REVIEW

Immunological Aspects of Atherosclerosis

A. KRÁLOVÁ[1], I. KRÁLOVÁ LESNÁ[1], R. POLEDNE[1]

[1]Laboratory for Atherosclerosis Research, Centre of Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague, Czech Republic

Summary

Atherosclerosis is a degenerative inflammatory disease of the vascular wall, which is characterized by the formation of atherosclerotic plaques that contain lipids, activated smooth muscle cells, immune cells, foam cells, a necrotic core and calcified sites. In atherosclerosis pathology, monocytes and macrophages play the most important role by accumulating redundant LDL particles in their oxidized form and producing proinflammatory cytokines. Atherosclerotic plaque macrophages reveal distinct phenotypes that are distinguished into M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages. Numerous environmental signals (cytokines, microbial cell molecules) that are received by macrophages drive their polarization, but it must be determined whether this classification reflects different macrophage subtypes or plasticity and phenotypic tissue changes, but the balance between subsets is crucial. M1 macrophages are dominant in symptomatic atherosclerotic plaques, while M2 macrophages are more frequent in asymptomatic plaques. Nevertheless, a positive correlation of both M1 and M2 macrophages with atherosclerotic lesion severity was also observed.

Corresponding author

A. Králová, Laboratory for Atherosclerosis Research, Centre of Experimental Medicine, Institute for Clinical and Experimental Medicine, Vídeňská 1958, 140 21, Prague, Czech Republic. E-mail: urba@medicon.cz

Introduction

Atherosclerosis is a degenerative inflammatory disease of the vascular wall, which is clinically manifested in ischemic heart disease, peripheral arterial disease, stroke or sudden death. It is characterized by the formation of atherosclerotic plaques, which contain lipids, activated smooth muscle cells, immune cells, foam cells, a necrotic core and calcified sites (Ross 1999).

The best description of pathological changes is represented by a classification that was published by Stary *et al.* (1994). In total, six stages of atherosclerotic lesions can be distinguished. The term "early lesions" is sometimes used for type I and II lesions, which are ordinarily already present in childhood. Both type I and II lesions are characterized by small lipid deposits in the arterial intima and minor endothelial changes. Type II is generally referred to as fatty streaks, which are characterized by increases in the number of monocytes, intimal macrophages and foam cells. Fatty streaks do not cross into the arterial lumen and therefore do not affect blood flow (Stary *et al.* 1994). Type II is the first visible lesion and is characterized by layers of macrophages, foam cells and lipid droplets within the intimal smooth muscle cells. Lipid-laden foam cells are derived mainly from macrophages, but smooth muscle cells and other vascular cells might also be involved (Shashkin *et al.* 2005). Type III lesions are intermediate and transitional, and they serve as a connection between type II and advanced lesions (Stary *et al.* 1994). Advanced atherosclerotic lesions can also be divided into three types – IV, V and VI. The type IV lesion is also known as atheroma, and it is characterized by severe intimal

disorganization caused by the lipid core. Type V lesions are defined by formation of new fibrous connective tissues. Disruptions of the lesion surface, hematoma, hemorrhage and thrombotic deposits can develop in these advanced lesions. Type IV or V lesions with one or more of these features are classified as type VI and may also be referred to as complicated lesions (Stary *et al.* 1995).

Theories of atherogenesis pathology

Atherosclerosis pathology research began more than 100 years ago with pioneering German and Russian studies recognizing lipid and inflammatory theories.

Further development of atherosclerosis patholology started in the 1950s by Keys documenting a crucial role of cholesterol concentration.

In 1985 Brown and Goldstein discovered that human cells have low-density lipoprotein (LDL) receptors on cell surfaces that remove cholesterol from the blood. If LDL receptors are not present in sufficient numbers or if genetic defects in LDL receptor structure occur, individuals develop hypercholesterolemia and become at risk for cholesterol related diseases (Goldstein and Brown 1973).

In the 1990s, Virchow's hypothesis was rediscovered by Russell Ross. He formulated the "response to injury" hypothesis (for summary see Ross 1999), which proposed that endothelial damage is the first step in the development of atherosclerotic lesions followed by monocyte and T-cell infiltration into the arterial wall (Jonasson *et al.* 1986). Although this protective effect of the intact endothelium to modify LDL atherogenic lipoprotein and monocyte influx into arterial wall has been questioned, the pioneer papers of Ross are still frequently referenced, and so-called endothelial dysfunction is supposedly the first sign of preclinical atherosclerosis development. It should be emphasized that under physiological conditions, transcellular LDL particle transport occurs through the endothelium (Rosengren *et al.* 2002).

Because it is perceived that the main function of monocytes and T cells is connected to local or systemic infection, several theories that proposed infectious disease as the cause of atherosclerosis appeared in the 1970s (for summary see Epstein *et al.* 2009). This concept is also supported by experiments performed by Fabricant *et al.* (1978), who found the presence of arterial lesions in chickens that had been infected with avian herpes virus. These lesions were similar to atherosclerotic

changes in humans. Later, several other infectious agents, which might be responsible for atherosclerotic changes in humans, began to appear gradually. These included Chlamydia pneumoniae, Cytomegalovirus, Helicobacter pylori, hepatitis A virus, Porphyromonas gingivalis, Herpes simplex virus and others, which most likely reflect a synergy of multiple infectious agents in the development of atherosclerosis. Antibodies against various pathogens were detected in atherosclerotic plaques. It was observed that in the case of Chlamydia pneumonia, the DNA of which was found in atherosclerotic plaques by polymerase chain reaction (PCR) (Farsak *et al.* 2000), there is invasion and damage in the arterial wall, increased LDL uptake by macrophages and foam cell formation in the neointima (Kalayoglu and Byrne 1998). Cytomegalovirus infection increased adhesion molecule expression, particularly intercellular adhesion molecule 1 (ICAM-1), on the endothelial surface (Altannavch *et al.* 2002). The animal model demonstrated cross immune response against heat shock protein 60 (HSP-60) – antigen located on the surface of Helicobacter pylori and on the endothelial cell surface (Ayada *et al.* 2009).

It is quite clear that viral and bacterial infections likely do not play a direct role in the process of atherosclerosis, as a sharp decrease of myocardial infarction mortality was not related to incidence of different infectious diseases (Dvořáková and Poledne 2004). Concurrently, a certain proinflammatory state, which is genetically determined, determines different monocyte behavior (Hubáček *et al.* 1999). The proinflammatory state marker could be C-reactive protein (CRP), as a number of studies have revealed that inflammatory markers including CRP and proinflammatory cytokines are strong predictors of atherosclerosis and other cardiovascular diseases (Ridker *et al.* 1998). The importance of the proinflammatory state in atherogenesis was highlighted by findings that increased CRP concentration in the "normal" range represents a significant marker of cardiovascular disease risk (de Maat and Trion 2004). It has been documented that in healthy men who later suffered a myocardial infarction (MI), the concentrations of IgA, IgE and IgG antibodies were significantly higher than in control (Kovannen *et al.* 1998).

The level of subclinical inflammation might be affected by several genetic and environmental factors including the effect of individual diet. There is considerable evidence that visceral fat in obesity plays

a crucial role in the inflammatory process as a chronic inflammatory disease with dysregulation of pro- and anti-inflammatory cytokine production (for summary see Lyon *et al.* 2003). It was also documented that a switch from a saturated fatty acid diet to a polyunsaturated fatty acid diet reduced CRP concentration (Králová Lesná *et al.* 2013). CRP concentration increased with increasing body weight and is related to visceral fat volume (Tintěra *et al.* 2004). A positive relation of CRP to body mass index (BMI), waist circumference and triglyceride concentration was also documented. Substantial sex differences were observed in the relationship of CRP to age. Whereas it is continuously increasing in men, this increase appears in women only after menopause. A decrease in body weight and visceral fat volume by increased physical activity was accompanied by a significant decrease of CRP levels in young obese women (Poledne *et al.* 2009).

Monocyte and macrophage participation in atherosclerosis development

Many studies about atherosclerosis have focused on monocytes and macrophages because they are involved in the inflammatory process (Libby *et al.* 2013). In atherosclerosis pathology, monocytes and macrophages play the most important role by accumulating of redundant LDL particles in their oxidized form and proinflammatory cytokine production (Fig. 1). Monocyte and macrophage presence in atherosclerotic plaque as well as neutrophils, dendritic cells and mastocytes has been well documented (Galkina and Ley 2007).
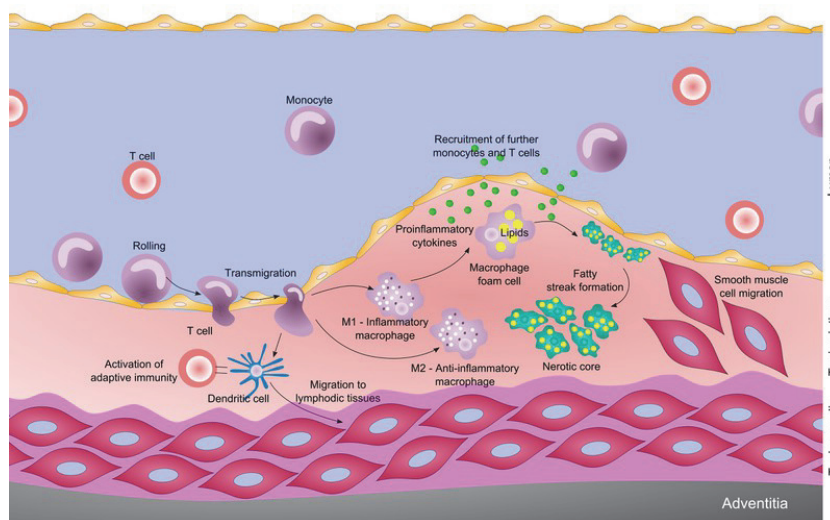

Fig. 1. The role of monocytes in the initiation and progression of atherosclerosis. Following initial injury, endothelial cells become activated and facilitate the rolling, attachment and transmigration of monocytes into the subendothelial space. Monocytes can differentiate into macrophages, which secrete proinflammatory cytokines. Engulfment of lipoprotein particles by macrophages can lead to fatty streak formation, which is the earliest ultrastructural alteration in atherogenesis. The migration of smooth muscle cells from the tunica media into the tunica intima further promotes atherogenic processes. Monocyte-derived cells turn this early lesion into an advanced atherosclerotic plaque that is comprised of a lipid-and macrophage-rich necrotic core that is finally destabilized, leading to plaque rupture (adapted from Heine *et al.* 2012).

Under physiological conditions, monocytes circulate in the blood, and they are responsible for maintaining homeostasis and removing dead or damaged cells (Auffray *et al.* 2009). During inflammation, blood monocytes migrate to lymphoid and nonlymphoid tissues in response to tissue-derived signals, where they could be activated by T cells (Geissman *et al.* 2008). Monocytes can differentiate into dendritic cells and macrophages which can phagocytose damaged cells and toxic molecules and produce cytokines (Tacke and Randolph 2006).

All of these adhesive and phagocytic processes are described in detail. The process starts with monocyte adhesion to the arterial endothelium. Some studies suggest the importance of ICAM-1 (Poston *et al.* 1992) and vascular cell adhesion protein – 1 (VCAM-1) (Cybulsky and Gimbrone 1991) expressed on endothelial cells for the interaction of monocytes with the endothelium.

Monocyte interactions with the arterial endothelium are most likely also induced by certain proatherosclerotic stimuli such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), angiotensin II and oxidized LDL (Álvarez *et al.* 2004). The above-mentioned risk factors may contribute to atherosclerosis development by initiating the inflammatory response and thereby trigger fibroproliferative inflammation.

An important molecule that regulates migration and infiltration into the subendothelial space of a damaged artery is monocyte chemoattractant protein 1

(MCP-1). Both MCP-1 and its receptor C-C chemokine receptor type 2 (CCR2) are induced and involved in various pathologies. Increased plasma levels of MCP-1 could constitute a risk factor in atherosclerosis development (Gosling *et al.* 1999). Mice deficient for MCP-1 or its receptor are resistant to atherosclerosis development (Boring *et al.* 1998).

Regarding the scavenger receptors expression it was shown, that increased scavenger receptors expression on the macrophages surface (SR – A and SR – B) leads to oxidized LDL uptake (Kunjathoor *et al.* 2002). During short-term exposure to excess lipids there are mechanisms maintaining control over LDL. These mechanisms include storage in lipid droplets, β-oxidation and cholesterol efflux. Prolonged exposure leads to failure of these mechanisms and various lipotoxic events (Tabas 2002). After transmigration to the subendothelial space, monocytes are transformed into macrophages under the influence of some stimuls from surrounding tissue.

In addition, macrophage activation through their Toll-like receptors (TLRs) produces a number of inflammatory cytokines, particularly IL-1β, TNF-α and MCP-1 into the extracellular matrix, which recruits more macrophages. Saturated fatty acids can also signal through TLR-2 and TLR-4 on macrophages *in vitro* and induce pro-inflammatory gene expression (Shi *et al.* 2006).

Macrophage egress is also a well-regulated process in which chemokine (C-C motif) ligand 19 (CCL19) and chemokine (C-C motif) ligand 21 (CCL21) play the most important role. Chemokines CCL19 and CCL21 and their receptor on macrophages C-C chemokine receptor type 7 (CCR7) are responsible for macrophage outflow from atherosclerotic lesions in hypercholesterolemia (Trogan *et al.* 2006). Netrin-1, which is important for neuronal guidance, is a molecule that inhibits macrophage egress from atherosclerotic plaques. During hypercholesterolemia, netrin-1 activates netrin receptor Unc5b on activated macrophages and inhibits macrophage outflow from atherosclerotic plaques. Genetic deletion of netrin-1 enhances macrophage egress from atherosclerotic plaques (van Gils *et al.* 2012).

It was further demonstrated that statin therapy can promote atherosclerosis regression *via* activation of the CCR7-dependent emigration pathway in macrophages (Feig *et al.* 2011).

Foam cells and other cells may significantly contribute to the formation of advanced atherosclerotic plaques that are susceptible to rupture. Necrotic death of foam cells has often resulted in release of intracellular components, leading to chronic inflammation (Baker *et al.* 2011). Subsequently, smooth muscle cells migrate into the atherosclerotic plaque and thus contribute to its development and subsequent rupture (Moore and Tabas 2011). Ruptured plaques have several histomorphological features that are different from stable plaques. Plaques that rupture tend to have a large lipid core, inflammatory cell infiltration of the fibrous cap and adventitia, and a thin cap depleted of smooth muscle cells. Depletion of matrix components from the fibrous cap caused by an imbalance between synthesis and breakdown leads to cap thinning (Ravn and Falk 1999). If the plaque ruptures, the thrombogenic lesion core is exposed to blood in the arterial lumen. Then, platelet adhesion and activation initiates the formation of an arterial thrombus.

Atherosclerotic plaque stability and vulnerability is also influenced by the activity of matrix metalloproteinases (MMPs), which are produced by inflammatory cells in atherosclerosis pathogenesis, as focally increased expression of several MMPs and MMP activity have been observed in experimental models of atherosclerosis (Brown *et al.* 1995). MMP activity may contribute to atherosclerosis pathogenesis by facilitating vascular smooth muscle cell migration through the internal elastic lamina into the intima of the vessel wall, where they proliferate and contribute to plaque formation (Ravn and Falk 1999).

Some studies suggest that diffuse intimal thickening is present in human arteries before atherosclerosis develops. Diffuse intimal thickening is believed to be related to atherosclerosis because it is consistently present in atherosclerosis-prone arteries, such as coronary arteries and abdominal aorta. Diffuse intimal thickening can be a depot for extracellular lipids in the earliest initial stages of atherosclerosis (type I lesions). It is assumed that extracellular proteoglycans located in diffuse intimal thickening play an important role in early stages of atherosclerosis by binding atherogenic lipoproteins followed by macrophages infiltration into the deeper layer of intima and forming of foam cells (for summary see Nakashima *et al.* 2008).

In addition to innate immune cells in atherosclerotic plaques, there are also adaptive immune cells from which CD4+ cells (particularly Th1 cells – T helper cells) are the predominant subpopulation. Th1 cells enhance the proinflammatory characteristics of

atherosclerosis by producing interferon-γ (IFN-γ) and soluble CD40 ligand (CD40L). Presently, B lymphocytes have not been documented in the atherosclerotic plaque, although autoantibodies have been found (for summary see Carbone *et al.* 2013).

Heterogeneity in monocyte subpopulation in atherosclerosis

In peripheral blood monocytes represent heterogenous population differing in their phenotypes and functions. They can be divided into functional subsets on the basis of their expression of CD14 and CD16 receptors. CD14 is an assistant receptor for bond of the lipopolysaccharide and CD16 represents a low affinity receptor for Fc parts of IgG antibody.

CD14+CD16- cells correspond with "classical" monocytes. These monocytes, which represent 85-90 % of monocytes in healthy subjects (Passlick *et al.* 1989), are characterizied by high phagocytic activity, they produce proinflammatory cytokines, and the migration of this subset strongly depends on MCP-1, which is produced by resident macrophages (Swirski *et al.* 2007).

CD14+CD16+ monocytes represent a minor population that can produce proinflammatory cytokines, but demonstrate lower scavenger receptor expression and higher antigen presenting capacity, do not express MCP-1 receptor and depend on fractalkine for migration into the subendothelial space. On the basis of CD14 expression, two groups of CD14+CD16+ monocytes can be further distinguished as CD14++CD16+ and CD14+CD16+. CD14++CD16+ monocytes (3-9 % of peripheral blood monocytes) are characterized by a combination of some markers typical for mature dendritic cells such as high production of IL-12, high expression of HLA-DR, CD11c and costimulatory molecule CD86. They maintain high expression of CD14 and high phagocytic activity (Grage-Griebenow *et al.* 2001). CD14+CD16+ monocytes (6-13 % of peripheral blood monocytes) are similar to mature tissue macrophages but they share some characteristics of dendritic cells. They are characterized by high expression of MHC I. and MHC II. molecules and antigen presenting capacity (Ziegler-Heitbrock *et al.* 1993).

The clinical significance of these monocyte subsets and their biological role in atherosclerosis pathogenesis is still under investigation, as correlations of monocyte subsets with clinical signs of atherosclerosis are not consistent (Dopheide *et al.* 2012). A very

interesting publication demonstrated that monocyte subpopulations can be influenced by cardiovascular risk factors (Hristov *et al.* 2010). In this study, subjects with a lower number of coronary artery disease risk factors had a higher ratio of CD14+CD16+/CD14++CD16+ monocytes compared with high-risk patients. Recently, the role of blood monocytes in atherosclerosis pathogenesis has been reviewed (Jaipersad *et al.* 2014). However, whether blood monocyte subsets reflect their participation in atherogenesis pathogenesis in the subendothelial space remains unclear.

Atherosclerotic plaque macrophages reveal distinct phenotypes that were distinguished into M1 (classically activated, proinflammatory) and M2 (alternatively activated, anti-inflammatory). M1 macrophages produce large amount of proinflammatory cytokines. Because of production of TNF-α, IL-1β and increased NO synthetase activity, M1 macrophages contribute to antimicrobial mechanisms activation (Hesse *et al.* 2001).

M2 macrophages can be further divided into three subsets based on their different function: macrophages involved in the healing process, regulatory macrophages and macrophages associated with tumor (Mosser 2003).

Numerous environmental signals (cytokines, microbial cell molecules, oxidative stress) received by macrophages drive their polarization, but it must be determined whether this classification reflects different macrophage subtypes or plasticity and phenotypic tissue changes, but the balance between subsets is crucial. Recent work (Cho *et al.* 2013) has demonstrated that M1 macrophages are dominant in symptomatic atherosclerotic plaques, while M2 macrophages are more frequent in asymptomatic plaques. Other authors (Stöger *et al.* 2012) found positive correlation of both M1 and M2 macrophages with atherosclerotic lesion severity.

Conclusion

Although a causal link between LDL cholesterol and atherosclerosis had been demonstrated (for example, by a significant decrease in atherosclerosis mortality after LDL cholesterol was decreased by statin treatment), the picture of atherosclerosis pathology is complete only after immunological influence is included. Although not all details of proinflammatory status regulation have been defined completely, the pivotal role of monocytes and macrophages in this pathology is clear.

Further research is necessary to understand all of

the detailed steps in proinflammatory processes in the subendothelial space and a genetic predisposition to myocardial infarction and stroke.

Conflict of Interest

There is no conflict of interest.

Acknowledgement

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