Role of Circulating Karyocytes in the Initiation and Progression of Atherosclerosis

Helena Chon, Marianne C. Verhaar, Hein A. Koomans, Jaap A. Joles, Branko Braam

Abstract—Cardiovascular disease is still hard to predict in an individual. The main focus in cardiovascular research has been on endothelial cells and vascular smooth muscle cells of the vessel wall and their interactions with the blood flow. Alterations in the properties of the blood have received a lot of attention in biochemical terms. Interestingly, alterations in the properties of circulating cells have received less attention. We propose that presence of 1 or more risk factors together with normal physiological stimuli induce redox-dependent changes in leukocyte gene transcription with pathophysiological responses. Thus, risk factors render leukocytes hypersensitive to normal stimuli. Risk factors can be subdivided into physical and chemical factors. Superimposed on physiological regulators of leukocyte function, these risk factors promote a cellular pro-oxidative state. Redox-sensitive transcription factors are activated, leading to responses involving inflammation, adhesion, migration, and additional reactive oxygen species generation. As a consequence, monitoring of individual gene expression signatures of these cells could well increase our understanding of the mechanisms by which leukocytes and, in particular, monocytes function. Furthermore, transcriptomes of these cells could be used to investigate the aggressiveness of the atherosclerotic process or to guide treatment in the patient with risk factors for atherosclerosis. (Hypertension. 2006;47:803-810.)

Key Words: gene expression ■ oxidative stress ■ atherosclerosis ■ leukocytes

Despite great advances in our knowledge about initiation and progression of atherosclerosis, progress with respect to detection of individuals at risk for cardiovascular events is still limited. The availability of sophisticated biochemical assays to quickly and routinely screen soluble factors in large numbers of patients with atherosclerosis has led to an ever growing number of recognized cardiovascular risk factors. Nevertheless, methodology to assess the stage and aggressiveness of atherosclerotic disease is not available for individual subjects. Therefore, cardiovascular events are not predictable and, conversely, adequacy of treatment cannot be measured in an individual. This concern has been expressed in a call for new definitions and risk assessment strategies that proposes to classify vulnerable patients by criteria other than plaque vulnerability.1

Virchow’s triad (1856) describes changes in blood flow, the vessel wall, and properties of blood as a model for the pathogenesis of thrombosis. This triad may be applicable to the process of atherogenesis. The main focus in cardiovascular research has been on the vessel wall, in particular, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) and their interactions with the blood flow. Alterations in the properties of the blood have received much attention in biochemical terms. Interestingly, alterations in the properties of circulating cells have received considerably less attention. In a lipid-feeding model, absence of endothelial intercellular adhesion molecule 1, P-selectin, CD18, or combinations of these molecules limited the size of atherosclerotic lesions but did not prevent atherogenesis,2,3 suggesting that activated ECs facilitate rather than initiate leukocyte infiltration and, thus, a more important role for circulating cells in atherogenesis. In this review, we discuss the pivotal role of the third component of the triad, that is, alterations in the properties of the components and the composition of blood, in particular, circulating karyocytes, in the initiation and progression of atherosclerosis. How are the leukocyte subpopulations and progenitor cells involved in the development and progression of atherosclerosis? Are leukocytes pivotal in the initiation of atherosclerosis or just secondary factors in atherogenesis? How is this related to the concept of dysfunctional stem cells?

We propose that circulating karyocytes form the driving force of the disease process. As such, circulating karyocytes can provide valuable information on the disease state of atherosclerosis. Because more and more information is appearing that all cells in the bloodstream with an active transcription apparatus can be relevant for the initiation and progression of atherosclerosis, the karyocyte is central. It also embodies that cells with highly different phases of differentiation are present, from stem cells to specialized lympho-
cytes. We consider the application of leukocyte gene expression in patients at risk for atherosclerosis for monitoring treatment of risk factors. In our view, correction of aberrant behavior of circulating karyocytes should be considered as a prime target in treatment of the cardiovascular patient.

**Circulating Cells in the Initiation and Progression of Atherosclerosis**

A variety of physicochemical stresses continuously endanger the integrity of the EC layer. Circulating karyocytes take part in maintenance and repair on the one hand but are also involved in lesion formation and progression on the other. We first consider regular maintenance and repair, with emphasis on progenitor cells, then the progression of microtrauma of the EC layer to more advanced lesions and plaque formation, and, finally, plaque rupture. For the evolution of microtrauma to plaque rupture we focus on leukocytes, in particular, monocytes and T lymphocytes. Additionally, the role of circulating karyocytes in EC maintenance and repair, with emphasis on vascular progenitor cells, is discussed.

**Initiation of Damage to the Vascular Wall**

ECs are constantly experiencing microinjury, which ultimately can lead to apoptosis and damage. Continuity of the EC layer is maintained by division of surrounding ECs and by incorporation of bone marrow–derived EC progenitor cells. Ongoing microinjury, facilitated by EC dysfunction and impaired endothelial regeneration and repair, eventually results in lesions evoking an injury response. Leukocyte adhesion and infiltration, the first histological sign of atherosclerosis, occurs as a response to injury. The inflammatory response particularly involves monocytes and T lymphocytes. Other than the occurrence of endothelial activation (increased expression of various leukocyte adhesion molecules), monocytes themselves can respond to physiological changes by increased adherence to ECs, as was observed in hypertension. Chemotaxis stimulated by monocytes leads to adhesion to ECs by the secretion of proinflammatory cytokines, such as transforming growth factor β and tumor necrosis factor (TNF) α, thereby inducing M-colony–stimulating factor and adhesion molecules. T lymphocytes can modulate EC function and, hence, induce EC dysfunction: T lymphocyte–derived cytokines, such as TNF-α and IFN-γ, inhibit the anticoagulant properties of EC, induce the expression of adhesion molecules, and stimulate the formation of gaps between ECs, enhancing leukocyte adhesion and infiltration. These cytokines also enhance EC antigen presentation by upregulation of major histocompatibility complex molecules and enzymes involved in antigen peptide processing and loading, which can be counterbalanced by NO. Thus, EC may induce a T-cell response and, in turn, activation of T cells may result in EC dysfunction.

**Foam Cell Formation, Matrix Deposition, and Proliferation in the Vascular Wall**

After the initial contact with the endothelium, monocytes infiltrate into the subendothelial layer. Once there, macrophage differentiation with concurrent induction of scavenger receptors facilitates uptake of oxidized low-density lipoprotein (oxLDL) leading to the formation of foam cells. The foam cells increase rapidly in number as they are trapped in the lesion by oxLDL. Even more oxLDL is formed via reactive oxygen species (ROS) produced by resident macrophages. T lymphocytes are attracted by inflammatory cytokines secreted by macrophages, such as inducible protein 10, monokine induced by IFN-γ, and IFN-inducible α-chemoattractant. Consequently, T lymphocytes are activated by oxLDL presented on major histocompatibility complex molecules in the lesion, which, in turn, causes the additional activation of macrophages, ECs, and VSMCs. Indeed, T-lymphocyte induction aggravates and T-cell inhibition attenuates the progression of atherogenesis in mice.

During lesion formation, macrophages and T lymphocytes secrete growth factors, such as platelet-derived growth factor and VEGF, and cytokines, such as IFN-γ and interleukin (IL) 1, that stimulate VSMC migration into the intimal layer of the vessel wall. VSMCs, in turn, also secrete growth factors and synthesize extracellular matrix, which forms a fibrous cap, thus becoming an atherosclerotic plaque. In addition, monocytes and T lymphocytes secrete CC motif chemokine receptor, which mediates VSMC proliferation. To summarize, fibrous lesions grow as blood-derived inflammatory cells, and VSMCs promote the proatherosclerotic actions of each other.

**Circulating Karyocytes and the Fate of Plaques**

In advanced lesions, foam and vascular cells damaged by oxLDL become apoptotic or necrotic and leave a reservoir of extracellular lipid and cell debris at the core of the lesion. Plaque erosion, a frequent cause of serious coronary thrombotic events, may result from local production of inflammatory mediators or by cytotoxic attack by activated killer T cells, leading to EC death. Also, activated ECs may degrade the matrix membrane to which they adhere. Other lesions may develop into vulnerable plaques. These are characterized by a large lipid reservoir, filled with many inflammatory cells, and covered with a thin fibrous cap. Newly formed vessels inside the plaque, stimulated by angiogenic factors produced by inflammatory cells, are prone to hemorrhage. Such neovascularization may also cause massive growth of VSMCs and matrix expansion leading to a much larger lesion. These vessels may also provide a conduit for entry of even more inflammatory cells, increasing plaque instability. The fibrous cap is maintained in a delicate way with an important role of inflammatory cells. Prolinflammatory cytokines, such as IFN-γ, are able to inhibit matrix production by VSMCs. Moreover, inflammatory cells can secrete collage-nases, such as matrix metalloproteinases, that break down matrix. The lesion edge, rich in foam cells, is, therefore, most prone to rupture. Thus, the fate of plaques is directed by inflammatory cells.

**Active Participation of Circulating Karyocytes in Atherogenesis**

How could circulating cells become active players in damage? There are 2 plausible routes that are not mutually exclusive. A first option is that circulating cells, predominantly monocytes, become hypersensitive under the influence
of physicochemical stimuli, including classical risk factors for atherosclerosis. Alternatively, circulating progenitor cells may become dysfunctional and fail to adequately participate in maintenance and repair of the vascular wall or even contribute to the formation of atherosclerotic lesions. Common to various injury pathways is the imbalance of NO and ROS. It is clear that NO can dampen many of the proinflammatory responses evoked by monocytes and ECs. Therefore, impaired NO production by ECs seems to be central in causing atherosclerosis.

The Hypersensitive Leukocyte Hypothesis

Different types of leukocytes process a wide variety of physiological stimuli, which act together to establish an adequate immune response and clearance of dysfunctional cells. We propose that the presence of 1 or more risk factors together with normal physiological stimuli induce redox-dependent changes in leukocyte gene transcription with pathophysiological responses (Figure 1). Thus, risk factors render leukocytes hypersensitive to normal stimuli. This concept translates into differentially affected function for each circulating cell type. The overall effect is that all leukocytes become more sensitive and more readily adhere to and infiltrate into the vessel wall. Risk factors can be subdivided into physical and chemical factors. Superimposed on physiological regulators of leukocyte function, these risk factors promote a cellular pro-oxidative state. Redox-sensitive transcription factors are activated, leading to responses involving inflammation, adhesion, migration, and additional ROS generation (Figure 1).

Physical Factors

Circulating cells traveling in the bloodstream are subject to different mechanical forces: hydrostatic pressure, strain through deformation, and shear stress. Interestingly, limited information is available about the relationship between leukocyte cell function and mechanical forces. Intraluminal pressure may have diverse effects on monocytes and other circulating karyocytes. Hydrostatic pressure has been shown to increase the proliferatory response to phytohemagglutinin A in monocytes, whereas amiloride-sensitive sodium channels in human B cells subjected to hydrostatic pressure are activated via the cytoskeleton. Furthermore, mechanical strain on monocytes induces c-fos and c-jun gene expression and activator protein (AP)-1 activity. Finally, because flow along the central axis of the vessel is higher than at the perimeter, circulating karyocytes sense differential flows and, thus, shear stress. The higher the flow, the greater the flow gradient across the cell surface, the higher the shear stress imposed on the cell. Very few studies have assessed the responses of monocytes or other leukocytes to shear stress. Leukocytes, including monocytes, display pseudopod (also called lamellipod) retraction in response to shear stress. Pseudopods are involved in adhesion and migration of monocytes. Pseudopod retraction is disturbed in endothelial NO synthase mice and enhanced by cGMP analogues, which suggests an additional mechanism for increased adhesion in NO deficiency. Taken together, there is scattered evidence that monocytes demonstrate a variety of responses to mechanical forces; however, studies on interactions between the deranged physical forces in hypertension and enhanced renin–angiotensin system, ROS, and sympathetic nervous system activity on monocytes are not available.

Chemical Factors, in Particular, Angiotensin II, ROS, and Inflammation

Inappropriate activation of the renin–angiotensin system is evident in hypertension, uremia, and diabetes. Moreover, expression of the angiotensin II (Ang II) type 1 (AT1) receptor is increased in atherosclerotic lesions. Interestingly, we observed upregulation of the AT1 receptor in leukocytes of patients with essential hypertension. Downstream signaling pathways of the AT1 receptor can mediate numerous proatherogenic effects, in particular, enhanced expression of adhesion molecules, activation of inflammation via nuclear factor κB (NF-κB) activation, and production of ROS. These proatherogenic effects occur in ECs and VSMCs and probably also in circulating karyocytes. Activation of AT1 receptor–dependent pathways may enhance adhesion by increased expression of macrophage antigen (Mac)-1 in granulocytes and migration in monocytes. Monocytes are very sensitive to Ang II stimulation, and Ang II favors differentiation of monocytes into dendritic cells,
implying a role for Ang II in the invasion of these cells into the vascular wall.\textsuperscript{37} Moreover, cross-linking of AT\textsubscript{1} receptor homodimers in monocytes, which is activated in hypertensive patients, enhances and prolongs signaling.\textsuperscript{38} In concordance, inhibition of Ang II generation or AT\textsubscript{1} receptor cross-linking activity reduced the progression of atherosclerosis in mice.\textsuperscript{38} Finally, a recent study indicated that Ang II strongly induced vascular lesions via enhanced macrophage infiltration in wild-type mice that received bone marrow from apolipoprotein E\textsuperscript{−/−} mice.\textsuperscript{39} Circulating karyocytes that have increased Ang II sensitivity are, thus, transformed into cells that are more prone to participate in adhesion, lesion formation, and inflammation, indicating their important role in atherogenesis.

There is also evidence that a shift in the NO/ROS balance toward oxidative stress can affect circulating karyocytes. Increased ROS production was observed in granulocytes in hypertensive rats\textsuperscript{55} and patients.\textsuperscript{60} All of the cell types in the vascular wall,\textsuperscript{41} as well as circulating karyocytes, such as neutrophils and monocytes, express reduced nicotinamide-adenine dinucleotide phosphate oxidase, a major source of ROS in cardiovascular disease. ROS-induced activation of NF-κB, peroxidase proliferator-activated receptor γ, and other redox-sensitive transcription factors can mediate numerous effects in circulating karyocytes. Moreover, ROS can amplify the actions of IFN-γ and NF-κB activation and enhance adhesion by upregulation of adhesion molecule Mac-1 on neutrophils.\textsuperscript{42} OxLDL is increased in overt cardiovascular disease and can enhance dendritic cell maturation.\textsuperscript{43} All in all, ROS and oxidative stress can render leukocytes hypersensitive, favoring the development of atherosclerotic lesions.

Inflammatory regulators, such as cytoplasmic repressor protein, TNF-α, IL-1β, IL-6, and IFN-γ, are elevated in cardiovascular disease. TNF-α and IL-1β, both strong inflammatory mediators secreted by activated macrophages, are potent inducers of NF-κB. Additionally, IL-1β can stimulate superoxide production directly by activating reduced nicotinamide-adenine dinucleotide phosphate oxidase in neutrophils and macrophages,\textsuperscript{44} whereas TNF-α also activates c-jun. IL-6 has widespread actions in inflammation and mediates these effects via 2 distinct pathways, that is, Janus-activating kinase/signal transducer and activation of transcription and extracellular signal regulated kinase/mitogen-activated protein kinase signaling.\textsuperscript{45} Finally, the proinflammatory cytokine IFN-γ is important in the progression of inflammatory responses associated with cardiovascular disease.\textsuperscript{46–48} Evidence that IFN-γ promotes atherosclerosis is based on the observation that apolipoprotein E\textsuperscript{−/−}, IFN-γ receptor double-knockout mice develop less atherosclerosis than apolipoprotein E\textsuperscript{−/−}, IFN-γ receptor\textsuperscript{−/−} mice.\textsuperscript{49,50}

**Progenitor Cell Dysfunction and Dysbalance and Atherosclerosis**

Subpopulations of mononuclear stem cells have the capacity not only to differentiate into hematopoietic cells also to differentiate into EC and VSMCs, myofibroblasts, cardiomyocytes, pancreatic cells, and so on, depending on the environment.\textsuperscript{12} Vascular progenitor cells can be isolated and cultured from peripheral blood, and many studies have demonstrated that endothelial progenitor cells (EPCs) contribute to neovascularization in response to limb or myocardial ischemia and to renal microvascular repair. The capacity of stem cells to develop into differentiated cell types may alter the course of disease.

As mentioned before, damage to the endothelium is repaired by replication of neighboring ECs and by incorporation of bone marrow–derived EPCs.\textsuperscript{51} Substantial evidence for a role in repair by EPCs has been derived from experiments applying hindlimb ischemia and studying repair by bone marrow–derived cells.\textsuperscript{51} Rookmaaker et al\textsuperscript{52} demonstrated that bone marrow–derived cells could also be found in the endothelial layer of renal vasculature of kidneys with glomerulonephritis. We studied the implications for vascular wall maintenance of the replacement of ECs by EPCs using a mathematical model.\textsuperscript{53} The competence of EPCs depends on the release from the bone marrow, homing to the site of injury,\textsuperscript{54} and the actual function, that is, proliferation and secretion of growth factors. Various conditions have been found to alter the release and angiogenesis of EPCs.\textsuperscript{55–57} It is becoming clear that number, function, and differentiation of progenitor cells can be affected by risk factors for atherosclerosis. For example, a decreased number and impaired function of EPCs have been reported in diabetes\textsuperscript{58,59} and in uremia,\textsuperscript{60} which may contribute to reduced endothelial regeneration and repair leading to vasculopathy. EPC numbers correlate with combined Framingham score and clinical outcome.\textsuperscript{61,62} Several reports have also suggested that vascular smooth muscle progenitor cells are present in the circulation, which may contribute to the development of atherosclerosis.\textsuperscript{54,63,64}

Although mechanisms underlying the changes in vascular progenitor cell function are emerging,\textsuperscript{65} little is known about the influence of chemical factors, such as pressure and shear stress, on EPCs. Chemical factors, such as inflammation, particularly cytoplasmic repressor protein and hyperglycemia, have known detrimental effects on EPC survival and angiogenic capacity.\textsuperscript{66,67} On the other hand, Ang II stimulates EPC proliferation and EPC-induced angiogenesis,\textsuperscript{68,69} in contrast to the generally proatherogenic functions of Ang II. Because these experiments were performed with healthy donors, it is conceivable that Ang II signaling is dysregulated in cardiovascular patients. This idea is supported by the finding that angiotensin-converting enzyme inhibitors and AT\textsubscript{1} receptor antagonists improve EPC numbers and function in patients with diabetes or coronary artery disease.\textsuperscript{70,71} In addition, Ang II may accelerate senescence via enhanced oxidative stress.\textsuperscript{72} Conversely, EPCs, or their pluripotent common precursor, may differentiate into cells with a vascular smooth muscle/myofibroblast phenotype that can have aggravating effects on plaque growth.\textsuperscript{73} It is, thus, clear that systemic factors also weaken repair mechanisms of the vessel wall.

**Synchronized Hypersensitive Response in the Initiation and Progression of Atherosclerosis**

Apparently, circulating karyocytes are affected by physicochemical stimuli just as much as EC, because they share the
same environment. Moreover, ECs are known to be important mediators of inflammation by cytokine secretion and by enhancing leukocyte infiltration. However, apart from cell characteristics, the response to the environment could well be different. On the one hand, ECs are fixed to the vascular wall, with the consequence that neighboring cells modulate their response. On the other hand, circulating karyocytes, being exclusively exposed to the circulation, may be even more sensitive to systemic changes. All in all, atherosclerosis is most probably to be accompanied by synchronization in the hypersensitivity displayed by ECs and circulating karyocytes.

**Circulating Karyocytes in Diagnosis and Treatment of (Pro)atherosclerotic Conditions**

Few studies have addressed the possibility of monitoring circulating karyocytes or monocytes in particular to assess cardiovascular patients. Individual gene expression signatures of these cells could well increase our understanding of the mechanisms by which monocytes function. Furthermore, transcriptomes of these cells could be used to investigate the aggressiveness of the atherosclerotic process or to guide treatment in the patient with risk factors for atherosclerosis.

As mentioned previously, we propose that hypersensitive karyocytes are crucially involved in the development of atherosclerosis. Hypersensitivity or dysfunction of circulating cells may be reflected in aberrant gene expression profiles of these cells. Studying these transcriptomes, for example, using microarrays, may ideally allow determination of the stage of atherosclerotic disease and the tendency for disease progression in an individual. In addition, treatment efficacy may be evaluated in the same fashion. Which information is currently available that supports the feasibility to establish a diagnosis based on gene expression profiles? There are several examples where gene expression profiles offered accurate diagnosis and prognostic data. Studies in the oncology field are particularly successful because of the obvious availability of frozen tissue and the severity of the disease. The best-known examples are in breast cancer and lymphomas. Profiling easily accessible biological material, such as circulating cells, may allow for application of such an approach to many more fields. For lymphomas, this is obviously feasible, but gene expression profiles in other conditions that do not as clearly involve circulating cells have also been studied (Table). Others have also suggested that circulating cells could be useful in cardiovascular disease.

We recently observed widespread changes in expression of inflammation-related genes in leukocytes from patients with essential hypertension. Microarray data were matched by conventional RT-PCR (Figure 2). This novel finding shows that essential hypertension is a complex systemic disease that

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PBMC indicates peripheral blood mononuclear cell; JRA, juvenile rheumatoid arthritis; MS, multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.
involves an inflammatory response, partly by downregulation of anti-inflammatory IL receptors. Leukocytes also displayed differential expression of genes related to blood pressure control, in particular, increased expression of AT1 receptor. Strikingly, effective treatment of high blood pressure strongly reduced the number of modulated genes. This finding couples blood pressure to complex transcriptional changes in circulating karyocytes, which seems to be correctable.

To reach successful implementation of gene expression profiling of circulating cells for clinical use, several conditions are required. Standardization of blood withdrawal, processing, and storage in all of the participating medical centers is essential for comparability. The microarray technique can be subject to external factors that influence quality; a robust profiling technique is, therefore, needed. A selection of a set of atherosclerosis genes that facilitates use in the clinic would be preferable. Furthermore, microarray data should be presented and made publicly available, for which MIAME provides an excellent format. More importantly, microarray data should be interpreted together with clinical parameters. To be able to perform hierarchical clustering and classification strategies that incorporate these clinical data, both continuous and categorical parameters could be scaled to sensible numbers with ranges comparable to those of microarray gene expression ratios. We expect that the powerful combination of expression profiling and the use of circulating karyocytes will set off revolutionary changes in the treatment of cardiovascular patients.

Conclusions
In contrast to the general concept of atherosclerosis, circulating karyocytes are anything but passive bystanders that come into play only when ECs capture them. Circulating cells are sensitive to pressure or flow, endothelial dysfunction, and thrombogenic properties. The study of circulating karyocytes is, therefore, important in further understanding the mechanism of atherosclerosis.

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