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ORIGINAL ARTICLE

Autoantibodies against the endothelial receptor of protein C are associated with acute myocardial infarction in young women

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Summary. *Background:* Acute myocardial infarction (AMI) is rare among young women. The search for unknown risk factors is warranted. Endothelial protein C receptor (EPCR) is largely present at the endothelial surface of large arteries. No studies about association of anti-EPCR autoantibodies (anti-EPCR) with AMI are available. *Methods:* Plasma IgA, IgM and IgG anti-EPCR levels were measured by enzyme-linked immunosorbent assay in 165 women younger than 45 years who survived a first AMI and 165 healthy women, matched by age and geographical origin. *Results:* Using the 90th percentile of IgA anti-EPCR in the control group, IgA anti-EPCR were independently associated with AMI after adjustment for cardiovascular risk factors (OR 5.1; 95% CI 1.7–15.6; $P = 0.004$). The risk apparently conferred by IgA anti-EPCR increased dose-dependently (P for trend $= 0.0002$). IgM anti-EPCR were less consistently associated with AMI: a significant increase in the risk was found when women above the 90th percentile were compared with those in the lowest quartile (OR 3.6; 95% CI 1.2–11.5; $P = 0.03$). IgG anti-EPCR were similar in patients and controls. A total of 145 patients underwent coronary arteriography. IgA or IgM anti-EPCR were not different among patients with different degrees of atherosclerotic lesion (ANOVA, $P = 0.77$ and 0.24, respectively). *Conclusions:* High levels of IgA and, to a lesser extent, IgM anti-EPCR, are associated with AMI in young women.

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Introduction

Acute myocardial infarction (AMI) is a rare event in young women. Traditional risk factors for atherosclerosis are usually less prominent in this population, and do not account for a large proportion of cases. For this reason, the search for new pathogenetic mechanisms is warranted. Coronary heart disease (CHD) is an inflammatory process which involves cellular and molecular responses to endothelial dysfunction [1], and recently autoimmune disorders have been demonstrated to increase the risk of premature atherothrombosis [2–7].

Endothelial protein C/activated protein C receptor (EPCR) is an endothelial membrane glycoprotein able to bind both protein C and activated protein C (APC) with high affinity [8]. Protein C, when bound to EPCR, is activated more efficiently by the thrombin–thrombomodulin complex on the endothelial surface [9]. APC is a major inhibitor of blood coagulation and strong evidence supports a role for APC in anti-inflammatory/antiapoptotic mechanisms [10,11]. Recently, we have demonstrated the presence of anti-EPCR autoantibodies in a group of patients with thrombosis and autoimmune diseases [12]. The fact that EPCR is mainly expressed in large vessels, especially arteries [13], prompted us to investigate the presence of anti-EPCR autoantibodies in AMI. As the occurrence of autoimmune disorders is more frequent in women than in men, the association of anti-EPCR autoantibodies with a first AMI event was investigated in women under the age of 45 years in a matched case–control study. We demonstrate that IgA and IgM anti-EPCR autoantibodies are observed with a higher frequency in women who had developed AMI than in controls, and that high levels of these autoantibodies are independent risk factors for AMI in young women.

Methods

Patients and controls

The participants in this study were from the ongoing Italian nationwide case–control study on premature AMI conducted by the Atherosclerosis, Thrombosis, and Vascular Biology Italian Study Group and described elsewhere [14]. Cases were 165 women hospitalized for a first AMI at an age of 45 years or less. They had no history of venous or arterial thrombosis. Controls were 165 healthy women unrelated to the cases, without thrombotic antecedents, individually matched with the cases by age and region of origin, within a range of ± 3 years. The cases and controls were enrolled between January 1998 and January 2003.

The Institutional Review Boards of the participating hospitals approved the study. All the participants gave their written informed consent and agreed to give blood samples for plasma measurements. Both cases and controls were administered a questionnaire on traditional cardiovascular risk factors. The collected data included age, height, weight, hypertension, diabetes, family history of CHD, age of menopause, estrogen use, smoking, total cholesterol, HDL cholesterol and triglycerides. The data on cases were collected from 3 to 12 months after their first AMI, while those pertaining to controls were obtained at the time of the hospital evaluation for study enrolment. Definitions of AMI and traditional risk factors have been described in detail [14]. The degree of coronary stenosis was evaluated by angiography and defined as follows: normal, when no narrowing was observed; non-significant stenosis, in case of a narrowing $< 70\%$ (50% in the case of the left main coronary artery); severe stenosis, when a narrowing $> 70\%$ (50% in the case of the left main coronary artery) was observed.

Blood collection

Blood collection took place from 3 to 12 months after AMI. Blood was withdrawn from an anticubital vein into evacuated tubes containing 0.129 mol L^{-1} trisodium citrate. Platelet-poor plasma was obtained by double centrifugation at 3000 *g* for 20 min and was kept at -70 °C until the assays were carried out.

Expression and purification of soluble EPCR

Soluble EPCR (sEPCR) fused with myc epitope and a six His tag at its C-terminal end was expressed in *Pichia pastoris* strain X-33 using the EasySelect *Pichia* expression kit (Invitrogen, Paisley, UK) and purified as described [12].

Assay for anti-EPCR autoantibodies detection

Anti-EPCR autoantibodies of IgM and IgG isotypes were measured using a previously described enzyme-linked immunosorbent assay [12]. To detect anti-EPCR of IgA isotype a peroxidase-conjugated murine Mo Ab antihuman IgA (BioTrend, Cologne, Germany) was used.

Ability of autoantibodies to prevent protein C activation on the endothelial surface

When enough volumes of samples displaying high levels of anti-EPCR were available, the immunoglobulins were purified and their ability to prevent protein C activation on endothelium was tested according to previously described procedures [12].

Statistical methods

In this matched case–control study, comparison between cases and controls for continuous and categorical variables was performed with a *t*-test for paired samples and McNemar test, respectively. Association between continuous variables and anti-EPCR antibodies was assessed with the Spearman correlation coefficient. A conditional logistic regression analysis with matched pairs of cases and controls was used to evaluate the risk of AMI associated with high levels of IgA, IgG and IgM anti-EPCR. The main independent variables were levels of IgA, IgG, and IgM anti-EPCR categorized according to the distribution of anti-EPCR in the control group. Univariate and multivariate analysis were performed adjusting for traditional risk factors for AMI. The levels of anti-EPCR autoantibodies were also divided in quartiles to assess whether or not there was dose–response relationship with the risk of AMI. Tests for trend in AMI across quartiles of anti-EPCR were evaluated in conditional logistic models using a continuous variable with the median values for each quartile of the antibodies. Product-terms were introduced in the conditional logistic models to analyze interaction (effect modification). Finally, the relationship between levels of anti-EPCR autoantibodies and coronary stenosis was assessed by ANOVA, to compare levels of anti-EPCR according to the categories of stenosis. We also dichotomized the degree of coronary stenosis as no stenosis vs. moderate or severe stenosis and used a non-conditional logistic regression model to seek for differences in anti-EPCR levels between groups.

Results

The case sample consisted of 165 women, who were 22–45 years old (median age: 40 years) at the time of the first AMI. Twenty-two percent had normal coronary arteriograms, 15% non-significant stenosis, and 63% significant stenosis.

IgA anti-EPCR

The median [inter-quartile range (IQR)] value of IgA anti-EPCR level was 10.7 (4.3–19.5) arbitrary units (AU) for controls and 15.7 (9.4–27.4) AU for patients. The 90th percentile of the IgA anti-EPCR in controls was 28.8 AU, and 36 of 165 patients (22%) had values exceeding this cut-off, compared with 17 of 165 controls (10%). The crude odds ratio (OR) for AMI in women with IgA anti-EPCR above the 90th

Table 1 Association of anti-EPCR autoantibodies and traditional risk factors with acute myocardial infarction (AMI) in young women

Characteristic	Patients, *n* (%)	Controls, *n* (%)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	*P*
IgA anti-EPCR	36 (22)*	17 (10)*	2.8 (1.4–5.6)	5.1 (1.7–15.6)	0.004
IgM anti-EPCR	26 (16)*	17 (10)*	1.8 (0.9–3.5)	2.0 (0.4–9.7)	0.080
Hypertension	38 (23)	12 (7)	4.2 (2.0–9.2)	5.4 (1.6–18.5)	0.007
Family history of CHD	49 (30)	14 (8)	5.0 (2.4–10.0)	4.4 (1.5–13.1)	0.008
Smoking	126 (76)	81 (49)	3.4 (2.0–5.6)	6.0 (2.5–14.2)	< 0.001
Diabetes	9 (5)	2 (1)	8.0 (1.0–64.0)	1.9 (0.2–19.5)	0.590
Menopause	11 (7)	1 (1)	11.0 (1.4–85.2)	12.1 (0.9–163.9)	0.060
Estrogen use	66 (40)	49 (30)	1.7 (1.0–2.8)	1.3 (0.5–2.9)	0.590
HDL/cholesterol	0.246 (0.095)†	0.302 (0.098)†	1.7 (1.3–2.2)‡	1.4 (0.9–2.2)‡	0.120
Triglycerides	133 (84)†	96 (41)†	2.1 (1.6–2.8)§	1.8 (1.2–2.7)§	0.003

EPCR, endothelial protein C receptor; CHD, coronary heart disease. Number of events (percentages in brackets) in patients and controls are indicated; odds ratios (OR) before and after adjustment for traditional risk factors associated with AMI are also shown. Anti-EPCR autoantibodies are categorized using the 90th percentile for the variable in the control group as the cut-off point. *P* corresponds to the adjusted OR, which are adjusted for all the variables presented in the table and BMI (including a lineal and quadratic term). *Number of patients (percentages in brackets) with values of anti-EPCR autoantibodies above the 90th percentile. †Mean (standard deviation). ‡Risk associated with decrease in each 0.1 units of the ratio. §Risk associated with increase in each 50 mg dL^{-1}.

Table 2 Association with acute myocardial infarction (AMI) according to the IgA anti-EPCR autoantibody levels

	Cases/ controls, *n*	Adjusted OR (95% CI)	*P* for trend
Q1, < 4 AU	19/42	1 (reference)	< 0.001
Q2, 4–10 AU	33/39	7.7 (2.0–29.6)	NA
Q3, 11–19 AU	44/43	8.1 (2.3–27.9)	NA
Q4, > 19 AU	69/41	13.7 (4.0–46.9)	NA

EPCR, endothelial protein C receptor; NA, not applicable. Patients and controls were stratified in quartiles according to the IgA anti-EPCR level. Odds ratios (OR) for AMI were calculated in the second (Q2), third (Q3), and fourth (Q4) quartiles, as compared with those in the first (Q1) quartile. Adjustment for risk factors associated with AMI was performed.

percentile was 2.8 [95% confidence interval (CI) 1.4–5.6; $P = 0.003$]. A multivariate analysis adjusting for traditional cardiovascular risk factors was subsequently carried out. Table 1 shows the frequency distribution and the unadjusted and adjusted ORs for anti-EPCR autoantibodies together with frequencies and ORs for the traditional risk factors. After adjustment for the latter, the OR for AMI in women with IgA anti-EPCR above the 90th percentile increased to 5.1 (95% CI 1.7–15.6; $P = 0.004$). Thus, IgA anti-EPCR levels above the 90th percentile were independently associated with AMI in young women. To analyze if the relative risk associated with IgA anti-EPCR increased in a dose-dependent fashion, patients were categorized in quartiles according to the IgA anti-EPCR levels in controls, and the OR for AMI was calculated for each of the three upper quartiles using the lowest quartile as the reference. Table 2 shows that the adjusted OR increased steadily with the IgA anti-EPCR levels, suggesting a dose–response effect (*P* for trend = 0.0002).

IgM anti-EPCR

The median (IQR) of IgM anti-EPCR levels was 20.7 (11.3–33.6) and 20.2 (11.2–39.9) for controls and cases, respectively. When the patients were categorized in quartiles, women with levels above the 90th percentile (> 49 AU) compared with those in the lowest quartile (< 11 AU) had a statistically significant increase in their risk (OR 3.6; 95% CI 1.2–11.5; $P = 0.03$). Thus, it appears that the association with AMI is only increased in women with high levels of IgM anti-EPCR (i.e. with a threshold effect).

There was no correlation between IgA and IgM anti-EPCR levels in controls (Spearman $\rho = 0.047$, $n = 165$), in patients (Spearman $\rho = 0.016$, $n = 165$), or when all the individuals included in the study were taken together (Spearman $\rho = 0.035$, $n = 330$). No interaction could be seen among the analyzed variables (likelihood ratio test, $P > 0.05$).

IgG anti-EPCR

High levels of IgG anti-EPCR autoantibodies could be detected in one patient and one control but there were no differences between cases and controls for IgG anti-EPCR autoantibodies (not shown).

Relationship to coronary stenosis

A total of 145 of 165 patients underwent coronary arteriography: 32 (22%) showed no lesion, 22 (15%) showed a non-significant stenosis and 91 (63%) showed a severe stenosis. We evaluated whether or not the presence of high levels of anti-EPCR autoantibodies was associated with the degree of coronary artery stenosis. Neither IgA nor IgM anti-EPCR levels were significantly different across the groups, as assessed by ANOVA ($P = 0.77$ and 0.24, respectively). The comparison between patients without stenosis ($n = 32$) and patients with stenosis ($n = 113$) showed also non-significant differences when adjusting for traditional risk factors (data not shown).

Effect of anti-EPCR autoantibodies on protein C activation

We observed no effect of the immunoglobulin fractions purified from the samples displaying the highest IgA ($n = 9$)

and IgM ($n = 11$) anti-EPCR levels on the activation of protein C on the endothelial surface (not shown).

Discussion

The association of anti-EPCR autoantibodies with AMI has been demonstrated for the first time in this study. We provide evidence that IgA anti-EPCR autoantibodies are independently associated with a first AMI in young women. This population of patients was chosen because autoimmune disorders are more frequently found in women than in men and because the prevalence of the classical risk factors is lower in this group [15]. In case of IgA anti-EPCR, levels above the 90th percentile increased fivefold the relative risk for AMI after adjustment for traditional risk factors (i.e. hypertension, familial history, smoking, diabetes, menopause, cholesterol and triglycerides) and estrogen use. The magnitude is similar to the risk conferred by hypertension and a family history of CHD and was dose-dependent. High levels of IgM anti-EPCR were also independently associated with AMI but less consistently, and more weakly than IgA. No differences could be seen in IgG anti-EPCR levels. The different association between different isotypes (IgA but not IgG) and the risk of coronary events has previously been described for anti-heat-shock protein 60 [16,17]. This apparently surprising finding could be explained by an infection of a mucosal tissue. Several lines of evidence associate infection, autoimmunity and arterial disease [18,19]. If the infective agent involved mucosal tissues, an IgA response would be triggered, which could display a cross-reaction with an autoantigen.

Unlike other autoantibodies like anticardiolipin or anti-β2-glycoprotein I, whose pathogenic mechanisms are poorly understood, anti-EPCR autoantibodies can reasonably be thought to play a causative role in arterial thrombosis. As EPCR is a receptor specifically located at the intima, immune complexes will deposit on the arterial wall. As a result antibody-dependent cellular cytotoxicity [20] or local activation of the complement system [21,22] may occur. The anti-EPCR autoantibodies may also impair the APC generation [12], which may lead to increased thrombin generation, inflammation and apoptosis [11,23], although this property does not seem to be the case in the present study. Finally, as anti-EPCR autoantibodies are not associated with the degree of coronary stenosis, they would be involved in the acute event rather than in the progression of atherosclerotic plaque.

This study has limitations. Even though the cases were studied and blood samples obtained at a distance from the acute episode, when they could be considered to be in a relatively stable condition, it cannot be ruled out that high anti-EPCR titers are the expression of a reaction to disease, and hence the consequence rather than the cause of AMI. Another limit of this retrospective study is that women who died after AMI could not be investigated, so that we cannot exclude the possibility that these patients may have had a specially high proportion of high anti-EPCR values. As this is the first attempt to study the anti-EPCR autoantibodies in patients with AMI, the possibility that the association has arisen by chance cannot be ruled out and new studies are necessary to confirm these findings. On the other hand, as we have studied a group of patients, women under 45 years, which might have an autoimmunity background, the possibility that other autoantibodies were present in this population should be taken into account.

In conclusion we found that high levels of IgA and, to a lesser extent, IgM anti-EPCR autoantibodies are independently associated with AMI in young women. It remains to be established whether or not these antibodies are associated with cardiovascular disease in other populations.

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