Beyond Cholesterol – New Cardiovascular Biomarkers

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Abstract

Atherosclerosis (AS) is the primary pathological result of obesity. Vulnerable AS plaques cause fatal clinical end points such as myocardial infarction and stroke. To prevent this, improvements in early diagnosis and treatment are essential. Because vulnerable AS plaques are frequently nonstenotic, they are preclinically undetectable using conventional imaging. Levels of blood lipids, C-reactive protein, and interleukin-6 are increased, but are insufficient to indicate the process of critical perpetuation before the end points present. More specific biomarkers (e.g. troponin, copeptin, natriuretic peptides, growth differentiation factor-15, or soluble ST2) indicate the acute coronary syndrome or cardiac insufficiency, but not a critical destabilization of AS lesions in coronary or carotid arteries. Thus, valuable time (months to years) that could be used to treat the patient is wasted. An improved management of this dilemma may involve better detection of variations in degrees of immune inflammation in plaques by using new biomarkers in blood and/or within the lesion (molecular imaging). Macrophage and T-cell polarization, and innate and adaptive immune responses (e.g. Toll-like receptors) are involved in this critical process. New biomarkers in these mechanisms include pentraxin 3, calprotectins S100A8/S100A9, myeloperoxidase, adiponectin, interleukins, and chemokines. These proteins may also be candidates for molecular imaging using nuclear (magnetic resonance) imaging tools. Nevertheless, the main challenge remains: which asymptomatic individual should be screened? At which time interval? Intense interdisciplinary research in laboratory medicine (biomarkers), nanomedicine (nanoparticle development), and radiology (molecular imaging) will hopefully address these questions.

Introduction

Despite therapeutic advances, cardiovascular events are the leading causes of death worldwide. This is due to the increasing prevalence of atherosclerosis (AS) caused by the obesogenic lifestyle that is increasingly practiced in the so-called
western, industrialized world. AS is a subacute immune-mediated inflammation of the vascular wall characterized by the infiltration of macrophages and T cells, which interact with one another and arterial wall cells [1, 2]. The ensuing chronic inflammatory process leads to the formation, progression, and rupture of vascular lesions called AS plaques [1, 3–5].

The discovery of early identification methods and techniques to follow the development of AS plaques is still an unsolved challenge, which is limited not only by the performance of blood biomarkers and imaging techniques at hand, but also by the availability of specific molecules for targeted recognition [1, 6]. Hence, AS is only diagnosed at advanced stages of the disease, either by direct assessment of the degree of stenosis or by evaluating the effect of arterial stenosis on organ perfusion [1, 7]. Nevertheless, so-called vulnerable AS plaques that contribute significantly to the end points myocardial infarction or ischemic stroke (carotid lesions) are frequently nonstenotic and thus not easily diagnosed before events occur [1].

Pathological Characteristics of the Vulnerable Atherosclerotic Lesion

Vulnerable or culprit AS plaques are vascular lesions that are prone to rupture. They are found more frequently in regions of nonuniform shear stress, e.g. around bifurcations of the carotid or coronary arteries. Vulnerable plaques are characterized by their thin fibrous cap, activated endothelium, strong infiltration of macrophages, large lipid core, immune activation, and increased production of proinflammatory mediators (cytokines, chemokines, and metalloproteinases). Adventitial neovascularization, an imbalance between clotting and bleeding, and less calcification are further features. The true role of calcification remains a matter of debate because sui generis stable calcified AS lesions may also mechanically destabilize highly inflamed plaques if they are located near these lesions.

Mechanisms Promoting Vulnerability

Inflammation
The Monocyte/Macrophage System
Immune-mediated inflammation is a key contributor to AS, and monocytes/macrophages are principal offenders. In recent years, a complex taxonomy of different macrophage subtypes (M1–M4) has been established [8]. All these subtypes contribute to the dynamic processes occurring in AS plaques. Ly6C^{high} M1 macrophages produce proinflammatory TNF-α, IL-1, IL-6, and nitric oxide; Ly6C^{low} M2 macrophages secrete anti-inflammatory IL-10. After lipid loading, both M1 and
M2 macrophages can develop into foam cells, which usually undergo apoptosis and, thus, create a necrotic center in the AS plaque. Induced by free hemoglobin/haptoglobin, M2 macrophages can develop into so-called M(Hb)-type macrophages, which produce more anti-inflammatory cytokines, show a decreased lipid uptake, an increased cholesterol efflux, and a less foamy nature. Thus, bleeding processes within plaques do not necessarily lead to destabilization. They may also alter the macrophage response to a more stabilizing one over time if an acute event does not interrupt this process [9]. Hence, complex interactions of influential factors with these macrophage types significantly affect the direction of pathological development: stabilization → destabilization → exacerbation, or chronification, respectively. Cytokines/chemokines (GM-CSF, M-CSF, LPS/IFN-γ, IL-4/IL13, and IL-10), immune complexes, metalloproteinases, and lipid peroxidation are some of the most important influential factors [10].

Adaptive Immune Responses (T-Helper Cells and B Cells)
After endothelial injury, low-density lipoprotein reaches the intima of the vessel wall and undergoes enzymatic/oxidative modifications. This modified lipoprotein is ingested by macrophages, where it supports foam cell formation and the production of proinflammatory TNF-α, IL-1β, monocyte chemoattractant-1, leukotriene B₄, and proteolytic matrix metalloproteinases (MMP). In this way, endothelial cells are stimulated to overexpress adhesion molecules like VCAM-1 and ICAM-1. Effector cells from adaptive immune responses [preferentially T-helper (Th)1/Th17 lymphocytes] accumulate in the subendothelial space around lipoproteins [11]. Macrophages and dendritic cells present various antigens (e.g. Apo B 100) through MHC class II molecules to CD4⁺ T cells. This mainly activates CD4⁺ T cells of the Th1 subtype, which releases the proatherogenic cytokines IFN-γ and TNF-α. Regulatory T cells counterbalance this activation, at least in part, by the local release of TGF-β and IL-10. If this process does not subside, extralesional amplification loops come into play. Antigen-loaded dendritic cells and soluble antigens are transported via lymphatic vessels to draining lymph nodes and/or the spleen. There, naïve T cells develop into effector T cells and reenter the bloodstream. When these cells reach the AS lesion, they augment B cells such that they develop antibody-specific responses. Thus, the stability of the lesion declines, procoagulant factors are expressed, and plaque rupture and thrombosis become more probable. The gut microbiome may also substantially contribute to the extralesional amplification processes [12]. For example, the synthesis of TMAO (trimethylamine-N-oxide) from dietary phosphatidylcholine by intestinal microbiota appears to play an important role. Increased blood and urine TMAO levels have been shown to correlate positively with an increased risk of incidental cardiovascular events [13].
Intercurrent infections (e.g. common colds and influenza) may also destabilize the AS process via T-cell activation loops. Thus, Th1-cell-derived IFN-γ can increase the macrophage MMP-9/TIMP-1 production ratio. More MMP-9 and less TIMP-1 can destabilize the lesion by increased proteolysis.

The Innate Immune Response (Toll-Like Receptors)
The Toll-like receptors TLR2 [14], TLR4 [15], and TLR7 [16] are centrally involved in AS. TLR7 deficiency was shown to accelerate AS in ApoE−/− mice and to promote more vulnerable plaque phenotypes with increased lesion size, fewer smooth muscle cells, and a lower collagen content, and allowed stronger infiltration of macrophages and larger lipid deposits [16]. Furthermore, increased TLR7 expression in human carotid plaques stimulated the expression of genes associated with a more stable plaque phenotype. These included the M2 macrophage markers IL-10, IL-1RN, CD163, CLEC4A, CLEC7A, MSR1, CD36, MS4A4A, CLEC10A, CLEC13A/CD302, and CD209. In addition, genes associated with thrombosis, such as CD40L, TF/CD142, PF4/CXCL4, vWF, GPIba/CD42b, GPIbβ/42c, GPIIb/CD41, GPIIIa/CD61, GPV/CD42d, and GPIX/CD42a are downregulated [16].

Calprotectins and Danger-Associated Molecular Patterns
The danger-associated molecular pattern proteins S100A8 and S100A9, which belong to the S100 calgranulin family, are increased by the traditional cardiovascular risk factors (smoking, obesity, hyperglycemia, and dyslipidemia). These proteins are endogenous ligands of TLR4 and the receptor for advanced glycation end products. In humans, S100A8 and S100A9 levels have been shown to correlate with the extent of coronary and carotid AS and, most importantly, with a vulnerable plaque phenotype [15]. Furthermore, Erbel et al. [17] recently found MMP7(+)S100A8(+)CD68(+) M4 macrophages in coronary artery plaque tissue of humans afflicted with increased AS instability indexes. Hence, these calprotectins represent an interesting new molecular biomarker for assessing plaque vulnerability, since they reflect the pathological process in a highly specific way.

Mast Cells
The number of mast cells and their tryptase content in human carotid AS plaques and neovascularization were positively associated with future cardiovascular events [18].

Pentraxin 3, Myeloperoxidase, and Adiponectin
In contrast to C-reactive protein, pentraxin 3, also a member of the C-reactive protein family, was not expressed in the liver, but using immunochemical analysis it was detected in the basement membrane, endothelial cells and
perivascular cells of carotid endarterectomy specimens from culprit AS lesions and emboli captured by distal protection devices [19]. This fact suggests that pentraxin 3 is potentially a specific marker which can be used for molecular imaging of plaque vulnerability.

Furthermore, myeloperoxidase (MPO) was found to be positively associated with carotid plaque inflammation. Higher baseline MPO values indicated a higher baseline carotid target-to-background ratio of the most diseased segment. This relationship was even observed during a 3-month follow-up [20].

In addition, peripheral blood adiponectin levels were found to be systemically lower in patients with vulnerable, as compared to patients with stable, AS plaques [19]. We investigated the role of adiponectin and its subfractions in patients with obesity-associated pre-AS lesions [21–25], and the potential of fluorescence-labeled globular adiponectin (gAd) and the full-length form of adiponectin (fAd) subfractions (fAd) to bind to AS lesions in ApoE-deficient mice [2, 26, 27]. We found only a low binding efficiency of fAd, but an inflammation-mediated strong accumulation of gAd in the fibrous cap of AS plaques [26, 27]. Therefore, gAd may be a promising target sequence for the molecular imaging of AS lesions [26]. Furthermore, we developed nanoconstructs between gAd and PEGylated stealth liposomes [27], which can deliver a high number of signal-emitting molecules to AS lesions [27]. Other nanoconstructs between gAd and protamine-oligonucleotide nanoparticles, called proticles [28–31], displayed a particular affinity for monocytes and macrophages, which may be of interest for sequential AS plaque targeting. Thus, our results indicate the potential of gAd-targeted nanoparticles for the molecular imaging of AS.

**Balance between Clotting and Bleeding**

*Intraplaque Hemorrhage*

Undoubtedly, intraplaque bleeding plays an important role in the AS destabilization process, which leads to clinical end points. The hemoglobin/haptoglobin scavenger receptor (CD163), IL-10, HO-1 (heme oxygenase 1), ferritin, and 4-hydroxy-2-nonenal (a major product of lipid peroxidation) were found to be strongly expressed in culprit lesions of patients with unstable angina pectoris [32]. Therefore, extracellular hemoglobin from intraplaque hemorrhage appears to induce oxidative tissue damage due to heme iron and the subsequent production of reactive oxygen species. The clearance mediated by macrophage hemoglobin scavenger receptors may alter the AS process to a more aggressive one. Thus, IL-10, iron content, and HO-1 activity are markers of intraplaque bleeding, which is associated with plaque destabilization [32].
Blood Cellular Microparticles

Microparticles (MPs) are anucleoid plasma membrane fragments with a size of 50 nm to 1 μm. They consist of oxidized phospholipids and specific proteins from the cells from which they originate and are induced by pathological processes. For example, endothelial MPs (EMPs) can be induced by shear stress, angiotensin II, TNF-α, or thrombin. MPs are not ‘cell debris’! Moreover, they exert endocrine and paracrine effector functions, which may play an important role in plaque vulnerability. Their levels peak during vascular remodeling when the activation of the inflammasome is greatest, and can be considered a biological danger signal in the organism. Furthermore, they indicate prothrombogenic activity [33]. EMP levels were found to be significantly elevated in patients with carotid artery disease compared to controls [33]. Nevertheless, although research to explore the use of EMPs as biomarkers of disease has progressed substantially over the past few years, further work is still required to develop well-standardized methods for their analysis and quantification. Validation of normal EMP ranges and a specific set of EMP markers for diagnostic testing remain to be established. When a methodology with higher precision is available, analysis of EMP levels and content can be combined with other markers of AS, including inflammatory, lipid, angiogenic, and metabolic profiling. These multiplex assays may help current clinical practitioners to rank patients according to their risk of carotid disease and stroke. Thus, patients who are still asymptomatic, but who have potentially dangerous vulnerable plaque lesions, may be identified and preclinically stabilized, e.g. by preventive carotid endarterectomy [33].

Conclusions

To diagnose a vulnerable plaque phenotype well before fatal clinical end points such as myocardial infarction and stroke occur is one of the most important challenges facing personalized medicine. Here, we have shown that local and systemic biomarkers can significantly assist practitioners in improving the diagnosis and treatment for these deadly vascular lesions. Promising candidates found in the peripheral blood are the calprotectins S100A8 and S100A9, pentraxin 3, and MPO. EMPs may also be important in this context. S100A8 and S100A9 probably have a high potential, because they are centrally involved in the fine-tuning and amplification of innate immune responses through endogenous danger-associated molecular patterns. Nevertheless, it is important for therapeutic approaches that more blockers are approved for clinical testing.
Although all herein discussed biomarkers may be interesting for a therapeutic intervention, the best candidate(s) for a blocking strategy remain to be found out. Undoubtedly, it is important to focus on the immune-inflammatory pathway and to integrate aspects of a disturbed balance between clotting and bleeding usually seen in vulnerable AS lesions.

Furthermore, the following main challenges remain unsolved:

(i) To catch the vulnerable lesion at the right time in the right ‘patient’, and
(ii) To act in a specific and effective manner without inducing side effects, because the ‘patient’ may still feel healthy at the time of the successful prediagnosis.

Further interdisciplinary cooperation between laboratory medicine (biomarker research), nanotechnology (nanocarriers for use as contrast agents and/or drugs), and radiology (molecular imaging) will pave the way for future success.

**Disclosure Statement**

This author has nothing to disclose.

**References**