Novel antibody markers of unstable atherosclerotic lesions

Donald B. Bloch,[1] Thomas J. Wang,[2] and Robert E. Gerszten[1,2]

[1]Center for Immunology and Inflammatory Diseases and [2]Cardiology Division, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA.

Detection of autoantibodies in human serum assists in the diagnosis of patients with autoimmune diseases. Diagnostic antibody signatures have also been proposed for colon, pancreatic, and breast cancers. In this issue of the *JCI*, Cleutjens et al. describe the application of a peptide array technique toward the development of antibody biomarkers of ruptured atherosclerotic lesions (see the related article beginning on page 2979). A phage-display library was prepared from mRNA derived from ruptured peripheral human atherosclerotic plaques, and the phages containing immunoreactive peptides were screened with serum from patients with ruptured atherosclerotic lesions. Antibodies reacting with 2 peptides, E1 and E12, were particularly sensitive for the early diagnosis of acute myocardial infarction. Further studies that include an adequate number of patients presenting very early after the onset of symptoms and additional control patient populations are warranted to compare the utility of these biomarkers to those in clinical use.

Cardiovascular disease is the leading cause of death in developed countries. Substantial advances in the treatment of cardiovascular disease have occurred in the past several decades, but translating these new therapies into clinical benefit relies on the ability to target the appropriate patients at the appropriate stage of disease development. Conventional clinical tools, such as physical examination and electrocardiogram, are insensitive for diagnosing cardiovascular disease and identifying patients at high risk for future events. This has led to growing interest in the use of circulating biomarkers to provide additional clinical information. Novel biomarkers may also suggest targets for therapeutic intervention.

In cardiology, biomarkers typically reflect existing cardiac injury (e.g., troponins and creatine kinase), wall stress (e.g., natriuretic peptides), or activation of coagulation or inflammatory pathways (e.g., C-reactive protein, plasminogen activator inhibitor, and myeloperoxidase) (1, 2). Consequently, available biomarkers often provide information that overlaps with what is already known about a patient, limiting their ability to provide new biological insight or be of incremental clinical utility. However, technologies are emerging to enable the systematic characterization of variation in genes, RNA, and proteins in individuals with and without disease, permitting the discovery of biomarkers in a manner that is unbiased by current knowledge.

Antibodies as markers of disease

Detection of autoantibodies in human serum assists in the diagnosis of patients with autoimmune diseases. For example, antibodies directed against Smith antigens (Sm) and dsDNA are specific for the diagnosis of SLE (reviewed in ref. 3). Similarly, the presence of anti-Ro and anti-La autoantibodies in sera suggests a diagnosis of Sjögren syndrome, while the detection of anti-Scl70 antibodies supports a diagnosis of diffuse systemic sclerosis (reviewed in ref. 3). It remains unknown why intranuclear autoantigens, which are protected from the extracellular environment by both nuclear and cellular membranes, become the target of the humoral immune system in patients with autoimmune diseases.

Recent studies suggest that autoantibodies may also assist in the diagnosis of patients with early malignancies. Wang and colleagues reported that potential peptides present in prostate cancer tissue could be used to detect antibodies directed against prostate cancer antigens (4). Similarly, peptide and protein arrays have been proposed to identify antibody signatures associated with colon (5), pancreatic (6), and breast cancers (7). The rationale for the development of such arrays for the detection of antibodies is that some tumor antigens may be considered foreign by the immune system, and antibodies directed against these tumor-associated antigens may serve as early, sensitive, and specific markers of malignancy. In terms of cardiovascular disease, prior studies have identified oxidized LDL–specific antibodies that reflect the presence of acute coronary syndromes and correlate with the extent of peripheral vascular disease and increased risk of cardiovascular events in prospective cohorts (8, 9).

Application of a phage-display technique for cardiovascular biomarker discovery

In this issue of the *JCI*, Cleutjens and colleagues describe the application of a peptide array technique for the identification and implementation of antibody signatures to identify individuals with ruptured, peripheral, inflammation-rich, atherosclerotic lesions (10). The authors generated a phage-display library prepared from mRNA preferentially expressed in ruptured peripheral human atherosclerotic plaques. The cDNAs were prepared and expressed as fusion proteins with the minor coat protein pVI of filamentous phage M13. Sera from patients with ruptured atherosclerotic plaques were then used to perform 4 rounds of selection for phages expressing immunoreactive fusion proteins. Two phages containing immunoreactive peptides (designated E1 and E12) fused to pVI were identified and used to screen sera for anti-E1 and anti-E12 antibodies. The E1 peptide contains the 16 amino acids present within a potential protein designated 1NFLS. The E12 peptide is a nonsense peptide derived from the untranslated 3′ end of a cDNA encoding PKCη. Antibodies directed against E1 were detected in a majority of patients with ruptured atherosclerotic plaques. Anti-E1 antibodies were not detected in sera from patients with

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stable plaques or from control individuals. Similarly, antibodies directed against E12 were detected in sera from patients with ruptured plaques, but not in sera from subjects with stable plaques or control individuals. The authors noted that the anti-E1 and anti-E12 antibodies were present and could be detected in patient sera before any measurable increase in troponin T levels was observed in the subjects who presented with acute coronary syndromes. Troponin T is a cardiac-specific protein that leaks into the plasma during myocardial injury and represents the most sensitive and specific clinical biomarker for myocardial infarction that is currently available in clinical practice (11). The authors thus suggest that these 2 biomarkers may be particularly useful in early patient triage in the emergency room.

What do these potential biomarkers tell us about the biology of unstable atherosclerotic lesions? The anti-E1 and anti-E12 antibodies described in the current article do not appear to be autoantibodies. Autoantibodies by definition are directed against self antigens. In SLE patients with anti-Sm antibodies, autoantibodies are directed against protein components of the human spliceosome, including proteins B, B′, D, and E (3). In contrast, the anti-E1 and anti-E12 antibodies identified by Cleutjens and colleagues appear to be directed against random peptides, not self antigens (10). As noted above, the E12 peptide corresponds to the artificially translated portion of the 3′ untranslated region of an mRNA. The E1 peptide is reported to be composed of the C-terminal 16 amino acids of potential protein 1NFLS, derived from expressed sequence tag (EST) BX_106432. However, this EST may in fact be a genomic fragment of chromosome 1 (NW_00183537), rather than a cDNA derived from mRNA. The E1 peptide, GQVRGFTMLTRLVLNL, is not part of any protein identified in GenBank. Therefore, it is possible that neither E1 nor E12 actually exists in vivo. Anti-E1 and anti-E12 antibodies must be considered anti-(random) peptide antibodies. The actual targets of the human antibodies that crossreact with E1 and E12 are unknown.

Another important question regarding the present study relates to the pathophysiology of antibody formation in patients whose cardiovascular disease is related to plaque instability. Clearly, anti-peptide antibodies do not develop on the day a patient presents with an acute myocardial infarction. The authors speculate that anti-peptide antibodies may form during the days or weeks prior to an acute episode, during periods of plaque instability. That is, there may be a substantial delay between plaque rupture and the onset of symptoms. Why do patients with stable atherosclerotic plaques fail to produce anti-peptide antibodies? Do they not have periods of plaque instability? Is there something fundamentally different about the pathophysiology of plaque formation in patients with stable plaques that does not induce anti-peptide antibody formation, and might these antibodies be useful in identifying distinct phenotypes among patients with atherosclerosis? These questions remain to be answered by future investigations.

Potential clinical significance

Biomarkers can aid in the risk stratification of patients without apparent disease (screening biomarkers), with suspected disease (diagnostic biomarkers), or with overt disease (prognostic biomarkers). The current biomarkers proposed by Cleutjens et al. (10) are diagnostic biomarkers, not screening biomarkers, because they identify patients only upon acute presentation. Diagnostic biomarkers are useful only if they are positive in individuals with disease (i.e., they are sensitive) and negative in individuals without disease (i.e., they are specific). In the present study, the sensitivities for the anti-E1 and anti-E12 antibodies were 40%–76% in patients with ruptured peripheral atherosclerotic plaques. The clinical utility of distinguishing stable from ruptured atherosclerotic plaques in patients presenting with vascular symptoms is unclear. Indeed, all patients in the study were undergoing vascular surgery for symptomatic peripheral vascular disease (either claudication, transient ischemic attack, or stroke) or enlarged abdominal aortic aneurysms. Would knowledge regarding plaque morphology alter the decision to proceed with surgery or alter subsequent medical treatment?

The sensitivity of anti-E1 and anti-E12 antibodies for acute myocardial infarction was much higher in the current study at 88%–93% (10). However, sensitivity was only 33% for detecting unstable angina, a condition also thought to be caused by plaque rupture, but without the myocardial necrosis that occurs in acute myocardial infarction. In contemporary practice, unstable angina and myocardial infarction are frequently approached similarly, by invasive angiography. Thus, poor sensitivity for detecting some forms of acute coronary syndrome reduces the utility of these putative biomarkers. Nonetheless, as the authors suggest, an early biomarker that is positive for myocardial infarction and measurable prior to the detection of cardiac troponin, the existing gold standard, would be of clinical value.

Although the biomarker set of E1 in combination with E12 showed 100% specificity in discriminating patients with ruptured lesions from patients with stable plaques, nearly two-thirds of rheumatoid arthritis patients had antibodies directed against E12 (10). Although only 1 rheumatoid arthritis patient had anti-E1 antibodies, the specificity of anti-E1 antibodies for ruptured plaques will have to be tested against additional control populations, including patients with other autoimmune or chronic infectious diseases. The observation that these biomarkers persisted for months after the acute myocardial infarction in some patients could also lower their specificity for detecting recurrent events.

Several recent studies have highlighted the limitations of presently available biomarkers, alone or in combination, in diagnosis and screening for cardiovascular disease, especially given their modest incremental benefit over conventional cardiovascular risk markers, including age, sex, lipid levels, diabetes, or hypertension (2, 12). As noted above, existing biomarkers have been derived from pathways known to be associated with atherosclerosis and its complications. The addition of correlated biomarkers in the same pathways are unlikely to provide incremental information. In contrast, biomarkers from uncorrelated or orthogonal pathways involved in disease pathophysiology are most likely to improve diagnostic or predictive performance. Some emerging proteomic technologies have promise for identifying such biomarkers, as evidenced by the work of Cleutjens and colleagues (10). Indeed, the authors' findings suggest that antibody reactivity against peptides E1 and E12 does not appear to correlate with general cardiovascular risk factors. Further studies, which must include an adequate number of patients presenting very early after the onset of symptoms, are warranted to compare these biomarkers with existing ones.

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Address correspondence to: Robert E. Gerszten, Cardiology Division and Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital East — 8307, 149 13th Street, Charlestown, Massachusetts 02129, USA. Phone: (617) 724-8322; Fax: (617) 726-5651; E-mail: rgerszten@partners.org.

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Fc receptors in immune thrombocytopenias: a target for immunomodulation?

Bethan Psaila[1,2] and James B. Bussel[1]

[1]Platelet Disorders Center, Division of Pediatric Hematology-Oncology, Weill Cornell Medical College of Cornell University, New York, New York, USA.
[2]Department of Haematology, Division of Investigative Sciences, Imperial College School of Medicine, Hammersmith Hospital, London, United Kingdom.

In autoimmune disease, Fc receptors (FcRs) form the interface between immune effector cells and their antibody-coated targets, and as such are attractive targets for immunomodulatory therapy. In this issue of the *JCI*, two highly novel studies of Fc–FcR interactions provide new insights into the role of FcRs in immune thrombocytopenia. Asahi et al. utilized a comprehensive platform of immunological assays to examine the mechanism underlying *Helicobacter pylori*–associated immune thrombocytopenic purpura, and Ghevaert et al. describe a specially designed antibody that saturates binding sites on fetal platelets without initiating FcγR-mediated platelet phagocytosis, preventing the binding of pathological maternal anti-HLA antibodies that cause fetomaternal alloimmune thrombocytopenia (see the related articles beginning on pages 2939 and 2929, respectively). These reports illustrate how a remarkably detailed molecular understanding of the FcR network may translate into new therapeutic strategies with high clinical impact.

A focus on the Fc receptor network present on macrophages

The interactions between immune cells and their target cells in autoimmune diseases have been the focus of much attention, and intense efforts have been made to manipulate the signaling pathways involved. The great majority of studies have examined T and B cells, and recently there has been increased interest in the role of Tregs as deft orchestrators of the immune response (1, 2). In contrast, macrophages have largely been investigated for their ability to execute intracellular killing and are considered to be the mobile but passive "clean-up men" of the host defense system. Part of their weaponry, which only devotees care to distinguish into subgroups, comprises the Fc receptors (FcRs). Seminal studies, initially from Ravetch's group and subsequently from the Lazarus laboratory, developed key insights into the network of FcRs expressed on macrophages, and the interactions between these phagocytic cells and antibodies emerged as attractive targets for immunomodulatory therapy (3, 4). Two articles in this issue of the *JCI* involve very different manipulations of the Fc-FcR interaction in order to increase our understanding of the pivotal role played by the FcR network in the pathogenesis of immune thrombocytopenia. Both reports are highly clinically relevant. In the first study, Asahi et al. examined the changes in the balance of FcRs expressed by patients with immune thrombocytopenia purpura (ITP) and *Helicobacter pylori* infection in order to explore the mechanism of platelet recovery that has been observed in these individuals following treatment to eradicate *H. pylori* (5). In the second study, Ghevaert et al. report the development and preclinical testing of a recombinant antibody designed to prevent FcR-mediated alloimmune destruction of platelets, which may have potential as a treatment approach for fetomaternal alloimmune thrombocytopenia (FMAIT) (6).

Inhibiting FcγR-mediated platelet clearance in ITP: a historical context

The central immunopathological disturbance in immune thrombocytopenia is the destruction of antibody-coated platelets by phagocytic cells in the reticuloendothelial system (7). Circulating monocytes and resident macrophages in the spleen and liver

Nonstandard abbreviations used: FcR, Fc receptor; FMAIT, fetomaternal alloimmune thrombocytopenia; HPA, human platelet antigen; ITP, immune thrombocytopenia purpura; IVIG, i.v. immunoglobulin.

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