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The role of selected chemokines and their receptors in the pathogenesis and destabilisation of atheromatous plaques in the carotid arteries

Rola wybranych chemokin i ich receptorów w patogenezie rozwoju i destabilizacji blaszki miażdżycowej w tętnicy szyjnej

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Abstract

Chemokines are cytokines that act selectively on cells and are capable of inducing selective migration of cells *in vitro* and *in vivo*. The term was first coined at the 3rd International Symposium on Chemotactic Cytokines in 1992. The name "chemokine" is a contraction of "chemotactic cytokine," meaning that these molecules combine features of both cytokines and chemotactic factors. They are a family of low-molecular-mass proteins acting on specific membrane receptors. A cell's overall sensitivity to chemotaxis depends on the expression profile of chemokine receptors. Atherosclerosis is essentially an excessive inflammatory and proliferative response to the damage of arterial walls. It takes place within the wall and leads to the formation of unstable atherosclerotic plaques. Many chemokines have been studied in terms of their role in the pathogenesis of an atheromatous plaque in the carotid arteries, both in animal models and with the use of human tissue. It seems that molecules that are the most involved in the formation of atheromas in the carotid arteries include: CCL2, CCL3, CCL4 and CCL5. However, reports are sometimes contradictory, and more research is needed. Finding a marker that could help predict the destabilisation of an atheromatous plaque would be a valuable addition to the standard diagnostic panel of tests used in both the diagnosis and monitoring of vascular pathologies.

Key words: chemokines, chemokine receptors, atheromatous plaque, mediators of inflammation

Streszczenie

Chemokiny są cytokinami, które działają na wybrane komórki i mają zdolność stymulowania migracji komórek *in vitro* i *in vivo*. Nazwa „chemokina" została utworzona na Trzecim Międzynarodowym Sympozjum Cytokin Chemotaktycznych w 1992 roku. Chemokiny są chemotaktycznymi cytokinami, czyli łączą w sobie cechy charakterystyczne dla czynników chemotaktycznych oraz cytokin. Są rodziną małocząsteczkowych białek, które działają poprzez pobudzanie swoistych dla nich receptorów błonowych. Profil ekspresji tych receptorów decyduje o wrażliwości komórek na bodziec chemotaktyczny. Miażdżyca zaliczana jest do procesu chorobowego, w którym mamy do czynienia z nadmierną, zapalno-proliferacyjną odpowiedzią na uszkodzenie ściany tętnicy. Proces zapalny toczący się w obrębie ściany naczynia wiąże się z rozwojem niestabilnych zmian miażdżycowych. Dotychczas przebadano wiele chemokin pod kątem ich udziału w rozwoju blaszki miażdżycowej w tętnicach szyjnych, zarówno na modelach zwierzęcych, jak i w badaniach na materiale ludzkim. Wydaje się, że największą rolę w rozwoju miażdżycy w tętnicach szyjnych odgrywają chemokiny CCL2, CCL3, CCL4 oraz CCL5. Jednakże doniesienia na ten temat są często niejednoznaczne i wymagają prowadzenia dalszych badań. Znalezienie markerów zapalnego podłoża destabilizacji blaszek miażdżycowych może stanowić istotne uzupełnienie badań diagnostycznych stosowanych w rozpoznawaniu i monitorowaniu leczenia niektórych chorób. Co ważne, szczegółowe poznanie roli wybranych chemokin i ich receptorów w rozwoju miażdżycy może przyczynić się do dokładniejszego zrozumienia mechanizmu powstawania niestabilnej blaszki miażdżycowej.

Słowa kluczowe: chemokiny, receptory chemokinowe, blaszka miażdżycowa, mediatory zapalenia

ATHEROMATOUS PLAQUE AND ITS DESTABILISATION

Atherosclerosis is a chronic inflammatory condition, manifested focally mostly in medium and large arteries. Environmental factors as well as genetic predisposition are involved in its aetiology. Many types of cells participate in the process, including monocytes, macrophages, T cells, vascular smooth muscle cells and endothelial cells. The disease manifests itself morphologically by the thickening within the inner layer of the arterial wall (the tunica intima). More specifically, numerous lipoprotein-laden macrophages (foam cells) become accumulated within the subendothelial layer. These deposits, known as fatty streaks, are the initial stage of atherosclerosis. A fully formed plaque consists of a fibrous cap and a core. The cap is a solid layer of connective tissue rich in collagen and vascular smooth muscle whereas the lipid core is soft, fragile and highly thrombogenic. The overall stability of an atheroma is a resultant of the ratio of its components as well as the presence of inflammatory cells.

FORMATION OF UNSTABLE ATHEROMATOUS PLAQUES IN CAROTID ARTERIES

Exacerbations of chronic inflammation of a plaque are responsible for its destabilisation (Opolski *et al*., 2002). Morphologically, such a plaque is more vascular, has a thinner fibrous cap that is prone to rupture and attracts more inflammatory cells. The lipid core swells up with liquid cholesterol esters (Kaźmierski *et al*., 2014; Opolski *et al*., 2002). The newly formed and expanding blood vessels are abnormal and prone to haemorrhages within and around the plaque, which may lead to its rupture. IFN-gamma plays an important role by inactivating myocytes (thereby inhibiting the synthesis of collagen) and by stimulating macrophages to release metalloproteinases (MMP) responsible for degrading connective tissue thus thinning the fibrous cap (de Boer *et al*., 1999; Nakajima *et al*., 2002). Both the macrophages as well as myocytes found in unstable atheromas show increased expression of MHC II molecules (Kaźmierski *et al*., 2014; Liuzzo *et al*., 2000). The presence of CD4+ T helper cells indicates that the disruption of endothelium leads to a subsequent immune reaction (Kaźmierski *et al*., 2014; Liuzzo *et al*., 2000). Activated lymphocytes release pro-inflammatory cytokines, chemokines and interleukins which bolster the inflammatory response within the vessel wall (Raines *et al*., 1996). Unstable plaques are susceptible to ruptures. Mural thrombi, forming over such areas, may rapidly occlude the vessel and cause acute ischaemia of the area supplied by the artery (Hennerici, 2004). The search for factors contributing to the destabilisation of atheromatous plaques continues.

INTRODUCTION TO CHEMOKINES AND THEIR RECEPTORS

Chemokines are cytokines that act selectively on cells and are capable of inducing selective migration of cells *in vitro* and *in vivo* (Koenen and Weber, 2010). The term was first coined at the 3rd International Symposium on Chemotactic Cytokines in 1992. However, the first description of a molecule belonging to this family, CXCL4, comes from 1961. It was not until the research on a similar molecule CXCL8 (Il-8) was conducted 20 years later that their chemotactic abilities were discovered. The name "chemokine" is a contraction of "chemotactic cytokine," meaning that these molecules combine features of both cytokines and chemotactic factors. In other words, they can induce movement (*-kinos* in Greek) in response to a chemical gradient (chemotaxis). They are a family of low-molecular-mass proteins acting on specific membrane receptors (Koenen and Weber, 2010). A cell's overall sensitivity to chemotaxis depends on the expression profile of chemokine receptors. In addition to chemotaxis, the molecules are also involved in activating adhesion molecules (they participate in the interaction between the endothelium and leukocytes), proliferation and apoptosis pathways (Wang *et al*., 2006). Chemokines may be subdivided into pro-inflammatory and lymphoid according to their function. Their involvement in angiogenesis and cell differentiation processes was documented as well, which explains their role in the pathogenesis of diseases such as myocardial infarction, ischaemic stroke, multiple sclerosis, Alzheimer's disease and some brain tumours (Fernandez and Lolis, 2002). Currently, there are not many studies on these issues. New clinical observations involving larger groups of patients are therefore necessary.

STRUCTURE OF CHEMOKINES AND THEIR RECEPTORS

Forty-eight human chemokines and 20 of their receptors are known (Murphy, 2002). Chemokines are very basic proteins of low molecular weight (8–12 kDa) and usually between 70 and 130 amino-acid-long. Despite being heterogeneous in terms of the amino acid sequence, they typically exhibit a similar tertiary structure (Murphy, 2002). Chemokines have been classified into four subfamilies: CXC (α-chemokines), CC (β-chemokines), C (γ-chemokines) and CX3C (δ-chemokines), with their respective receptors (CXC-R, CC-R, C-R, CX3C-R). The classification is based on different ways that cysteine residues can be spaced, i.e. in CXC the two cysteines are separated by an amino acid, while in the CC class the cysteines are adjacent. The letter "L" stands for ligand, and a number completes the name (e.g. CXCL12). The letter "R" stands for receptor (e.g. CXCR4) (Baggiolini, 2001). Chemokines are made up of at least three distinct elements – an N-terminus, a β-sheet and a C-terminal α-helix.

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The N-terminus typically contains four cysteine residues, with the exception of lymphotactins (XCL1, XCL2) which contain only two cysteines. However, their structure as well as functionality is similar to other chemokines. There are fewer human chemokine receptors than individual molecules – only 20 have been identified thus far. They are "promiscuous" – various chemokines can bind to a given receptor. Also, one chemokine exhibits affinity to several receptors. Based on their amino acid sequence, chemokine receptors belong to the class A of rhodopsin-like receptors. Chemokine receptors, despite their heterogenicity, share many similarities. They are between 340 and 370 amino-acid-long. They form 7 transmembrane loops of which the cytoplasmatic fragments of the second and third loops are coupled with G proteins (G protein-coupled receptors, GPCR). All chemokine receptors contain two conservative cysteines – one in the N-terminus and the other in the third extracellular loop. The two amino acids are linked by a disulphide bond (Baggiolini *et al.*, 1997). Another characteristic feature is the presence of multiple serine and threonine residues in the C-terminus, which become phosphorylated upon binding to the ligand. The receptors may also interact with one another. CXCR2, CCR2 and CCR5 receptors form homodimers. CXCR4 and CCR2 dimerize upon binding to a chemokine, which may be necessary for further signal transmission into the cytoplasm (Mellado *et al.*, 2001) (Fig. 1).

FUNCTIONAL CLASSIFICATIONS OF CHEMOKINES

Functionally, chemokines may be divided into proinflammatory and lymphoid molecules. Some share characteristics of both groups. Proinflammatory chemokines are released in the presence of pathogens or other stimuli that induce inflammation. Their receptors are expressed on phagocytic cells such as neutrophils, monocytes, immature dendritic cells, and in some stages of T cell development. These molecules play an important role in defending the organism from external pathogens, as part of the innate immunity as well as in non-infectious inflammatory diseases. Lymphoid chemokines, sometimes referred to as "homeostatic chemokines," are expressed in primary (thymus, marrow) and secondary lymphoid organs (spleen, lymphatic nodes) (Ohl *et al.*, 2003). Some lymphoid chemokines are equally expressed in diffuse lymphoid tissue – in the skin (SALT), the gut (GALT) and mucous membranes (MALT) (Cupedo and Mebius, 2003). Receptors for these particular chemokines are found on the surface of T and B lymphocytes and mature dendritic cells. Regulating interactions between T cells, B cells and dendritic cells, as well as their migrations within the lymph nodes, play a crucial role in the development of appropriate immune responses, therefore certain chemokines appear to be equally important in physiological conditions.

SELECTED CHEMOKINES AND THEIR RECEPTORS IN THE PATHOGENESIS OF ATHEROSCLEROSIS OF CAROTID ARTERIES

Many chemokines have been studied in terms of their role in the pathogenesis of an atheromatous plaque in the carotid arteries, both in animal models and with the use of human tissue. It seems that molecules that are the

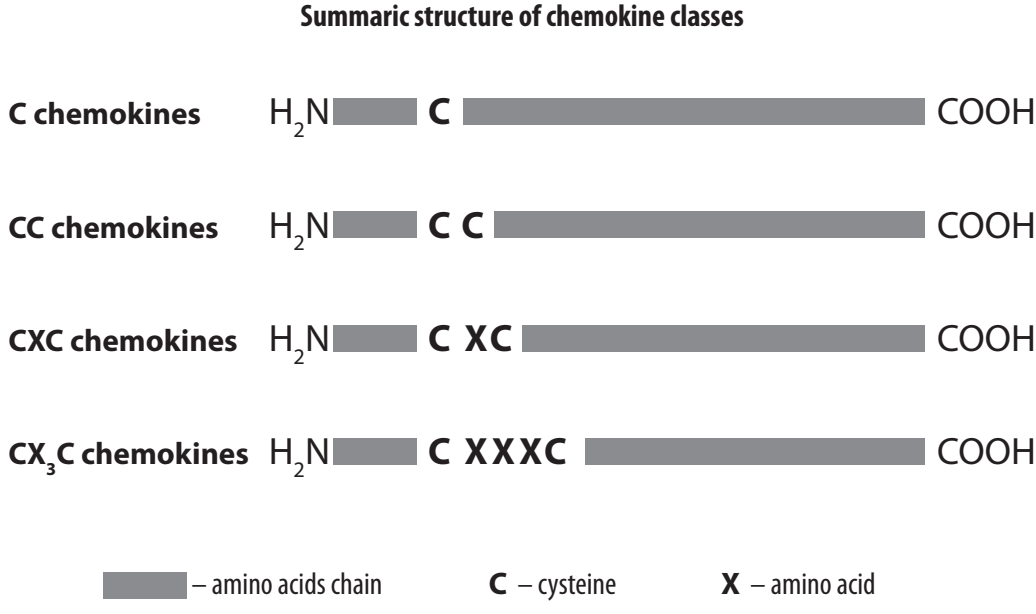

Fig. 1. Structure of chemokine classes

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most involved in the formation of atheromas in the carotid arteries include: CCL2 (monocyte chemoattractant protein-1, MCP-1), CCL3 (macrophage inflammatory protein-1α, MIP-1α), CCL4 (macrophage inflammatory protein-1β, MIP-1β) and CCL5 (regulated on activation, normal T cell expressed and secreted, RANTES). However, reports are sometimes contradictory, and more research is needed. Several studies in human beings and animal models have suggested an active role of CCL2 in atherosclerotic plaque vulnerability (Wahlgren *et al.*, 2009). It is produced by monocytes, endothelial cells, fibroblasts and vascular smooth muscle cells. Increased concentrations of this chemokine have been found in atheromas with high macrophage content. The type of leukocyte recruited by a chemokine depends on the expression profile of specific receptors on its surface. In this case an interaction between CCL2 and its receptor CCR2 found on monocytes stimulates their migration and activation within an atheromatous plaque (Ikeda *et al.*, 2002). CCL2 is also at least partially responsible for the hyperplasia of vascular smooth cells in the intima, which leads to the formation of a thickened intima-media complex. Concentrations of CCL2 on the surfaces of these cells (vascular smooth muscle cells, mesenchymal cells of the intima, mononuclears) in atheromas may be studied in samples obtained during carotid endarterectomies. In transgenic animal models, the silencing of genes encoding CCL2 and CCR2 produced a decrease in the frequency and severity of atherosclerosis (Boring *et al.*, 1998). CCL3 is another chemoattractant of monocytes and neutrophils to inflamed plaques (Montecucco *et al.*, 2008). It exerts its proinflammatory properties by binding to transmembrane receptors (CCR1 and CCR5) found on inflammatory cells (Ottonello *et al.*, 2005). CCL3-mediated neutrophil recruitment has been shown in the presence of other proinflammatory molecules, such as TNF-α or insulin (Montecucco *et al.*, 2008). These studies indicate that CCL3 could influence the migration of different inflammatory cell subsets within both injured brain and atherosclerotic plaques. CCL4 (MIP-1β) induces monocyte recruitment towards the site of inflammation by binding to a transmembrane CCR5 receptor. Recent evidence has shown that CCL4 mRNA expression was upregulated in downstream portions of human carotid plaques as compared to upstream in both symptomatic and asymptomatic patients with ischaemic stroke (Montecucco *et al.*, 2010). Tatara *et al.* (2009) studied whether CCL4 was a risk factor of ischaemic stroke in patients with hypertension. In their work, conducted on a group of 551 patients, they concluded that CCL4 plasma concentrations were an independent risk factor of ischaemic cerebro- and cardiovascular incidents in patients with hypertension. The involvement of the proinflammatory chemokine CCL5 (RANTES) in atherosclerosis and ischaemia/reperfusion syndrome has been investigated in recent years. It is secreted by macrophages, platelets and T cells. RANTES selectively binds three transmembrane receptors: CCR1, CCR3 and CCR5. The CCR5 receptor is expressed on macrophages, endothelial cells and vascular myocytes. In humans, CCL5 is expressed on macrophages and T cells. Therefore, a CCL5/CCR5 interaction recruits these cells to a plaque. Several studies have confirmed the role of CCL5 in the promotion of atherogenesis in humans and mice (Koenen *et al.*, 2009). Braunersreuther *et al.* (2007) demonstrated that the genetic deletion of Ccr5, but not Ccr1, inhibits the development of atherosclerosis in ApoE-knockout mice fed a high-cholesterol diet. The authors of the study were hopeful that the promising results in mice would be applicable to humans, and suggested that the CCR5 receptor could be a target of atherosclerosis treatments. When Montecucco *et al.* (2010) compared symptomatic and asymptomatic patients, who had undergone a carotid endarterectomy procedure, they found the CCL5 plasma concentrations to be much higher in the symptomatic group 30 days after ischaemic stroke. The differences in the levels of other serum inflammatory markers (such as CRP, CCL2, CCL3, CCL4) were statistically insignificant. Conversely, the increase in the serum levels of CCL2 and CCL5 was not confirmed by Zaremba *et al.* (2006), who measured these chemokines in the first week after ischaemic stroke. In contrast, serum CCL3 levels on days 1, 2 and 3 of stroke were significantly higher than in the control group. In *in vitro* tests, CCL5 has been shown to induce migration of peripheral blood mononuclear cells across the activated blood-brain barrier (BBB). On the other hand, CCL5 could also increase cerebral damage through the secondary induction of other potent proinflammatory cytokines (such as IL-6) (Shahrara *et al.*, 2006).

ROLE OF SELECTED CHEMOKINES AND THEIR RECEPTORS IN PLAQUE DESTABILISATION

Animal models have been a great aid in the studies on the potential link between chemokine expression levels and plaque progression to the unstable state. Winnik *et al.* (2011) studied whether plasma CCL5 levels could be indicative of the plaque's condition, and serve as markers of plaque vulnerability in the carotid arteries. They established that circulating RANTES levels may help identify the extent of atherosclerosis but appear to be of a limited value for the identification of unstable lesions. However, Lv *et al.* (2014) reached a different conclusion. Their study revealed that serum RANTES levels were significantly higher in the rabbit models of the vulnerable atherosclerotic plaque (VAP) group than in the AS (atherosclerosis) group and in the controls.

The role of RANTES as a biomarker for unstable atherosclerotic plaques has also been suggested by Böger *et al.* (2005), who measured RANTES levels in the cells and extracellular matrix of unstable plaques in type 2 diabetes

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mellitus haemodialysis patients and correlated it with RANTES gene polymorphism. The variants with higher chemokine expression were associated with a higher all-cause mortality, mostly due to cardiac events. CXCL16, a chemokine of the CXC family, has been proposed as an important mediator in atherosclerosis. The characteristics of the cellular expression of CXCL16 shows that it is abundant in human monocyte-derived macrophages, dendritic cells, B cells, smooth muscle cells, T cells and endothelial cells. Yi's (2011) investigations indicate that circulating CXCL16 becomes much higher in atherosclerosis, and it may be an atherogenic marker in the plasma. Furthermore, research on knockout mice has proven that CXCL16 overexpression promoted the evolution of preexisting lesions to vulnerable plaques. Segers *et al*. (2011) focused on CXCL10. The team proved that the molecule plays a functional role in the destabilisation of atherosclerotic plaques in mice and is specifically upregulated in vulnerable plaques in humans. In human carotid arteries, CXCL10 levels determined the morphology of lesions. This was evidenced by an increased number of atheromatous plaques with increasing CXCL10 concentrations, and by the association with unstable plaque characteristics, such as macrophage dominance and reduced presence of smooth muscle cells and collagen.

SUMMARY

Filling the gaps in our understanding of the role of inflammation in the formation of plaques would perhaps yield a clinically applicable marker of plaque instability, which would be a useful addition to presently available imaging modalities. Pioneer studies using transgenic models involving gene silencing and the use of chemokine receptor agonists show promising results and give hope for finding new targets for the treatment of vascular pathologies.

Author contributions

Maria Konarska-Król and Magdalena Justyna Kacperska contributed equally to this work.

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Conflict of interest

The authors declare that there are no conflict of interest.

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