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Noninvasive diagnosis of ruptured peripheral atherosclerotic lesions and myocardial infarction by antibody profiling

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Technical Advance

Novel biomarkers, such as circulating (auto)antibody signatures, may improve early detection and treatment of ruptured atherosclerotic lesions and accompanying cardiovascular events, such as myocardial infarction. Using a phage-display library derived from cDNAs preferentially expressed in ruptured peripheral human atherosclerotic plaques, we performed serological antigen selection to isolate displayed cDNA products specifically interacting with antibodies in sera from patients with proven ruptured peripheral atherosclerotic lesions. Two cDNA products were subsequently evaluated on a validation series of patients with peripheral atherosclerotic lesions, healthy controls, and patients with coronary artery disease at different stages. Our biomarker set was able to discriminate between patients with peripheral ruptured lesions and patients with peripheral stable plaques with 100% specificity and 76% sensitivity. Furthermore, 93% of patients with an acute myocardial infarction (AMI) tested positive for our biomarkers, whereas all patients with stable angina pectoris tested negative. Moreover, 90% of AMI patients who initially tested negative for troponin T, for which a positive result is known to indicate myocardial infarction, tested positive for our biomarkers upon hospital admission. In conclusion, antibody profiling constitutes a promising approach for noninvasive diagnosis of atherosclerotic lesions, because a positive serum response against a set of 2 cDNA products showed a strong association with the presence of ruptured peripheral atherosclerotic lesions and myocardial infarction.

Noninvasive diagnosis of ruptured peripheral atherosclerotic lesions and myocardial infarction by antibody profiling

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Novel biomarkers, such as circulating (auto)antibody signatures, may improve early detection and treatment of ruptured atherosclerotic lesions and accompanying cardiovascular events, such as myocardial infarction. Using a phage-display library derived from cDNAs preferentially expressed in ruptured peripheral human atherosclerotic plaques, we performed serological antigen selection to isolate displayed cDNA products specifically interacting with antibodies in sera from patients with proven ruptured peripheral atherosclerotic lesions. Two cDNA products were subsequently evaluated on a validation series of patients with peripheral atherosclerotic lesions, healthy controls, and patients with coronary artery disease at different stages. Our biomarker set was able to discriminate between patients with peripheral ruptured lesions and patients with peripheral stable plaques with 100% specificity and 76% sensitivity. Furthermore, 93% of patients with an acute myocardial infarction (AMI) tested positive for our biomarkers, whereas all patients with stable angina pectoris tested negative. Moreover, 90% of AMI patients who initially tested negative for troponin T, for which a positive result is known to indicate myocardial infarction, tested positive for our biomarkers upon hospital admission. In conclusion, antibody profiling constitutes a promising approach for noninvasive diagnosis of atherosclerotic lesions, because a positive serum response against a set of 2 cDNA products showed a strong association with the presence of ruptured peripheral atherosclerotic lesions and myocardial infarction.

Introduction

In recent years, it has become apparent that inflammation plays a key role in the onset and progression of atherosclerosis (1–4). The vast majority of its acute clinical manifestations are not the result of progressive luminal narrowing by a slowly growing stable atherosclerotic plaque, but are caused by the formation of an occluding thrombus on the surface of a ruptured or fissured plaque. Despite increasing knowledge of the pathogenesis of atherosclerosis and the identification of several circulating markers associated with cardiovascular disease, biomarkers for noninvasive diagnosis of ruptured atherosclerotic lesions and markers that have prognostic value for the identification of individual patients at high risk of future cardiovascular events are not yet available (5–11). Based on the hypothesis that (auto)immune mechanisms contribute significantly to the inflammatory state and subsequent plaque rupture (12–14), we report here the identification and initial characterization of (auto)antibody-binding cDNA products. These cDNA products were isolated by serological antigen selection (SAS) on a phage-display library derived from cDNAs preferentially expressed in ruptured peripheral human atherosclerotic plaques. Phage display is a technique in which bacteriophages are engineered to produce and to expose or display a foreign peptide fused to one of their capsid proteins at the external surface of the phage. The phage can then be used to screen which ligand, antibody, or antigen binds to the expressed fusion protein.

Here, we explored the use of the antibody signature — a molecular fingerprint of antibodies present in a particular disease state — against selected cDNA products as a biomarker for noninvasive detection of ruptured peripheral plaques and myocardial infarction. Using serological antigen selection, we identified 2 cDNA products that could distinguish pooled ruptured plaque sera from pooled sera from patients with stable plaques and sera from healthy controls. Subsequent validation on substantial panels of individual sera showed that our biomarker set was able to discriminate between patients with peripheral ruptured lesions and patients with peripheral stable plaques with 100% specificity and 76% sensitivity. Furthermore, 93% of patients with an acute myocardial infarction (AMI) tested positive for our biomarkers, whereas all patients with stable angina pectoris tested negative. In addition, 90% of AMI patients who initially tested negative for troponin T (TnT) tested positive for our biomarkers upon hospital admission. Thus, antibody profiling constitutes a promising approach for noninvasive diagnosis of atherosclerotic lesions, because a positive serum response

Nonstandard abbreviations used: AMI, acute myocardial infarction; SAP, stable angina pectoris; SAS, serological antigen selection; TnT, troponin T; UAP, unstable angina pectoris.

Conflict of interest: The authors have declared that no conflict of interest exists.

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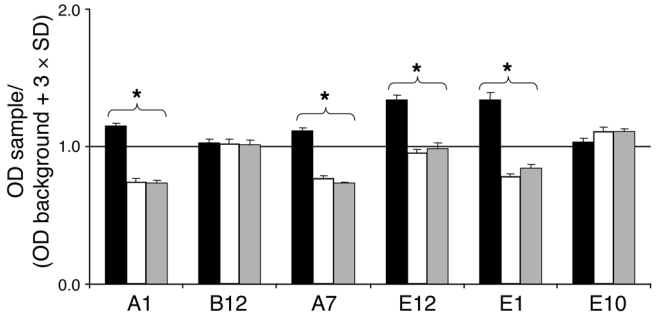

Figure 1
Autoantibody signature of 4 phage-displayed peptides in pooled sera of patients with proven peripheral ruptured (black bars) or stable (white bars) lesions and in pooled control sera (gray bars). Reactivity is represented as the ratio of OD_{450} sample/(mean OD_{450} + 3SD) for empty phage. Clones A1, A7, and E1 had identical inserts. Data are mean ± SD. *$P < 0.05$.

against a set of 2 cDNA products showed a strong association with the presence of ruptured peripheral atherosclerotic lesions and myocardial infarction.

Results

Isolation of antigenic phage-displayed cDNA products. In the discovery phase of this study, in order to create an expression library of cDNAs predominantly expressed in ruptured peripheral atherosclerotic lesions, a suppression subtractive hybridization (SSH) library of more than 3,000 ruptured plaque–enriched cDNAs previously generated in our laboratory (15) was recloned into phage-display vectors pSP6A, pSP6B, and pSP6C (16). Using these vectors, inserted cDNA fragments are expressed, along with minor coat protein pVI of filamentous phage M13, in the form of a fusion protein. The resulting surface complex functioned as bait to capture antibodies in sera from patients known to have peripheral ruptured atherosclerotic plaques, referred to herein as ruptured plaque sera. To enrich for cDNA products binding to (auto)antibodies in ruptured plaque sera, we carried out 4 successive rounds of affinity selection (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI32767DS1), the process of affinity purification used in the identification and isolation of phages carrying recombinant peptides with high affinity and specificity to the desired target molecule. Briefly, a library of phage-displayed peptides is incubated in an immunotube coated with the target. Unbound particles are washed away, while the bound phages are eluted and used for amplification and/or further purification. The selection process can be repeated several times, as done in the present study.

We assessed the antibody signature against 96 randomly selected antigens. While the majority of the phage-displayed cDNA products showed a positive antibody signature in pooled ruptured plaque sera, sera from patients with stable plaques, and sera from healthy controls, the antibody signature of 4 clones could distinguish pooled sera from patients with ruptured peripheral lesions from both stable plaque sera and control sera (Figure 1). Sequence analysis revealed that 3 clones encoded the same cDNA products, designated antigen E1, while the fourth clone encoded the unrelated antigen E12.

Detailed serological analysis of antigens E1 and E12. Next, in the validation phase of the study, antigens E1 and E12 were tested on large panels of individual sera from patients with either peripheral or coronary atherosclerotic lesions (see Methods, Table 1, and Supplemental Table 1 for detailed patient characteristics). The peripheral lesion cohort consisted of 45 patients with proven ruptured atherosclerotic lesions, 30 patients with proven stable atherosclerotic plaques, and 40 age- and sex-matched healthy controls. The coronary lesion cohort consisted of 40 patients with AMI, 29 patients with stable angina pectoris (SAP), and 30 patients with unstable angina pectoris (UAP).

In the peripheral lesion cohort, antigen E1 showed reactivity (calculated as described in Methods) to 28 of 45 (62%) ruptured plaque sera, whereas no reactivity to stable plaque and control sera was observed (Figure 2A). In addition, antigen E12 showed reactivity in 18 of 45 (40%) ruptured plaque sera, while none of the stable plaque or control sera tested positive (Figure 2B). Furthermore, there were remarkable correlations between the clinical condition of patients (Supplemental Table 1) and both the plaque phenotype and the anti-E1 and anti-E12 response. Combination of the serum reactivity against antigens E1 and E12 further increased the sensitivity to 76%, while the specificity remained at 100% (Figure 2C). As shown in Figure 2D, there was partial overlap between E1 and E12 positivity in individual patients.

Screening of the coronary lesion cohort showed that 35 of 40 (88%) AMI sera tested positive for E1, while all 29 patients with SAP tested negative (Figure 2E). Moreover, 34 of 40 (85%) AMI sera tested positive for E12, while all 29 patients with SAP tested negative (Figure 2F). Combination of the serum reactivity against clones E1 and E12 increased the sensitivity to 93% (Figure 2G). Of the 30 patients with UAP, 10 patients with TnT-negative

Table 1
Baseline characteristics of participating peripheral and coronary artery disease patients

	Peripheral vascular disease		Coronary artery disease		
	Ruptured ($n = 45$)	Stable ($n = 30$)	AMI ($n = 40$)	UAP ($n = 30$)	SAP ($n = 29$)
Age (yr)	65.1 ± 1.8	64.8 ± 2.0	65.0 ± 2.0	65.3 ± 2.4	62.9 ± 1.9
Male (%)	73	63	65	55	93
Hypertensive (%)	44	50	25	55	31
Smoker (%)	52	46	50	37	73
Diabetic (%)	48	53	6	23	10
Statin use (%)	50	52	21	60	90
CRP (mg/l)	9.2 ± 2.1	6.2 ± 2.2	19.2 ± 9.0	8.6 ± 2.6	7.3 ± 2.9
Cholesterol (mmol/l)	5.7 ± 0.2	6.0 ± 0.3	5.7 ± 0.2	4.9 ± 0.2	4.3 ± 0.2
LDL (mmol/l)	4.0 ± 0.2	3.8 ± 0.3	3.8 ± 0.2	3.0 ± 0.1	2.1 ± 0.2
HDL (mmol/l)	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
Triglycerides (μmol/l)	2.1 ± 0.2	2.0 ± 0.4	2.0 ± 0.1	2.1 ± 0.3	2.1 ± 0.4
TnT upon admission (μmol/l)	NA	NA	0.16 ± 0.06	0.00 ± 0.00	0.00 ± 0.00
Peak TnT (μmol/l)	NA	NA	7.15 ± 2.19	0.00 ± 0.00	0.00 ± 0.00

Data (except percentages) are mean ± SEM. NA, not applicable.

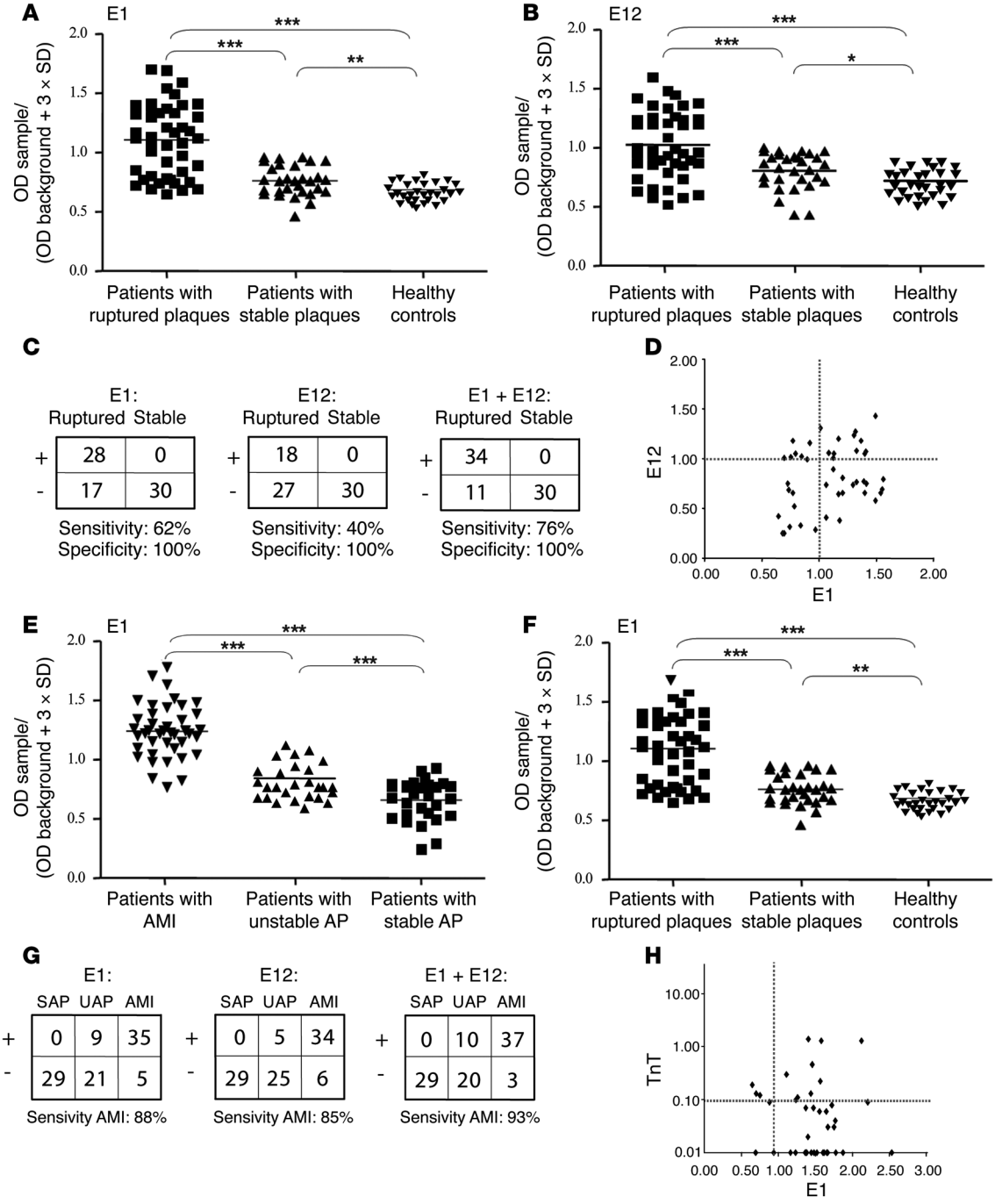

Figure 2

Detailed serological analysis of antigens E1 and E12. Antibody profile of clone E1 (**A**, **E**, and **H**) and clone E12 (**B** and **F**); sensitivity and specificity of clones E1, E12, and E1 and E12 combined (**C**, **D**, and **G**); and correlation of the response to clones E1 and E12 (**D**) and E1 and TnT levels (**H**) in patients with peripheral vascular disease (**A**–**D**) and coronary artery disease (**E**–**H**). Reactivity is represented as the ratio of OD_{450} sample/(mean OD_{450} + 3SD) for empty phage. (**A**, **B**, **E**, and **F**) Data points and horizontal bars represent reactivity of individual sera and mean reactivity, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$.

Braunwald classification (17) of IIIB ($n = 7$) and IIB ($n = 3$) UAP were reactive to either E1 or E12, while the remaining 20 patients (Braunwald classification I, $n = 17$; IIA, $n = 3$) tested negative.

Moreover, the anti-E1 and anti-E12 antibody response preceded the increase in the level of troponin, the current best available biomarker, in patients with AMI, because at presentation 10 of 40 AMI patients showed a positive TnT response compared with 35 with a positive anti-E1 response ($P = 0.0000523$; Figure 2H), 34 with a positive anti-E12 response ($P = 0.000136$), and 37 with a positive anti-E1 and/or anti-E12 response ($P \leq 0.000126$). In serum samples

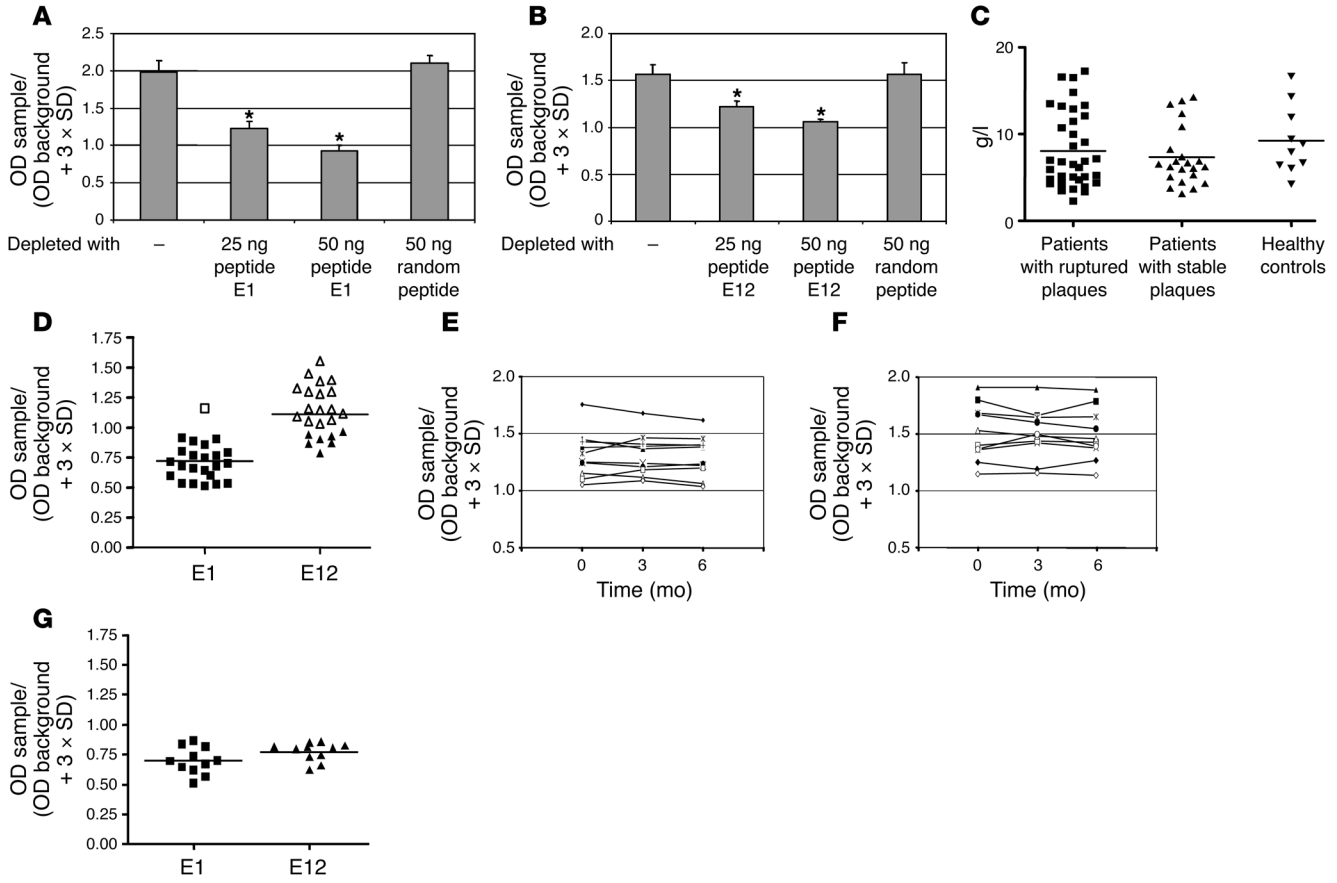

Figure 3

Specificity of the E1 and E12 autoantibody signature. (**A** and **B**) Ability of synthetic peptides E1 and E12 to deplete human ruptured sera from autoantibodies directed against phage E1 (**A**) and E12 (**B**), respectively. Data are mean ± SD. (**C**) Serum IgG content in patients with peripheral vascular disease. (**D**) Serum reactivity of 23 age- and sex-matched RA sera against clones E1 and E12. Filled symbols represent nonreactive sera; open symbols represent reactive sera. (**E** and **F**) Temporal pattern of the antibody profile to clones E1 (**E**) and E12 (**F**) in 10 patients with AMI. (**G**) Serum reactivity of 11 patients with non–atherosclerosis-related cardiac diagnoses. (**A**, **B**, and **D**–**G**) Reactivity is represented as the ratio of OD_{450} sample/(mean OD_{450} + 3SD) for empty phage. (**C**, **D**, and **G**) Data points and horizontal bars represent reactivity of individual sera and mean reactivity, respectively. *$P < 0.05$ versus nondepleted.

taken 8–24 h after presentation, all 40 patients showed a positive TnT response. However, we did not observe a statistically significant correlation between the anti-E1 and/or anti-E12 response at presentation and peak troponin levels (data not shown).

In addition, in both the peripheral and coronary lesion cohorts, reactivity against clones E1 and E12 showed no significant correlation with general cardiovascular risk factors (age, sex, lipid levels, hypertension, CRP levels, diabetes, smoking) or medication (statins, β-blockers, and ACE inhibitors; data not shown).

Specificity of the anti-E1 and anti-E12 response. To determine the specificity of the observed positive antibody signature, 5 individual reactive ruptured plaque sera were preincubated with synthetic peptides representing the cDNA products of clones E1 and E12 and with nonspecific (random) peptides. As shown in Figure 3, preincubation with E1 and E12 peptides inhibited the formation of specific IgG antibody/phage complexes. In contrast, the serum reactivity against clones E1 and E12 was not inhibited by depletion with both random peptides. We also tested whether there was a correlation between total serum IgG content and the antibody signature against clones E1 and E12. As shown in Figure 3C, there was no significant difference in the IgG levels in the groups tested, and there was no evidence for a correlation between total serum IgG and the anti-E1 and anti-E12 IgG content.

We next tested whether the positive E1 and E12 antibody signatures were atherosclerosis specific or were also present in other chronic inflammatory diseases (Figure 3D). In a panel of 23 age- and sex-matched RA patients, 1 serum showed a positive anti-E1 signature, while 15 of 23 sera tested positive with antigen E12. After detailed analysis of the medical history of the included RA patients by examining medical records and taking patients' histories, the only RA patient with a positive anti-E1 signature had a previous AMI. Evaluation of the medical history of the RA patients further revealed 5 additional patients with signs of advanced but stable atherosclerotic disease, 1 each with SAP, intermittent claudication, aorta stenosis, prior percutaneous transluminal coronary angioplasty, and peripheral artery angioplasty. However, there was no correlation between the presence of a positive anti-E12 response and the presence of stable atherosclerotic disease. Thus, evaluation of the antibody signatures against E1 and E12 in RA patients further strengthened the

Table 2
Detailed characteristics of clones E1 and E12

Clone	Insert size (nt)	cDNA identity (accession no.)[A]	Translated aa sequence of the insert[B]	Insert size (aa)	Homology on aa level (accession no.)[C]
E1	284	100% 1NFLS (BX106432)	GQVRGFTMLTRLVLNL*	16	91% (12 aa) NKG2E (NP_002252)
E12	270	100% protein kinase C, eta (NM_006255)	GQVHIIRSSLIYALFTRSISNY*	22	None to eukaryotic proteins

Asterisks indicate stop codons. [A]Homology of the cDNA inserts to annotations in public databases. [B]Translated peptide sequence of the cDNA insert, translated according to the reading frame of the phage vector. [C]Highest significant homology of the synthetic peptide insert to a protein annotation in the public domain. All sequences were annotated using Blast analysis.

ruptured plaque specificity of the anti-E1 autoantibody response. In contrast, the observed reactivity against clone E12 suggests cross-reactivity across chronic inflammatory diseases.

Triggered by the observation of the positive anti-E1 signature of the RA patient with a previous AMI, we tested the temporal patterning of the anti-E1 and anti-E12 response in 10 AMI patients. As shown in Figure 3, E and F, the anti-E1 and anti-E12 response remained elevated at 3 and 6 months after hospital admission, at levels comparable to those upon admission ($P < 0.001$, Spearman test).

Finally, all 11 patients with non–atherosclerosis-related cardiac diagnoses (atrium fibrillation, $n = 9$; long QT syndrome, $n = 1$; and AV-node tachycardia, $n = 1$) showed a negative anti-E1 and anti-E12 response (Figure 3G), while their anti-E1 and anti-E12 levels were comparable to those of healthy controls ($P < 0.01$, Spearman test).

Characterization of antigens E1 and E12. Clone E1 contained an insert of 284 nt, showing 100% homology with the human fetal liver/spleen–derived cDNA clone 1NFLS (Table 2). On the protein level, the insert encodes a 16-aa peptide fused to the C-terminal end of phage protein pVI. Detailed bioinformatics revealed a high level of homology with the human natural killer cell lectin-like receptor NKG2E. Furthermore, although the 270-nt insert of clone E12 showed 100% homology with the 3′ untranslated region of human protein kinase C, eta, no significant homology with any eukaryotic protein was found. However, the 22-aa clone–specific part of the fusion protein showed 81% homology in a stretch of 11 aa and 75% in a stretch of 16 aa with both *CMV* US14 protein and *Candida albicans* MRP1p protein. To test the possibility that the serum response against antigen E12 originated from a *CMV* and/or *C. albicans* infection, we determined *CMV* viral load (by real-time PCR) and IgG antibody titers against both pathogens (by ELISA) in our patient population. However, no correlation with *CMV* and/or *C. albicans* status and serum reactivity against antigen E12 was found (data not shown).

Discussion

In this study, we used SAS to isolate cDNA products specifically interacting with serum samples of patients with ruptured peripheral atherosclerotic lesions. In our cross-sectional study, a set of 2 antigens had 100% specificity and 76% sensitivity in discriminating between patients with proven ruptured peripheral atherosclerotic lesions and patients with proven stable peripheral plaques and healthy controls. Moreover, 93% of patients with an AMI tested positive for our new biomarkers, while all patients with SAP tested negative.

The anti-E1 and anti-E12 antibody response preceded the increase in TnT levels in patients with AMI. Whereas TnT, as a marker of myocardial necrosis, takes at least 3–6 hours following myocardial injury by arterial obstruction to rise to measurable circulating levels, our biomarkers were highly predictive for the presence of AMI within 2 h of symptom onset, even in patients initially negative for TnT. The observation of an antibody response so soon after onset of symptoms, even before markers of myocardial infarction can be detected, is puzzling. On one hand, Rittersma et al. (18) previously reported that plaque instability frequently occurs days or weeks before occlusive coronary thrombosis. Thus, if we assume episodes of plaque rupture to occur days or weeks before the clinical symptoms, there should be ample time for raising a significant antibody response even before markers of myocardial necrosis are elevated. Our finding that only the sera from the most severely affected UAP patients were reactive, while the remaining patients with UAP all tested negative, suggests that E1 and E12 may be positive even prior to myocardial infarction.

Because the anti-E1/anti-E12 response remained elevated at 3 and 6 months after admission, several alternative explanations need to be raised. The anti-E1/anti-E12 response might also reflect (a) a persisting antibody response even after healing of the ruptured plaque; (b) an association with plaque composition or plaque activity rather than plaque rupture, or (c) continuous production of antibodies by other vascular sites.

In addition to the uncertainty at present of the clinical value of our biomarkers, the present study has several other limitations. First, although we observed a strong association between a positive anti-E1/anti-E12 response and the presence of a peripheral ruptured plaque, myocardial infarction, or severe UAP in this cross-sectional study, we need prospective data to prove the correlation between plaque rupture and myocardial infarction. Second, despite the high level of homology of clone E1 with the human natural killer cell lectin-like receptor NKG2E (Table 2), there is a lack of pathophysiological knowledge of E1/E12 peptides and interacting antibodies.

Several authors have previously provided evidence for the prognostic value of circulating biomarkers for plaque instability and rupture — including CRP, type II secretory phospholipase A2, PAPP-A, and myeloperoxidase (MPO), recently reviewed by Koenig and Khuseyinova (9) and by Morrow et al. (11). The chemokines CCL5 and CCL18 were also recently identified as markers of refractory UAP (19). Although the levels of several of the above-mentioned circulating markers may carry important prognostic information, they are, at best, moderate predictors (maximum odds ratio, 4.2) for future development of deleterious clinical complication of atherosclerosis; currently, none can be recommended for routine clinical use. In addition, as was recently reported, even measurement of a set of 10 contemporary cardiovascular biomarkers added only moderately to standard risk factors in assessing risk in individuals (10, 20).

It will therefore be interesting to test whether the anti-E1 and anti-E12 response will increase the sensitivity and/or positive predictive value for the presence of ruptured plaques and/or major adverse cardiovascular events in individual patients. In this respect, we stress the importance of our initial finding of a positive anti-E1 antibody signature only in patients with proven ruptured peripheral atherosclerotic lesion or UAP/AMI; this observation is important not only with respect to patients with advanced atherosclerotic disease, but also with respect to healthy controls and patients with extensive RA. In contrast, the anti-E12 response indicated cross-reactivity across chronic inflammatory diseases, while no significant correlation between the presence of advanced but stable atherosclerotic lesions and positive anti-E12 antibody signature was observed. However, in our cohort of 23 RA patients, 4 of 5 patients with advanced but stable atherosclerotic disease had E12 reactivity values in the top half of the range, and 3 of 5 were in the top tertile. Additional experiments are needed to further substantiate these initial observations.

To explore the (auto)antibody repertoire of patients, the novel SAS approach (16) has been applied recently to successfully identify a panel of candidate colorectal (21), prostate (22), and lung (23) tumor–related and MS-related (24, 25) antigens. In the present study, we combined our library of cDNAs preferentially expressed in ruptured peripheral human atherosclerotic plaques with the strong selective power of the SAS procedure in order to isolate ruptured plaque–specific antigens. Because the antigen library used contains peptides derived from fusion of inserted cDNA fragments with phage coat protein pVI in 3 reading frames and 2 orientations, each cDNA fragment theoretically results in 6 different recombinant peptides. This characteristic of the pVI display system was reflected in our panel of identified antigens. Although both clone inserts were 100% homologous to known cDNAs, as a result of the use of an alternative reading frame (clone E1) or translation of a normally untranslated region (clone E12), both clones reflect so-called "mimotopes" (peptide mimicking a known epitope, but not homologous at the sequence level).

In conclusion, the antibody response against clones E1 and E12 forms a potential new biomarker for the noninvasive diagnosis of ruptured peripheral atherosclerotic lesions as well as UAP and AMI. Longitudinal studies are needed to determine its diagnostic and predictive value for the identification of patients experiencing cardiovascular events or those at high risk for future events.

Methods

Serum and tissue samples. The study was approved by the Medical Ethical Committee of the University of Maastricht and Maastricht University Hospital, and all patients included provided written informed consent. Serum and peripheral atherosclerotic plaque samples were obtained from 81 patients undergoing vascular surgery (Department of General Surgery, Academic Hospital Maastricht). All patients with femoral, iliac, popliteal, and carotid artery disease were symptomatic, defined as intermittent or severe claudication accompanied by a decreased Ankle-Brachial pressure index (ABPI <0.9) in the case of femoral, iliac, and popliteal artery disease and as the occurrence of at least 1 transient ischemic attack or minor stroke within the 3 months before surgery and a stenosis between 70% and 99% in the case of carotid artery disease. Patients with abdominal aortic disease had abdominal aortic aneurysms (AAAs) and permanent dilatation of the aorta, with a diameter of at least 50 mm. In addition, patients with comorbidities including autoimmune or chronic inflammatory disease, patients with a previous vascular event, and patients with multiple vascular territories affected were excluded from the peripheral and coronary atherosclerotic disease cohorts. Immediately after resection, tissue samples were routinely fixed, processed, and embedded in paraffin. Hematoxylin and eosin–stained sections were classified according the morphological criteria of Virmani et al. (26). Ruptured plaques were defined as thin fibrous cap atheromas with a luminal thrombus in communication with the lipid-rich necrotic core or as thin fibrous cap atheromas with intraplaque hemorrhage, while stable plaques were defined as thick fibrous cap atheromas without any sign of intraplaque hemorrhage or luminal thrombosis.

In addition to the surgical group, serum samples from 99 patients admitted to the first heart aid facility of Academic Hospital Maastricht and diagnosed with AMI, SAP, or UAP were included. AMI was defined by TnT levels of at least 0.1 μg/l, ST segment elevation, and onset of symptoms between 30 min and 12 h prior to admission. Baseline serum samples were obtained within 30 min of admission. All AMI patients included had no previous history of clinical cardiovascular disease. UAP was diagnosed and classified according to Hamm and Braunwald (18). SAP was diagnosed and classified according to guidelines adopted by the Canadian Cardiovascular Society (27). Control sera used in the discovery phase were derived from 10 age- and sex-matched blood donors from the Dutch blood bank (Sanquin).

In the discovery phase of the study (SAS and ELISA), pooled sera from patients with peripheral ruptured plaques or stable plaques and from healthy controls were used (n = 10 per group). To evaluate isolated antigens, we determined corresponding antibody signatures on a validation set of individual serum samples of 45 patients with ruptured peripheral atherosclerotic lesions (73% male; mean age, 65.1 ± 1.8 yr), 30 patients with stable plaques (63% male; mean age, 64.8 ± 2.0 yr), and 30 additional healthy controls (individuals of the general population older than 45 years but free of cardiovascular disease and other serious illnesses; 63.3% male; mean age, 65.0 ± 1.8 yr). A second validation cohort consisted of 40 AMI patients (65% male; mean age, 65.0 ± 2.0 yr), 29 patients with SAP (93% male; mean age, 62.9 ± 1.9 yr), and 30 patients with UAP (55% male; mean age, 65.3 ± 2.4 yr).

In addition, sera of 11 patients (64% male; mean age, 61.5 ± 3.5 yr) with other cardiac diagnosis (atrium fibrillation, n = 9; long QT syndrome, n = 1; AV-node tachycardia, n = 1) and 23 age- and sex-matched RA patients (68% male; mean age, 63.0 ± 1.9 yr) diagnosed in accordance with the criteria of the American College of Rheumatology (28) were analyzed to test the specificity of the anti-E1 and anti-E12 response.

Antibody profiling. Using a phage-display library containing cDNAs preferentially expressed in ruptured peripheral human atherosclerotic plaques, we performed SAS to isolate cDNA products specifically interacting with serum of patients with ruptured peripheral atherosclerotic lesions. In the validation phase, we tested whether the antibody signature of the identified cDNA products could distinguish patients with ruptured peripheral lesions, UAP, or AMI from patients with stable lesions and healthy controls. Antibody profiling was performed by an investigator blinded to plaque phenotype and clinical presentation. Details concerning the construction of the library, SAS, and ELISAs are provided in Supplemental Methods and Supplemental Figure 1.

Serum depletion to determine specificity of anti-E1 and anti-E12 reactivity. Sera of 5 individual patients (15 μl) with a positive anti-E1 and anti-E12 response were preincubated for 20 min at 37°C, 20 min at room temperature, and 20 min at 4°C in the presence of synthetic peptide E1 (Ac-VRGFTMLTRLVLNLK-NH2), E12 (Ac-VHIIRSSLIYALFTRSISNYK-NH2), or random peptides. Subsequently, peptide/IgG complexes were precipitated and discarded.

Statistics. Statistical analysis was performed with GraphPad Prism software (version 4). Statistical differences between 2 groups were assessed by unpaired Student's t test ($n \geq 25$) or Mann-Whitney test ($n < 25$); differences between more than 2 conditions were tested by 1-way ANOVA. Statistical differences between TnT and anti-E1/anti-E12 scores were assessed by Wil-

coxon matched-pairs test. Correlation analysis between the anti-E1 and anti-E12 response in patients with AMI at presentation and after 3 and 6 months, as well as between healthy controls and patients with non–atherosclerosis-related cardiac diagnoses, were performed by Spearman's rank correlation test. Data in text and tables are mean ± SEM; data in figures are mean ± SD. A *P* value less than 0.05 was considered significant.

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