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Review

Monocytes in Metabolic Disorders – Opportunities for Flow Cytometry Contributions

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A B S T R A C T

Chronic inflammation has arised as a major underlying cause of atherosclerosis, obesity and diabetes. It is mediated by cells of innate immune system like macrophages but also by their antecedents, circulating monocytes. Roles of monocyte subsets and different markers of monocyte activation in the context of metabolic disorders have been reviewed. Applying cell based approach through flow cytometry in this field has resulted with new understanding of pathophysiologic mechanisms. Possible implications of these insights in diagnosis, prognosis and revealing of therapeutic targets in metabolic disorders remain a challenge for future.

Key words: *monocytes, metabolism, atherosclerosis, obesity, flow cytometry*

Introduction

Chronic inflammation has arised as a major underlying cause of atherosclerosis, obesity and diabetes, most important contemporary metabolic disorders that directly contribute to development of cardiovascular diseases[1]. Inflammatory processes in atherogenesis have been involved from endothelial activation to the rupture of the atherosclerotic plaque. Recent years have offered new insights in the active role of adipose tissue as a source of endocrine but also of many inflammatory factors[2]. Moreover, chronic inflammation preceeds insulin resistance and in that way it has been suggested to be a crucial link between obesity and atherosclerosis[3].

Innate Immunity in Metabolic Disorders

In an effort to understand inflammatory mechanisms in metabolic disorders, a hypothesis has been proposed that they can be considered as disorders of the innate immunity. This type of immune answer is phylogenetically older than the adaptive immunity. It is the first line of host defense against infections and trauma and brings an advantage in evolutional surviving in a corresponding environment. However, when the answer is too strong, unproportional to the offending cause or continuous (like in overfeeding), immune overreaction could provoke illness instead of preventing it[4].

Ten years ago, when Pickup et al. suggested this hypothesis, they build their theory considering high level of multiple inflammatory cytokines in metabolic disorders. In last years, reconsidering this thesis, the attention has been drawn to the mononuclear-phagocyte system. This system includes macrophages, monocytes and their bone marrow precursors, cells of the innate immunity that are the main source of inflammatory mediators. In that context, it was recently suggested that macrophages have central role in inflammatory mechanisms of several metabolic diseases. Macrophages might be »at the crossroads of insulin resistance and atherosclerosis« and monocytes »main effectors of inflammatory actions in these disorders«[5,6].

The main subject of our interest, monocytes make about 5 to 10% of peripheral leucocytes in human. Their half-life in peripheral blood is about 3 days. Having phagocyte and antigen presenting capabilities, monocy-

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tes take part in innate and adaptive immunity. Inflammatory, metabolic and immune stimuli promote recruitment of monocytes in peripheral tissues where they differentiate in macrophages and dendritic cells, although some macrophages might also develop by local multiplication[7].

Atherogenesis

Monocytes are the dominant inflammatory cell involved in the early stages of atherogenesis. Following endothelial injury, the coordinated interaction with endothelial cells results in the activation and extravasation of monocytes. This process could be divided in three basic steps consisting of subsequent involvement of adhesion molecules, chemokines and cytokines. The activation of endothelial cells triggers secretion of chemokine monocyte chemoattractant protein-1 (MCP-1) and attracts monocytes. MCP-1 prompts activation of monocytes by binding to its receptor CCR2. The first step of their adhesion is rolling which is performed by a family of selectins. Selectins allow leucocytes to weakly and reversibly bind on endothelial cells and start the adhesion cascade. The second step, the firm binding of monocytes occurs following activation and interaction of integrins with their endothelial ligands from immunoglobulin family, precisely intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The third step is the diapedesis of leucocytes through endothelial junctions between cells[8,9].

In atherosclerosis, from the moment of recruitment in subendothelial space, monocytes are continuously loaded with lipids by means of scavenger receptors and develop into foam cells. Their accumulation builds the fatty streaks, precursors of atherosclerotic plaques. These cells also secrete inflammatory mediators that stimulate proliferation and infiltration of smooth muscle cells, maturing of the complex lesion and finally participate in the rupture of the plaque and consequent thrombosis[8,9].

Obesity and Insulin Resistance

The role of mononuclear-phagocyte system also emerged recently in obesity and insulin resistance. Adipose tissue in obesity undergoes multiple structural and functional changes, involving adipocytes hypertrophy, switch in adipocytokines secretion pattern but also macrophages infiltration[10]. In fact, besides adipocytes, adipose tissue contains cells of the so-called stromovascular compartment. Adipose tissue in thin persons contains only about 10% of macrophages but in obesity they rise up to 40%. The expression of macrophage marker CD68 has correlated with body mass index and this level falls with reduction of weight, for instance after bariatric operations or with insulin sensitizing medication[11,12]. Studies confirmed that 30 to 59% of enhanced genes expression in adipose tissue in obesity are typical for macrophages. Fluorescent sorting of cells of adipose tissue showed the source of several key inflammatory molecules like tumor necrosis factor α (TNFα), interleukin-1b (IL-1b) and inducible nitric oxide synthase (iNOS) are macrophages unlike other stromovascular cells and adipocytes[11,12]. In experiments these cells have been shown to secrete about 90% of inflammatory adipocytokines like MCP-1 or TNFα[13]. Step by step, macrophages have become the focus of the link between obesity and insulin resistance. In vitro experiments proved that macrophages block insulin action in adipocytes by changing expressions of glucose transporter 4 (GLUT4) and insulin receptor supstrate-1. Animals with disabled nuclear factor κB (NFκB) inflammatory path in myeloid lineage were protected from developing insulin resistance and glucose intolerance[14–16]. On the other hand, animals were prone to develop these disorders by deletion of nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) in macrophages[17]. Although the definitive confirmation of the causal relation between macrophages and insulin resistance has been still lacking because macrophages might have been just a bystander in this mechanism, there is an enormous challenge to pursue further this hypothesis[18]. Its appealing value consists in the fact that macrophages might be possible site for intervention in order to prevent metabolic consequences of obesity.

Monocytes and Metabolic Disorders

Macrophages in adipose tissue, just like those in arterial wall, are derived from circulating monocytes originating from myeloid progenitors in bone marrow[11]. The mechanism of the recruitement of monocytes to inflammatory tissues, their activation and differentiation in macrophages is similar in atherosclerosis and obesity. The specificity of atherogenesis is that this process involves big arterial vessel walls and arterial shear forces, unlike all other cases where it occurs at the capillary level. The trigger of atherosclerosis is endothelial injury and in obesity it has still been undetermined.

Experimental evidences prove that monocytes substantially take part in inflammatory processes of atherogenesis and obesity but several studies demonstrated that this reflects also on their circulation level in clinical settings. In large prospective studies on representative sample of American population, circulating monocytes were related with measures of subclinical peripheral atherosclerosis[19]. In asymptomatic men of medium age statistically significant difference was found in the number of monocytes between groups divided by the body mass index[20]. Recently published study in healthy subjects revealed also that among leucocytes, the highest cardiovascular risk is brought by monocytes. Although the direct link with insulin resistance has not been investigated in this study, it could be recognized indirectly through positive correlation of monocytes with body mass index, waist circumference, systolic pressure, high density lipoprotein (HDL) cholesterol and triglycerides, components of the so-called metabolic syndrome[21].

Monocyte Subsets

The more precise characterization and insight in the functional role of monocyte was enabled by the discovery of their heterogenous phenotype[22]. The specific surface marker for monocyte population is CD14, a part of the lipopolysacharide receptor. Based on the intensity of CD14 expression, monocytes could be differentiated in a large majority of cells that express high levels of CD14, $CD14^{++}$, and about 10–15% of monocytes with lower expression of CD14 surface marker, $CD14^{+}$. The additional separation of monocytes is defined by the surface expression of CD16 antigen, also known as Fc receptor γIII. Four different subsets could be differentiated from combinations of these two antigens, but the two best defined are $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$. $CD14^{++}CD16^{-}$ subset is considered as classic monocytes because it is the closest to the original definition of monocytes as cells that migrate to inflamed areas. The subset $CD14^{+}CD16^{+}$ seems to be more mature, to be more potent antigen presenting cells and has more similarities with macrophages. $CD16^{+}$ monocytes are also more important source of TNFα in the blood[7,8,23,24].

In different studies high levels of $CD14^{+}CD16^{+}$ monocytes have been related with states of chronic inflammation. This subset is expanded in infectious diseases like AIDS or sepsis, but also in coronary heart disease. $CD14^{+}$ $CD16^{+}$ monocytes have been related with atherogenic lipids and inversely correlated with HDL cholesterol[26]. In a study with renal disease patients, levels of $CD14^{+}CD16^{+}$ cells were independently related with subclinical atherosclerosis measured by intima-media thickness[27].

In contrast to that, another study has not found difference in the number of $CD14^{+}CD16^{+}$ cells between groups of patients with diabetes, with diabetes and cardiovascular disease and control group. However, the expression of CD14 on monocytes has been significantly higher in the group with diabetes, and even higher in the group with diabetes and cardiovascular disease. Higher number of $CD14^{++}$ monocytes has been shown in one more study in patients with diabetes mellitus[28,29]. What is the role of these different monocyte subsets in atherosclerosis or obesity is still to be determined.

Monocyte Activation

Further characterisation of monocyte subsets could be assessed from the changing phenotype during the monocyte activation and extravasation. As their interaction with endothelium involves expression of adhesion molecules, chemokine receptors, intracellular free radicals or proinflammatory cytokines, this is reflected in the different profile of functional phenotype markers. Our aim was to present several of the most representative examples of activation markers.

For instance, different expression of chemokine receptors is also distinctive for the already mentioned monocyte subsets. The inflammatory $CD14^{++}$ subset, express high levels of CCR2, chemokine receptor for MCP-1, low levels of CCR5, receptor of CCL3 and medium levels of CX3CR1, receptor for fractalkine. The $CD16^{+}$ subset is CCR2 negative, but express high levels of CX3CR1 and CCR5 receptors[7].

CCR2

The key promigratory signal of MCP-1 to monocytes is transferred by chemokine receptor CCR2[30]. Strong reduction of macrophage infiltration, foam cell formation and atherosclerosis progression was found in the model of apoE-/- mouse with deletion of CCR2[31]. This has confirmed its causal relationship with atherosclerosis on experimental level.

In studies in humans expression of CCR2 on monocytes has positively correlated with levels of low density lipoprotein (LDL) cholesterol and negatively with HDL cholesterol[32,33]. However, suppression of CCR2 on monocytes was found in models with high oxidized LDL cholesterol that is usually formed in the intima of arteries[34–36]. It has been considered that suppression of CCR2 stimulates retention of recruited monocytes in the vessel wall and that might represent a step in the differentiation of monocytes to macrophages or foam cells. In the presumed two step model, this chemokine receptor first mediates recruitment of monocytes and then, suppressed by oxidized LDL, promotes the pathologic accumulation of these cells in the intima[37,38].

Recent studies have shown also a role of CCR2 in the development and maintaining of obesity. In animals fed by high-fat diet it was determined that macrophages express higher levels of markers related to the migration of cells like CCR2[39]. In mouse with deletion of CCR2, the number of macrophages in fat tissue was reduced, just as inflammatory markers and systemic insulin resistance. Pharmacologic CCR2 antagonist reduced the quantity of macrophages in fat tissue and raised insulin sensitivity in mouse with preexisting obesity. However, a study on animals with CCR2 deficiency form different genetic background did not show the same effect of this mutation, so infiltration of macrophages might be mediated also by CCR2 independent influences[40,41].

Gene expression of CC chemokines involved in monocyte chemotaxis and their receptors, particularly CCR2 was higher in subcutaneous and visceral adipose tissue of obese patients. The expression levels of CCR2 on circulating monocytes were significantly increased in diabetic patients and correlated with poor blood glucose control[42,43].

According to these data, the system MCP-1/CCR2 has probably direct role in recruitement and retention of monocytes in fat tissue and atherosclerotic plaques[44]. Exloring these contributions would probably have impliations on effectivness of treatement with CCR2 antagonists.

CX3CR1

CX3CR1 is a receptor for fractalkine, a recently discovered chemokine with distinctive structural and func-

tional properties[45]. Fractalkine is present as membrane bound molecule or a soluble glycoprotein. Besides being a chemokine, fractalkine functions as an adhesion molecule for monocytes. CX3CR1 is expressed on leucocytes and fractalkine is expressed by endothelial cells in inflammatory conditions, but also on smooth muscle cells and platelets. The expression of fractalkine and its receptor has been also documented inside the atherosclerotic plaque[46]. Experimental data confirmed decreased atherosclerosis in animals with CX3CR1 deletions[47] and several human studies have concluded that polymorphisms of CX3CR1 have been related with reduced risk for atherosclerotic cardiovascular disease[48].

Considering the surface expression of CX3CR1 on monocytes, no difference between coronary artery disease patients and controls and no effect of statin therapy was found in one study[49] and a second suggested higher levels of $CX3CR1^+$ cells among peripheral blood mononuclear cells, especially monocytes in coronary artery disease patients[50].

Although the role of CX3CR1 in migration of $CD16^+$ subset of monocytes has been assumed, the precise role of CX3CR1 in monocyte recruitment is still to be determined[51]. In the process of monocyte adhesion and transmigration CX3CR1/CX3CL1 system seems to have also an indirect role. Experimental data show that endothelial fractalkine first binds to CX3CR1 of platelets and that increases platelet P-selectin expression. Activated platelets then trigger monocyte adhesion[51,52].

The most recent experimental study has shown that the absence of the CX3CR1 receptor results in increased apoptosis of monocytes and foam cells. Together with previously described potential effects on monocyte recruitment, that would lead to reduced foam cell load and the inhibition of plaque progression[53]. Inhibition of CX3CR1/CX3CL1 interaction during early atherogenesis stages might be beneficial, however since CX3C signals are likely to be required for foam cell survival too, such inhibition during later atheroma stages could result in disease exacerbation. These results suggested that possible therapeutic approach would require careful consideration and profound understanding of the system[53].

CD11b

Different subsets of monocytes have different expression of adhesion molecules, but in most studies adhesion molecules were investigated independently from monocyte subsets. CD11b on cell surface is a well investigated marker of activation and monocyte adhesion. CD11b is one of several α subunits of $\beta 2$ integrin receptor CD11/CD18[28]. Strong and permanent adhesion of monocytes on endothelial cells occurs only with activation of integrin. Ligand of CD11b on endothelial cells is ICAM-1. Levels of soluble form of this endothelial adhesion molecule, sICAM-1, are recognized as useful predictor of cardiovascular events in healthy persons and related with different stages of cardiovascular diseases. Raised levels of endothelial adhesion molecules were found in men with abdominal obesity[54,55].

Besides studies on adhesion molecules on activated endothelial cells important number of studies has shown stronger expression of their receptor CD11b on monocytes in pathophysiologic states that are linked with development or advanced atherosclerosis. In several studies raised expression of this marker was found in patients with coronary heart disease, especially in unstable angina[56]. Increased expression of CD11b together with CD14 was determined in patients with hypercholesterolemia, and they were lowered with simvastatin treatment[57].

Experimental studies point to the raised expression of CD11b on circulating monocytes in obesity even before development of diabetes and hypertension[58]. Higher levels of CD11b and increased adhesion on endothelial cells was shown in vitro on monocytes under the prolonged influence of free fatty acids[59]. Leptin also positively influenced expression of CD11b on monocytes[60]. It was shown that MCP-1 induces expression of CD11b on peripheral monocytes in the model of experimental atherosclerosis in apoE-/- mouses[61]. Similar direct influence of MCP-1 on the expression of CD11b on monocytes was obtained in the experiment with overfed mouses[62].

These experimental models support strong correlation of CD11b with measures of obesity but in clinical data this relationship has not been consistently recognized. In female patients with extreme obesity before bariatric operation and a control group with normal body mass index, there was no difference in expression of CD11b on monocytes. In a prospective analysis, CD11b on monocytes has not changed expression after bariatric operation[63]. On the other hand, when stratified by CD11b expression on circulating monocytes, obese hypertensives had markedly increased expression of the macrophage marker CD68 in adipose tissue[64].

Results are somehow clearer in clinical studies that have dealed with postprandial changes on monocytes. Raised expression of CD11b on monocytes was found after glucose load in patients with diabetes, but also in control group[65]. In the study that evaluated expression of CD11b on monocytes in relation with elevated triglycerides after fat meal, significant increase of CD11b was found 6 hours postprandially[66]. In a different study raised expression of CD11b on monocytes was found even 10 hours after fat meal[67].

Toll-like receptors

An essential step further in understanding innate inflammatory mechanisms was the recent discovery of toll-like receptors (TLR). They recognize pathogen-associated molecular patterns on exogenous pathogens like bacteria and viruses but also on possible endogenous ligands[68–70]. Being the crucial recognition and signaling receptors in the host defense, toll-like receptors are also linking innate and adaptive immunity[71,72]. The discovery of the expression of several toll-like receptors, particularly TLR1, TLR2 and TLR4, on endothelial cells and ma-

crophages in atherosclerotic lesions, has implied their critical role in atherogenesis[73,74]. Once again, the strongest evidence for the involvement of TLR signaling has been documented from experimental models of atherosclerosis prone mouse. Animals with genetic deficiency of TLR4 had significantly reduced atherosclerotic plaque size and macrophage infiltration[75]. Moreover, TLR4 polymorphisms that weaken the receptor function and inflammatory response have been associated with lower risk for atherosclerosis and acute coronary events[76,77].

Several endogenous pathways of TLR activation are associated with metabolic disorders. TLR4 in macrophages was upregulated by oxidized LDL. Activation of TLR4 by free fatty acids provided a possible inflammatory mechanism that link nutrition, obesity and atherosclerosis[78]. Expression of TLR4 in monocytes was induced by high glucose and inhibited by pioglitazone[79,80]. Flow cytometry analysis of circulating monocytes determined that patients with coronary artery disease and acute coronary syndromes had increased expression of TLR4[81,82]. The importance of toll-like receptors consists in the fact that their involvement for the first time suggested a site where inflammatory mechanisms in atherosclerosis might be initiated[72].

Oxidative Burst

Oxidative stress is an imbalance in oxidative-reduction processes because of overproduction of reactive oxidative species (ROS) or a deficit in antioxidative activity. Free radicals have important physiologic roles. Organisms use it first of all as an antimicrobial tool in host defense during phagocytosis. This event is called oxidative burst and could be measured by various fluorescent probes in flow cytometry. Most cells can produce small oxidative burst under the influence of different cytokines, growth factors and hormones. That has led to the knowledge that free radicals serve as signal molecules in various cell functions. Physiologic cell radicals are produced in controlled way and in low concentrations because their toxic capabilities limit their role as second messengers[83].

Free radicals are admitted to have important functions in the pathogenesis of endothelial dysfunction and development of atherosclerosis. Two most important mechanisms involve capturing of nitric oxide and oxidation of LDL. Main site of free radicals production in vessel wall is in endothelial, smooth muscle cells and fibroblasts. According to some investigations, blood cells are also an important source of ROS, especially phagocytes, neutrophils and monocytes. ROS easily pass membrane and may affect other leucocytes but also the close vascular endothelium. Through the transcription factor sensitive on oxidative stress, NFκB, free radicals stimulate expression of adhesion molecules and chemokine receptors on monocytes[84,85].

Experimental evidence points to the oxidative burst in mononuclear cells as one of possible pathophysiologic mediators of chronic inflammation in atherosclerosis and obesity. Oxidation of LDL in vitro was shown to be much faster in obese persons than in non-obese[86]. Further analysis confirmed that higher production of free radical was located in adipose tissue and it was suggested that it could be related with macrophage infiltration because of their high capacity of free radical production[87]. In vitro measurement confirmed stimulative effect of leptin on oxidative burst in monocytes[88].

Hyperlipidemic patients presented significantly higher rates of monocyte ROS generation and positive correlation between monocyte ROS generation and oxidized LDL concentrations was found[89]. Oxidative burst in monocytes has been related with intima-media thickness as a surrogate of atherosclerosis in patients with hypertension[84]. Increased reactive oxygen species were detected also in monocytes from obese patients suggesting that these cells are already committed to an inflammatory phenotype in peripheral blood[90].

Flow Cytometry Contributions

Active role of inflammatory mechanisms in metabolic disorders has supported a new, cell based approach in their research. We have presented a considerable amount of data that was provided by applying the succesful experience of flow cytometry analysis in characterizing immune cells. Advantages of flow cytometry are that it offers a multiparametric and dynamic understanding of cell biology. Diversity of cell-specific markers helps in discovering sequences of events in cells activation and signalling pathways. Integration of these data enables insight in functions of cell systems and understanding sources of pathophysiological processes[91].

This method is inevitable in *in vitro* and *in vivo* experimental studies, but interesting results have been obtained by moving from the basic science to clinical applications. The challenge of possible implications in diagnosis, prognosis and revealing of therapeutic targets in metabolic diseases remains for future. Still lacking are prospective large scale studies that present the well-known method of confirming biomarkers in metabolic diseases. In that context the recently published flow cytometry study in the framework of Atherosclerosis Risk in Communities (ARIC) Carotid magnetic resonance imaging study has been an important step towards this direction, especially because it was precedeed by efforts to validate reliability of multicenter studies in this field[92,93].

Lot of data support the hypothesis that monocytes are representative and easily accessible model to study the inflammation in metabolic disorders and future advances in this research might provide clues to recognize the metabolic risk even in subclinical stage[94].

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MONOCITI U METABOLIČKIM POREMEĆAJIMA – DOPRINOSI PROTOČNE CITOMETRIJE

S A Ž E T A K

Kronična upala smatra se temeljnim uzrokom ateroskleroze, debljine i šećerne bolesti. Posredovana je stanicama urođenog imunološkog sustava poput makrofaga, ali isto tako i njihovim prethodnicima, monocitima u cirkulaciji. Razmotrili smo uloge podskupina monocita te različitih monocitnih aktivacijskih biljega u kontekstu metaboličkih poremećaja. Primjena metode protočne citometrije u tom području doprinijela je novom razumijevanju patofizioloških mehanizama. Moguća značenja tih spoznaja u dijagnozi, prognozi i razotkrivanju terapijskih ciljeva u metaboličkim poremećajima preostaju kao izazov u budućnosti.