2 has an impact on the migration and/or proliferation of SMCs following acute arterial injury (18). Furthermore, a number of population genetic studies have been conducted evaluating susceptibility to atherosclerosis in subjects carrying polymorphic variants of the MCP-1 or CCR2 encoding genes. A G to A substitution in the human CCR2 gene causing a Valine to Isoleucine substitution (CCR2-V64I) has been identified and mostly evaluated (19). Szalai *et al* evaluated the frequency of the CCR2-V64I polymorphism in 318 CAD patients compared to 320 controls and suggested that patients with the CCR2-V64I polymorphism were at reduced risk for CAD, based on the complete absence of individuals with the rare I/I genotype in the CAD group (20). In a similar cohort of ours conducted in 210 CAD patients and 165 controls, no association between CCR2-V64I and the presence, angiographic severity or clinical presentation of CAD was established (21). Two polymorphisms have been also identified in the 5' region of the MCP-1 promoter (-2518

Table I. Chemokines implicated in atherosclerosis.

Chemokine family	Systemic name	Alternative name	Receptor	Main cellular sources	Main targets	References
CC	CCL1	I-309	CCR8	Endothelial cells	Monocytes/macrophages	36
	CCL2	MCP-1	CCR2	Monocytes/macrophages, endothelial cells, smooth muscle cells	Monocytes/macrophages, activated T cells	9-17
	CCL3	MIP-1α	CCR1, CCR5	Monocytes/macrophages, T-cells	Monocytes/macrophages, activated T cells	31-33
	CCL4	MIP-1ß	CCR5	Monocytes/macrophages, T-cells	Monocytes/macrophages, activated T cells	31-33
	CCL5	RANTES	CCR1, CCR3, CCR5	Monocytes/macrophages, T cells, platelets	Monocytes/macrophages, activated T cells	23,24,47
	CCL11	EOTAXIN	CCR3	Smooth muscle cells	Monocytes/macrophages, mast cells, B cells	27
	CCL13	MCP-4	CCR2, CCR3	Endothelial cells, monocytes/ macrophages, T cells	Monocytes/macrophages, activated T cells	38
	CCL17	TARC	CCR4	Monocytes/macrophages, dendritic cells	Th2 T cells, dendritic cells	35
	CCL18	PARC	Unknown	Monocytes/macrophages, dendritic cells	Resting T cells	34
	CCL19	ELC	CCR7	Monocytes/macrophages, smooth muscle cells	Activated T cells	34
	CCL22	MDC	CCR4	Monocytes/macrophages, dendritic cells	Activated T cells	35
CXC	CXCL1	GRO-α	CXCR2	Monocytes/macrophages endothelial cells	Monocytes/macrophages	44,45
	CXCL4	PF4	Unknown	Platelets, monocytes/macrophages	Endothelial cells	50-54
	CXCL8	IL-8	CXCR1, CXCR2	Monocytes/macrophages, endothelial cells, smooth muscle cells	Endothelial cells, monocytes/ macrophages, smooth muscle cells, T cells	40-43
	CXCL9	MIG	CXCR3	Endothelial cells, monocytes/ macrophages	Activated T cells	46
	CXCL10	IP-10	CXCR3	Endothelial cells monocytes/ macrophages, smooth muscle cells	Activated T cells	46
	CXCL11	I-TAC	CXCR3	Monocytes/macrophages, endothelial cells	Activated T cells	46
	CXCL12	SDF1-α	CXCR4	Monocytes/macrophages, smooth muscle cells	T cells, platelets, smooth muscle cell progenitors	48,49
CX3C	CX3CL1	Fractalkine	CX3CR1	Endothelial cells, smooth muscle cells	Monocytes/macrophages, activated T cells, smooth muscle cells	56-59

MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; TARC, thymus and activation-regulated chemokine; PARC, pulmonary and activation-regulated chemokine; ELC, Epstein-Barr virus-induced molecule 1 ligand chemokine; MDC, macrophage-derived chemokine; GRO, growth related oncogene; PF platelet factor; IL, interleukin; MIG, monokine induced by IFN-γ; IP-10, 10-kDa IFN-γ-inducible protein; I-TAC, IFN-γ-inducible T cell α-chemoattractant; SDF, stromal cell-derived factor.

A to G and -2076 A to T). The -2518 polymorphism has been reported to have functional effects (22). In support of these, Szalai *et al* investigated the prevalence of the -2518G polymorphism in coronary artery diseased (CAD) patients and healthy controls and reported a significantly higher frequency of the -2518G homozygote variant of the MCP-1 promoter in CAD patients than in controls, thus implicating the MCP-1/CCR2 pathway in atherosclerosis (20).

RANTES and CC receptor 5. RANTES (regulated on activation, normal T cell expressed and secreted,) or CCL5 is expressed by T lymphocytes in advanced lesions and is

highly produced in human transplant-associated accelerated atherosclerosis by macrophages, lymphocytes, myofibroblasts and endothelial cells. Pattison *et al* investigated the expression of RANTES using *in situ* hybridization and immunohistochemistry in coronary arteries of patients undergoing accelerated atherosclerosis compared to normal coronary arteries (23). They demonstrated that RANTES mRNA and protein were detected in the lymphocytes, macrophages, myofibroblasts and endothelial cells of arteries undergoing accelerated atherosclerosis but not in normal coronary arteries, concluding that RANTES may be a pivotal mediator of the cellular infiltrate seen in graft atherosclerosis. In a completely different setting, Veillard *et al* reported that blocking *in vivo* RANTES-mediated signalling using the CC chemokine antagonist Met-RANTES, reduced the progression of atherosclerosis in a hypercholesterolemic mouse model, indicating that blockade of chemokine receptor/ligand interactions could become a novel therapeutic target decelerating the progression of atherosclerosis (24).

The role of CCR5/RANTES pathway was also investigated by population-based genetic studies. Genetic changes in the CCR5/RANTES system may influence the development of CAD. A 32-base-pair deletion in the CCR5 receptor (CCR5 Δ32) and two promoter polymorphisms in RANTES (-28 C to G and -403 G to A) have been identified and thoroughly investigated. Studies based on screening the 32-base-pair deletion in the CCR5 receptor gene and its association with CAD, have produced conflicting conclusions. Szalai *et al* [20] reported that the CCR5 Δ32 genotype has an atheroprotective effect, since they found a higher frequency of CCR5-Δ32 homozygotes in controls than in CAD patients. Similarly, Gonzalez *et al* (25) demonstrated that non-carriers of the CCR5-Δ32 allele had a three-times greater risk of MI under 55 years of age. Finally, in a study of ours, the frequency of CCR5-Δ32 was evaluated in 210 angiographically-assessed CAD patients and 165 controls with negative coronary angiography. No differences were observed. However, the frequency of deletion in the population studied was relatively low, limiting the power of a negative association (21). The effect of polymorphisms of RANTES has also been investigated by several cohorts. Szalai *et al* found no association between RANTES polymorphisms -28G and -403A and CAD (20). In a recent study by Boger *et al*, contacted in type 2 diabetics in end-stage renal disease, patients carrying the RANTES -403A or In1.1C allele of the intronic In1.1T/C polymorphism had a significantly higher "all cause" mortality risk, mainly due to cardiac events (26).

Eotaxin and CC receptor 3. Haley *et al*, using DNA microarray technology and subsequent Northern analyses, demonstrated a marked increase in eotaxin (CCL11) mRNA in cultured human aortic SMCs treated with tumor necrosis factor-α (TNF-α). In the same study, immunohistochemical analysis demonstrated overexpression of eotaxin protein and its receptor, CCR3, in human atheromas, with negligible expression in normal vessels, suggesting that eotaxin participates in vascular inflammation (27). The evaluation of circulating levels of eoataxin as a marker of atherosclerosis revealed inconclusive results. In a study by Emanuele *et al*, increased eotaxin circulating levels were associated with presence and angeographic severity of CAD (28), while Mosedale *et al* evaluated by ELISA eotaxin and MCP-1 circulating levels in patients with atherosclerosis and normal subjects and conclude that, although there may be a transient increase in circulating chemokine levels following coronary angioplasty, there is no difference in the levels of circulating MCP-1 or eotaxin in subjects with or without atherosclerosis (29).

MIP-1 chemokines. Macrophage inflammatory protein-1α (MIP-1α) was identified 15 years ago. It was the first of the currently known four members of the MIP-1 chemokine subfamily. These proteins are produced by many cells, particularly macrophages, dendritic cells, and lymphocytes. Despite their structural similarities, MIP-1 subtypes show diverging signalling capacities (30). Several studies in the literature imply a role for the MIP chemokines in atherogenesis. Lutgens *et al*, in a study involving microarray analysis on mRNA of aortic arches of ApoE-/- mice fed normal chow or Western-type diet revealed important functions for genes involved in inflammation, especially the small inducible cytokines, MCP-1, MCP-5, MIP-1α, MIP-1ß, MIP-2, and fractalkine. Further expression and *in vivo* validation studies showed that this gene cluster mediates plaque progression and stability (31). Additionally, Holven *et al* demonstrated, among others, significantly higher levels of spontaneously-released MIP-1α and MIP-1ß by peripheral blood mononuclear cells (PBMCs) in patients with familial hypercholesterolemia (FH). The authors concluded that one of the pathophysiological consequences of FH is enhanced chemokine responses which, in turn, may promote recruitment and activation of leukocytes within the vessel wall, contributing to atherosclerosis (32). Additionally, drugs with anti-inflammatory anti-atherogenic properties, such as hydroxymethylglutaryl (HMG)-CoA reductase inhibitors, have been shown to down-regulate CC chemokine ligands and receptors. Veillard *et al* used human vascular endothelial cells and human primary macrophages, stimulated with tumor necrosis factor (TNF)-α or interferon (IFN)-γ, respectively. They demonstrated by ELISA that 1 mM simvastatin significantly reduced MCP-1 in endothelial cells and macrophages. Further mRNA analysis revealed that expression of the chemokines, MCP-1, MIP-1α and MIP-1ß, as well as the chemokine receptors, CCR1, CCR2, CCR4 and CCR5, was decreased by simvastatin, both in endothelial cells and macrophages (33).

Novel CC chemokines. Novel members of CC chemokines and potent chemoattractants for lymphocytes, such as pulmonary and activation-regulated chemokine (PARC) or CCL18, Epstein Barr virus-induced molecule 1 ligand chemokine (ELC) or CCL19, liver and activation-regulated chemokine (LARC) or CCL20 and secondary lymphoid-tissue chemokine (SLC) or CCL21 have been also evaluated in atherosclerosis. Reape *et al*, using reverse transcriptase-polymerase chain reaction and *in situ* hybridization methodologies, demonstrated gene expression for PARC and ELC but not for LARC or SLC in human atherosclerotic plaques. Immunohistochemical staining of serial plaque sections with specific cell markers revealed highly different expression patterns of PARC and ELC. They further demonstrated, *in vitro*, up-regulation of

ELC mRNA in aortic SMC stimulated with TNF-α and IFN-γ but not in SMCs stimulated with serum. Both PARC and ELC mRNA were expressed by monocyte-derived macrophages but not by monocytes (34).

Greaves *et al* analyzed the expression of the linked chromosome 16q13 genes that encode macrophage-derived chemokine (MDC/CCL22), thymus- and activation-regulated chemokine (TARC/CCL17), and the CX3C chemokine, fractalkine (CX3CL1), in primary macrophages and human atherosclerotic lesions by reverse transcription-polymerase chain reaction and immunohistochemistry. Expression of all the studied chemokines was up-regulated in interleukin-4- and interleukin-13 treated macrophages. Immunohistochemistry showed that MDC, fractalkine, and TARC were expressed by a subset of macrophages within regions of plaques that contain plaque microvessels. Greaves *et al* concluded that MDC, fractalkine, and TARC could play a role in mononuclear cell recruitment into atherosclerotic lesions and influence the subsequent inflammatory response (35).

Haque *et al* demonstrated that I-309 (CCL1), a novel CC chemokine produced by T lymphocytes and stimulated monocytes, was produced by endothelial cells and was responsible for the monocyte chemotactic activity induced by Lp(a) in human umbilical vein endothelial cells. The identification of endothelial cells as a source for I-309 suggests that this chemokine may participate in vessel wall biology and particularly in Lp(a)-mediated effects in atherosclerosis (36). Haque *et al* further demonstrated that I-309 stimulates chemotaxis of human vascular smooth muscle cells (VSMCs) and that this chemotaxis is blocked by murine monoclonal antibody against the I-309 receptor (CCR8) and by the G-protein inhibitor, pertussis toxin, concluding that induction of CCR8 and CCL1 under conditions associated with VSMC proliferation and migration raises the possibility that CCR8 may play an important role in vessel wall pathology (37).

Finally, Berkhout *et al* described and characterized the novel human CC chemokine, monocyte chemotactic protein (MCP)-4. MCP-4 induced a potent chemotactic response in peripheral blood monocytes but not in neutrophils. Binding studies in monocytes showed that MCP-4 interacts with the CC chemokine receptor-2 (MCP-1 receptor). Furthermore, expression of MCP-4 protein was demonstrated by immunohistochemistry in human atherosclerotic lesions and found to be associated with endothelial cells and macrophages. The authors concluded that MCP-4, like MCP-1, may be involved in the recruitment of monocytes into the arterial wall during atherosclerosis (38).

3. The CXC chemokines and chemokine receptors

The CXC chemokines are the second largest class. As mentioned before, CXC chemokines have a single amino acid residue separating their two amino-terminal cysteins. They can be further structurally subdivided into two groups based on the presence or absence of an ELR (Glu-Leu-Arg) amino acid motif in their amino-terminal domain. Examples of ELR^+ and ELR^- CXC chemokines are interleukin (IL)-8 and 10 kDa IFN-γ inducible protein (IP)-10, respectively (39). Since CXC chemokines are mainly neutrophil chemoattractants, they have received less attention concerning their role in the pathogenesis of atherosclerosis. However, regarding IL-8 (or CXCL8), IP-10 (or CXCL10) and stromal cell derived factor (SDF)-1 or CXCL12, in particular, there is convincing evidence in the literature supporting their role in atherogenesis.

IL-8 and CXC receptors 2. Since the discovery of IL-8, the prototype of CXC chemokines, in 1987, the knowledge on its role in leukocyte infiltration has advanced rapidly with increasing interest in the implications of IL-8 in vascular pathology. Apostolopoulos *et al* demonstrated the expression of IL-8 in human atherosclerotic plaques by *in situ* hybridisation (40). IL-8 has been shown to contribute in SMC proliferation and migration (41) and, despite the fact that IL-8 has been thought to act predominantly on neutrophils, Gerszten *et al* demonstrated that it induced firm adhesion of rolling monocytes to endothelial monolayers expressing E-selectin (42). Additional supporting evidence for a potential role for CXC chemokines in atherogenesis came from an experiment by Boisvert *et al*, in which LDL receptor knockout mice which were irradiated and repopulated with bone marrow cells lacking the murine homologue of IL-8 receptor CXCR2 had less extensive lesions and fewer macrophages than those mice receiving bone marrow cells expressing the receptor (43). Schwartz *et al* reported that another ligand of CXCR2, growth-related oncogene (GRO)-α or CXCL1 may contribute to adhesion of monocytes to minimally modified-LDL stimulated endothelial cells similarly to IL-8 (44) and, in a more recent study by Hue *et al*, using isolated carotid artery from ApoE-/- mice demonstrated that GRO-α but not MCP-1 plays a role in monocyte arrest on the endothelium of atherosclerosis-prone vessels (45). The above studies clearly suggest an important role of CXCR2-mediated pathways in the accumulation and migration of monocytes into the intima, and suggest that multiple ligands of CXCR2 may be key participants in the pathogenesis of atherosclerosis.

IP-10, I-TAC, MIG and CXC receptor 3. The chemokine receptor, CXCR3, has multiple high-affinity CXC chemokine ligands including IP-10, monokine-induced by IFN-γ (MIG) or CXCL9 and IFN-γ inducible T-cell α-chemoattractant (I-TAC) or CXCL11. Mach *et al* demonstrated different levels of expression of IP-10, MIG, and I-TAC by atheroma-associated cells and suggested a potential role for these 3 IFN-γ-inducible CXC chemokines in the recruitment and retention of activated T lymphocytes observed within the vascular wall during atherogenesis (46).

SDF-1 and PF4. Platelets represent both a source and a target for chemokines that may be involved in the process of atherogenic recruitment. Platelets secrete both CXC chemokines, such as platelet factor (PF) 4 or CXCL4 and epithelial neutrophil-activating protein (ENA)-78 or CXCL5, and CC chemokines, such as MIP-1α and RANTES (47). Abi-Younes *et al* reported that, of the 16 chemokines tested, only SDF-1 induced platelet aggregation and proposed an involvement of SDF-1 in the pathogenesis of atherosclerosis and thrombo-occlusive diseases (48). Additionally, Schober *et al* investigated the involvement of SDF-1 in neointimal formation after vascular injury. SDF-1 was detected in the carotid arteries of ApoE-/- mice after wire-induced injury and was mostly located

at SMCs. Furthermore, treatment of ApoE-/- mice after carotid injury with a neutralizing SDF-1 monoclonal antibody reduced neointimal lesion area, and decreased neointimal SMC content without influencing the relative content of neointimal macrophages. Thus, the authors concluded that SDF-1 plays an active role in neointimal formation after vascular injury in ApoE-/- mice by regulating neointimal SMC content (49). The role of SDF-1 in atherogenesis has also been investigated by population genetic surveys. Nevertheless, both studies that evaluated the effect of the common single nucleotide polymorphism, G801A, of the SDF-1A gene on susceptibility to CAD have concluded no association (20,21).

As mentioned previously, activated platelets secrete a number of chemokines of both CC and CXC subgroups. The role of platelet-derived chemokines in atherosclerosis has been the subject of several studies in recent literature. PF4, a CXC-chemokine member secreted by platelets, induces up-regulation of monocyte activation, firm adherence of neutrophils on shear stressed endothelium, and release of neutrophil granule components (50,51). Pitsilos *et al* detected PF4 in the cytoplasm of luminal and neovascular endothelium, in macrophages and in regions of plaque calcification. The presence of PF4 in macrophages and neovascular endothelium significantly correlated with lesion grade (52). Furthermore, in a recent study, Yu *et al* demonstrated that E-selectin, an adhesion molecule involved in atherogenesis, is up-regulated in human umbilical vein endothelial cells exposed to PF4 (53). Finally, Nassar *et al* showed that PF4 bound to oxidized (ox)-LDL directly, and also increased ox-LDL binding to vascular cells and macrophages, thus demonstrating an alternative mechanism by which platelet activation at sites of vascular injury may promote the accumulation of deleterious lipoproteins (54).

4. The CX3C chemokine and chemokine receptor

CX3C chemokines include an amino-terminal domain with a novel arrangement of three amino acids separating their first two cysteines. Fractalkine or CXCL1 is the only currently known member of the CX3C chemokine subfamily. It exists as membrane-bound and in soluble form. Membrane-bound fractalkine consists of an extracellular domain of 76 amino acids connected to an extended mucin-like stalk, followed by transmembrane and intracellular domains of 34 amino acids. Soluble fractalkine is released, presumably by proteolysis, at the membrane-proximal region by TNF-α-converting enzyme. Soluble fractalkine has been proved to be an efficient chemoattractant for monocytes and natural killer cells. The receptor of fractalkine (CX3CR1) is a seven-transmembrane domain G protein-coupled receptor, and fractalkine binds to it with high affinity, activating intracellular signalling and directly mediating monocyte adhesion (55,56).

Through its unique structural and functional characteristics, fractalkine displays properties of both chemokine and adhesion molecules (55,56). It also possesses chemoattractant activities for both monocytes and T cells and is up-regulated in cytokine-stimulated endothelial cells (56). Fractalkine is, therefore, a "perfect" candidate for an exceptional role in the pathogenesis of atherosclerosis. Indeed, fractalkine and its receptor, CX3CR1, have been implicated in atherogenesis by several studies under different settings. Immunochemical studies have confirmed its expression in monocytes/macrophages, endothelial and SMCs within human atherosclerotic coronary arteries (57). Additionally, Lucas *et al* reported that SMCs in the neointima of human atherosclerotic plaques express CX3CR1. They further demonstrated that primary cultured human coronary artery SMCs migrate toward fractalkine, suggesting that CX3CR1 also induces migration of SMCs to atherosclerotic lesions (58). Combadiere *et al*, by testing the potential role of fractalkine in double knockout mice (ApoE-/-, CX3CR1-/-), showed a significant decrease in lesion size in animals lacking the fractalkine-CX3CR1 signalling mechanism (59). Recent studies have also implicated fractalkine in platelet stimulation and activation. Activated platelets have been shown to exacerbate atherosclerosis in murine models of atherogenesis. Schafer *et al* demonstrated that platelets from rats, pre-incubated with fractalkine, had increased P-selectin surface expression. Also, pre-incubation with fractalkine enhanced platelet adhesion to collagen and fibrinogen (60).

In support of this evidence, several population-based genetic studies conducted in different population samples evaluated the effect of genetic variations of the CX3C receptor on susceptibility to coronary artery disease. Two polymorphisms were identified in the CX3CR1 gene, one which causes a codon change from valine to isoleucine at position 249 (CX3CR1-V249I), and another that causes a codon change from threonine to methionine at position 280 (CX3CR1-T280M). These changes are located in the sixth and seventh transmembrane domains, respectively (61). Several studies conducted in different population samples revealed an atheroprotective effect of these common single nucleotide polymorphisms of the CX3C receptor gene which have been shown to reduce the activity of the fractalkine-CX3CR1 pathway (21,62-64). Furthermore, subjects carrying these polymorphisms not only had mononuclear cells with decreased numbers of fractalkine binding sites (62) but also showed improved endothelium-dependent vasodilation (63).

The structural and functional uniqueness of fractalkine, demonstrated by multiple studies under different settings have clearly established a key role for fractalkine and its receptor in atherogenesis.

5. The C chemokines and chemokine receptors

The C chemokine family is represented by two chemokines, XCL1/lymphotactin-α and XCL2/lymphotactin-ß. Human lymphotactins recruit T lymphocytes and natural killer cells by interacting with their specific receptor, XCR1, in normal immune function and chronic inflammatory conditions (65). Nevertheless, there is currently no evidence in the literature supporting a critical role for C chemokines in atherosclerosis.

6. Discussion

Recent literature provides convincing evidence that chemokines are involved in all aspects of atherosclerosis; orchestration, migration, proliferation and accumulation of the cell populations participating in atherosclerosis. It is well established that atherosclerosis is a chronic inflammatory process taking place in the vascular wall. Chemokines are important mediators

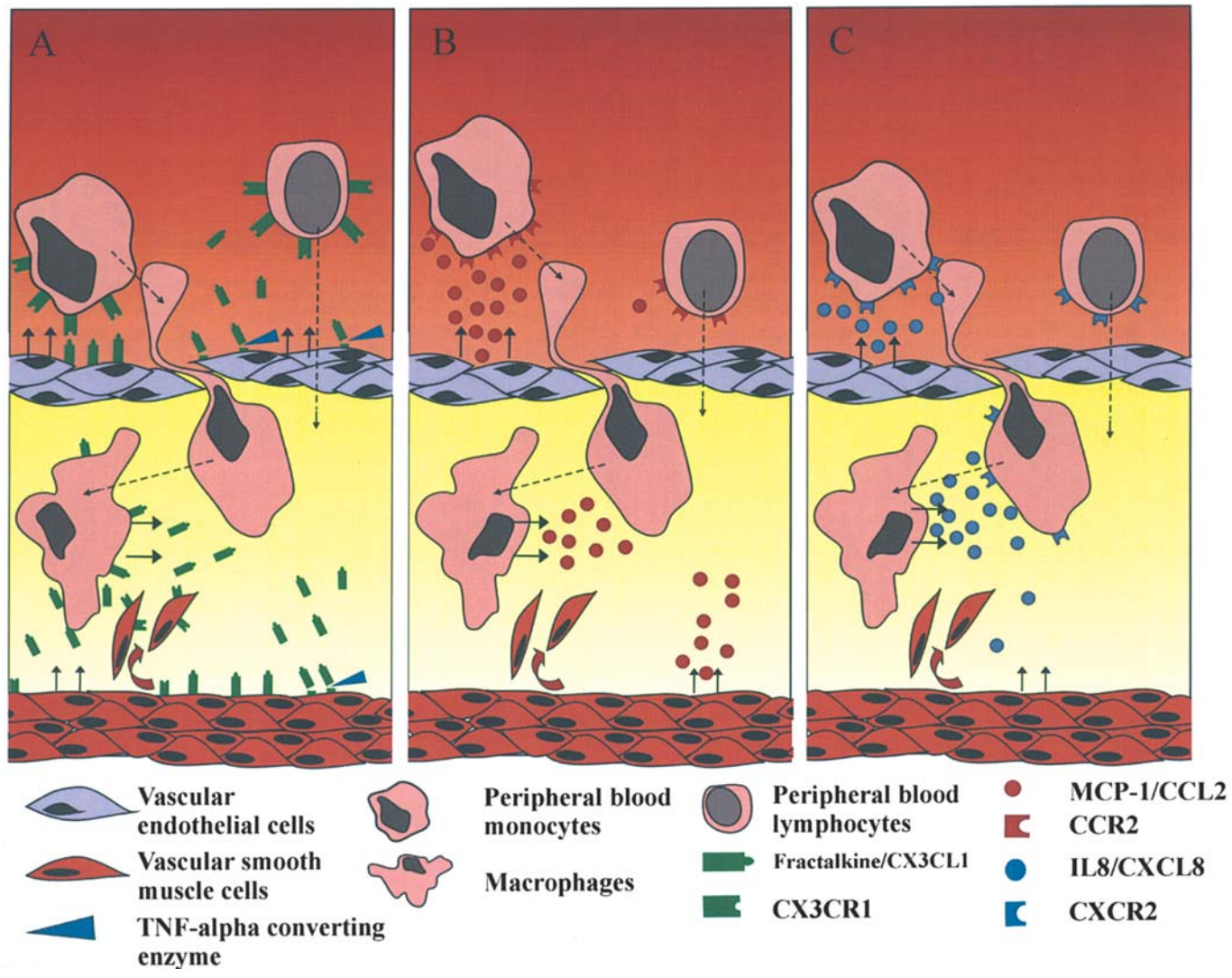

Figure 1. Schematic presentation of the main chemokine-mediated pathways involved in atherosclerosis. Fractalkine is expressed by vascular endothelial cells, smooth muscle cells and presumably by macrophages of the intima and acts either as a chemotactic cytokine or as an adhesion molecule. In both forms fractalkine promotes the accumulation and migration of monocytes and T cells in atherosclerotic lesions. It has been also implicated in vascular smooth muscle cell migration in the process of advanced-lesion formation (A). MCP-1 is highly expressed by lesions' macrophages but also by endothelial cells and smooth muscle cells and is a major chemoattractant for circulating monocytes and activated T cells. MCP-1/CCR2 pathways have also been implicated in the accumulation and migration of smooth muscle cells in atherosclerotic lesions. (B). Finally IL-8 is expressed by vascular endothelial cells, smooth muscle cells and macrophages and is a potent chemoattractant for circulating monocytes and T cells. It has been also shown to contribute in smooth muscle cell proliferation and migration (C).

of inflammation and have been implicated in a specific or non-specific manner in almost every inflammatory disease. Several chemokine ligands and receptors have been extensively investigated and have been proved to be involved in more disease-specific ways in atherosclerosis. We consider MCP1/CCR2, IL-8/CXCR2 and fractalkine/CX3CR1 as the key chemokine-receptor pathways most definitely participating in atherogenesis (Fig. 1). Their role has been demonstrated by studies conducted in *in vitro* systems, in animal models of accelerated atherosclerosis, and in genetic epidemiology studies. Nevertheless, an important issue is whether knowledge derived from indirect investigations can be applied in disease management practices. Further clinical evaluations are clearly required to establish the diagnostic or prognostic potential of these chemokines in atherosclerosis and, perhaps, to rationally design strategies for chemokines and their receptors as therapeutic targets.

Chemokines as markers of atherosclerosis. The inflammatory process participates in the pathogenesis of atherosclerosis and affects clinical presentation of atherosclerotic disease. Several mediators of inflammation have been tested as potential markers of atherosclerosis. Elevation of plasma C-reactive protein, using new sensitive assays, predicts an increased risk of cardiovascular events in patients with unstable and stable angina pectoris. An association between elevated plasma levels of intercellular adhesion molecule (I-CAM) and risk of coronary heart disease has also been established (66). Circulating levels of chemokine ligands and receptors have also been investigated as potential markers of atherosclerosis with controversial results. The most promising evidence comes from studies evaluating serum levels of MCP-1. In a large population-based study by Deo *et al*, plasma levels of MCP-1 were associated with traditional risk factors for atherosclerosis. The results support the hypothesis that MCP-1 may mediate

some of the atherogenic effects of these risk factors and underlie the potential role of MCP-1 as a biomarker and target for drug development (67). In a more recent study by Hoogeveen *et al*, mean plasma MCP-1 levels were found to be significantly higher in peripheral artery disease patients than controls. MCP-1 levels correlated significantly with other inflammatory markers in comparison subjects. Furthermore, incident coronary heart disease was significantly associated with increased MCP-1 levels, independent of other cardiovascular risk factors, suggesting that MCP-1 is associated with atherosclerotic disease in two different vascular pathologies (68). Finally and most impressively, in a large cohort, De Lemos *et al* demonstrated that, in patients with acute coronary syndromes, an elevated baseline level of MCP-1 was associated with both traditional risk factors for atherosclerosis as well as an increased risk of death or myocardial infarction, independent of baseline variables. Thus, MCP-1 appears to play a crucial role at multiple stages of atherosclerosis, and is a potent alternative biomarker and a possible therapeutic target (69). Similarly to the above, Romuk *et al* evaluated levels of CXC chemokine IL-8 and demonstrated significantly higher levels of IL-8 in unstable coronary heart disease patients in comparison to stable coronary heart disease patients and controls, concluding that a soluble form of IL-8 may be a useful clinical predictor of unstable coronary heart disease (70).

Nevertheless, there are still several limitations in the use of circulating chemokines as markers of atherosclerosis, mainly due to the low specificity of such tests. However, reports on circulating levels of chemokines as markers of atherosclerotic activity under experimental conditions are encouraging. For instance, Troseid *et al* evaluated the effect of physical exercise on vascular inflammation using peripheral markers of inflammation in subjects with metabolic syndrome and demonstrated a significant reduction in MCP-1 and IL-8 in the combined exercise groups compared to the combined non-exercise groups, proposing that the protective effect of exercise might, in part, be due to suppression of the inflammatory process (71).

Additionally, prognostic genotyping evidence from recent literature seems promising. According to several studies, the presence of certain chemokine polymorphisms independent of other established risk factors has been associated with increased prevalence of CAD, acute coronary syndromes, and other cerebrovascular pathologies (21,62-64,72). Thus, such polymorphisms could potentially be applied to identify patients with a high risk of developing atherosclerosis and render a more precise estimation of the individual's overall cardiovascular risk.

Chemokine ligands and receptors as therapeutic targets. Chemokines are important therapeutic targets. Most of the efforts in this area have been directed towards the development of chemokine receptor antagonists. Currently, there are several studies available in the literature evaluating chemokine receptor antagonists as therapeutic targets in several diseases. For instance, regarding CC chemokines, the efficacy of CCR1 antagonists is being evaluated for the treatment of rheumatoid arthritis and multiple sclerosis. A monoclonal antibody blocking the binding of MCP-1 to CCR2 is also being tested for the treatment of rheumatoid arthritis. CCR5 antagonists that block HIV entry into cells (AOP-RANTES, met-RANTES) are being evaluated in advanced clinical trials as adjuvant treatments for AIDS. Furthermore, small-molecule inhibitors of CX3CR1 are being tested as a potential treatment for psoriasis and rheumatoid arthritis, and CXCR4 antagonists are being evaluated for efficacy in rheumatoid arthritis and cancer (73,74). Chemokine receptors have thus proven to be attractive therapeutic targets, especially regarding traditional inflammation-mediated diseases.

Chemokine antagonists have been proposed for potential treatment of vascular disease. Ni *et al* demonstrated a new strategy for anti-MCP-1 gene therapy to treat atherosclerosis by transfecting an N-terminal deletion mutant of the human MCP-1 gene into the skeletal muscle in ApoE-/- mice. This strategy effectively blocked MCP-1 activity and inhibited the formation of atherosclerotic lesions. Furthermore, it increased the lesional extracellular matrix content. Authors demonstrated that anti-MCP-1 gene therapy may serve not only to reduce atherogenesis but also to stabilize vulnerable atheromatous plaques and proposed that this strategy could be a useful and plausible form of gene therapy against atherosclerosis in humans (16). Additionally, Inoue *et al* reported that blockade of MCP-1 by transfecting an N-terminal deletion mutant of the MCP-1 gene limited progression of pre-existing atherosclerotic lesions in the aortic root in hypercholesterolemic mice and changed the lesion composition into a more stable phenotype. This strategy decreased expression of CD40 and the CD40 ligand in the atherosclerotic plaque and normalized the increased chemokine and cytokine gene expression, suggesting that MCP-1 is an essential mediator in the progression and destabilization of established atheroma (17).

Restenosis may be a more promising target for chemokine antagonists than atherosclerosis. Prevention of restenosis after coronary intervention is a major clinical problem, which highlights the need for new therapeutic options. Usui *et al* demonstrated that transfection of an N-terminal deletion mutant of the MCP-1 gene into skeletal muscles suppressed monocyte infiltration/activation in the injured site and markedly inhibited restenotic changes after balloon injury of the carotid artery in rats and monkeys. This strategy also suppressed the local production of MCP-1 and inflammatory cytokines. The authors concluded that monocyte infiltration and activation mediated by MCP-1 are essential in the development of restenotic changes after balloon injury and suggested this strategy as a potential form of gene therapy against human restenosis (75).

Although studies in mice and rats have established the importance of chemokines, particularly that of MCP-1, in the development of atherosclerosis, large-scale studies evaluating end points such as acute coronary syndrome or cardiovascular cause of death, are essential to substantiate the use of chemokine antagonists in the treatment of cardiovascular disease in humans.

It might be some time before chemokine antagonists are available for the treatment or prevention of atherosclerosis. However, there is evidence that several agents already in use as a treatment of either atherosclerosis or its risk factors modify chemokine expression. For instance, HMG-CoA reductase inhibitors have been demonstrated by several studies to reduce,

in vitro and *in vivo,* the expression of several markers of vascular inflammation, including chemokines (33). Similar findings have been demonstrated for angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockade and glitazones (76-78). Thus, some widely used anti-atherogenic drugs could mediate their beneficial actions partially through inhibition of certain chemokine pathways.

In conclusion, in the past few years we have witnessed a rapid increase in our understanding of the role of chemokines and their receptors in cardiovascular pathologies. Nevertheless, all investigators agree on the fact that the precise mechanism of the chemokine pathways involved in the establishment and progression of atherosclerosis is not fully elucidated and much more information is needed before chemokine-based therapies can be applied in clinical practice.

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