Autoantibody against the amino acid sequence 661-680 in apo B-100 is associated with decreased carotid stenosis and cardiovascular events

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Abstract

Immunization with malondialdehyde (MDA)-modified peptides corresponding to the amino acid sequence between 661 and 680 in apo B-100 (p45) inhibits atherosclerosis in apo E knockout mice. The same effect can be obtained by treating the mice with recombinant anti-MDA-p45 IgG, suggesting that these antibodies have atheroprotective effects. In the present study we analyzed if autoantibodies against p45 and MDA-p45 are related to carotid atherosclerosis and acute cardiovascular events in humans. Using a nested case control design we determined plasma levels of IgG recognizing native and MDA-modified p45 in baseline samples from 75 subjects with acute myocardial infarction or sudden cardiac death and 148 matched controls. The control group was found to have significantly higher levels of p45 IgG than the cases. Moreover, an independent association was found between high levels of MDA-p45 IgG and a low degree of carotid stenosis ($p=0.006$). There was a high degree of co-variation between IgG binding to native p45 and MDA-p45 ($r=0.68$, $P<0.0001$) The associations between lower levels of autoantibodies against the apo B-100 p45 sequence and cardiovascular disease are in agreement with previous experimental studies demonstrating that these antibodies have atheroprotective effects. Our findings support the notion that the p45 sequence of apo B-100 is a potential target for immunomodulatory treatment of atherosclerosis in humans.

Keywords: apolipoproteins, antibodies, carotid stenosis, peptide, ultrasound

Activation of adaptive immunity plays an important role in the development of atherosclerosis [1,2]. T cells in human atherosclerotic plaques recognize epitopes in oxidized LDL when presented by macrophage MHC class II molecules [3] and autoantibodies against oxidized LDL are commonly expressed in man, suggesting that oxidized LDL is an important antigen in atherosclerosis [4,5]. Several lines of evidence favour the concept that adaptive immune responses are activated as part of the disease process and promote inflammation and plaque growth [1,2]. However, immunization with oxidized LDL has also been shown to reduce atherosclerosis, demonstrating the existence of an atheroprotective immune reaction to oxidized LDL [5-11]. Oxidized phospholipids [12] and aldehyde-modified peptide sequences in apo B-100 [13] are the major targets for the immune system in oxidized LDL. We have previously demonstrated that high IgM levels against a number of different aldehyde-modified peptide sequences in apo B-100 are associated with increased severity of carotid disease and risk for development of acute myocardial infarction [13]. Immunization of apo E knockout (KO) mice with some of these native and aldehyde-modified apo B-100 peptide sequences induces an immunoglobulin switch from IgM to IgG that is accompanied by an inhibition of atherosclerosis [14-16]. To study the possible atheroprotective effects of this IgG we produced recombinant human IgG1 specific for a malondialdehyde (MDA)-modified peptide corresponding to the sequence between amino acids 661 and 680 in apo B-100 (p45) [17]. Subcutaneous immunization with MDA-p45 peptide has previously been shown to inhibit atherosclerosis by about 50% in apo E KO mice [15]. A similar inhibition of atherosclerosis was observed in apo E KO mice following three weekly injections of recombinant anti-MDA-p45 IgG. Taken together these results suggest that IgG recognizing the MDA-modified peptide sequence between amino acids 661 and 680 in apo B-100 may protect against atherosclerosis.

The aim of the present study was to investigate plasma levels of p45 IgG autoantibodies in subjects with and without acute cardiac events as well as the association between these autoantibodies and the severity of atherosclerosis in the carotid artery as determined by B-mode ultrasound in humans.

Material and Methods

Study population

The study subjects, born between 1926 and 1945, were recruited from the "Malmö Diet and Cancer (MDC)" study cohort as previously described [13] Participants who had a history of myocardial infarction or stroke prior to enrolment were not eligible for the present study. The study population consisted of 223 subjects, 75 cases that developed acute cardiac events, i.e. fatal or non-fatal myocardial infarction or deaths due to coronary heart disease during follow-up and 148 controls matched for age, sex, smoking habits, presence of hypertension, month of participation in the screening examination and duration of follow-up. For two of the cases it was only possible to find one instead of two matching controls. The ethical committee of Lund University, Sweden approved the study.

Laboratory analyses

After overnight fasting blood samples were drawn for the determination of serum values of total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol and whole blood glucose. LDL cholesterol in mM was calculated according to the Friedewald formula. Oxidized LDL was measured using ELISA (Mercodia, Uppsala, Sweden) in EDTA plasma supplemented with the antioxidants DTPA and BHT.

B-mode ultrasound vasculography

An Acuson 128 Computed Tomography System (Acuson, Mountain View, California) with a 7 MHz transducer was used for the assessment of percent carotid stenosis as described previously [18].

Determination of p45 and MDA-p45 IgG

A 20 amino acid long peptide corresponding to the sequence between amino acids 661 and 680 in human apo B-100 (p45; IEIGLEGKGFEPTLEALFGK) was produced (KJ Ross Petersen AS, Horsholm, Denmark) and used in ELISA. The peptide was modified by treatment with 0.5 M MDA [19] for 3 h at 37 °C. The MDA-modified peptide was dialyzed against PBS containing 1 mM EDTA with several changes for 18 h at 4 °C. The MDA modification of peptide was assessed using the thiobarbituric acid reactive substances (TBARS) assay as described [13]. The aldehyde content of the modified peptide was 0.022 nmol per µg peptide. The native and MDA-modified peptides were diluted in PBS pH 7.4 (20 µg/ml) and absorbed to microtiter plate wells (Nunc MaxiSorp, Nunc, Roskilde, Denmark) in an overnight incubation at 4 °C. After washing with PBS containing 0.1% Tween-20 (PBS-T) the coated plates were blocked with SuperBlock in TBS (Pierce, Rockford, Illinois) for 5 min at room temperature followed by an incubation test plasma, diluted 1/100 in TBS-0.1% Tween-20 containing 10% Superblock (TBS-T) for 2 h at RT and overnight at 4 °C. After rinsing, deposition of autoantibodies directed to the peptides was detected using mouse anti-human IgG antibodies (Sigma, St Louis, MO) appropriately diluted in TBS-T. After incubation for 3 h at room temperature the plates were washed and the bound antibodies were detected by alkaline phosphatase conjugated goat anti-mouse IgG (Sigma), incubated for 2 h at room temperature. The colour reaction was developed by using phosphatase substrate kit

(Pierce) and the absorbance at 405 nm was measured after 2 h of incubation at room temperature.

Statistics

Statistical analysis was done using SPSS. The results are presented as median and range and as proportions when appropriate. Spearman rank correlation coefficients were calculated to evaluate associations between p45 IgG, risk factors and carotid stenosis. Mann-Whitney test was used to assess differences between groups. Chi-square test was used for comparing proportions. Multiple regression analysis was used to study independent correlations between carotid stenosis and other variables.

Results

Using a nested case control design we selected 75 subjects with coronary events (acute myocardial infarction or death due to coronary heart disease) and 148 controls matched for age, sex, smoking and hypertension from the Malmö Diet Cancer Study. The median time from inclusion to the acute coronary event was 2.8 years (range 0.1-5.9 years) among cases. The baseline characteristics of the study groups are shown in the table. The cases were characterized by having higher plasma triglycerides ($P<0.05$) and lower levels of IgG against native p45 ($P<0.05$). There was no association between plasma levels of native or MDA-p45-IgG and time from inclusion to the acute event among the cases. There were no significant differences in lipoprotein lipids, oxidized LDL or glucose between cases and controls.

Associations between p45 IgG, oxidized LDL and cardiovascular risk factors

There was a high degree of co-variation between IgG binding to native p45 and MDA-p45 ($r=0.68$, $P<0.0001$, figure1), suggesting that these antibodies to some extent may recognize

the same epitope. There was a weak association between high levels of MDA-p45 IgG and decreased levels of oxidized LDL in plasma (r=-0.17, P<0.05). Both p45 and MDA-p45 IgG levels showed inverse association with fasting glucose (r=-0.13, P<0.05 and r=-0.21, P<0.005), but otherwise there were no significant associations between antibody levels and age, gender, BMI, systolic blood pressure, diastolic blood pressure, LDL cholesterol, HDL cholesterol and triglycerides. The plasma level of oxidized LDL increased with age (r=0.34, P<0.0005) and was also significantly associated with LDL cholesterol (r=0.44, P<0.0005), HDL cholesterol (r=-0.22, P=0.001), triglycerides (r=0.35, P<0.0005), insulin (r=0.21, P<0.005) and BMI (r=0.14, P<0.05)

Associations between p45 IgG, oxidized LDL and carotid stenosis

The severity of carotid disease was assessed using B-mode ultrasound by determining the percent carotid stenosis at the baseline investigation. Cases were characterized by a more severe degree of carotid stenosis (*P*<0.05; Table). Low levels of MDA-p45 IgG were associated with a higher degree of carotid stenosis both among controls (r=-0.19, P<0.05) and cases (r=-0.31, P<0.05), as well as in the study group as a whole (r=-0.23, P<0.001). Figure 2 depicts the percent of carotid stenosis in the whole study group divided into tertiles according to MDA-p45 IgG levels. The mean percent carotid stenosis was almost twice as high among those in the lowest tertile of MDA-p45 IgG as compared to those in the highest tertile (14.6±13.7 % versus 7.6±9.6%, P=0.001). The inverse association between MDA-p45 IgG and degree of carotid stenosis remained statistically significant also after adjusting for age, gender, lipoprotein lipids and blood pressure in multiple regression analysis (P=0.006). There was no significant association between plasma oxidized LDL, native p45 IgG and carotid stenosis.

Discussion

The present observations suggest that subjects with low levels of IgG autoantibodies against the p45 sequence of apo B-100 have increased severity of carotid disease and risk for development of myocardial infarction and sudden cardiac death. These results are in good agreement with our previous experimental studies demonstrating that immunization of apo E KO mice with MDA-p45 peptide results in an increase in specific IgG associated with a decrease in aortic plaque area and plaque content of inflammatory cells [15] . Moreover, treatment of apo E KO mice with human recombinant IgG specific for the MDA-p45 sequences also reduced aortic plaque area and decreased plaque inflammation [17]. Taken together these observations support the notion that the p45 sequence of apo B-100 (amino acids 661-680) is a potential target for immunomodulatory treatment of atherosclerosis.

We have previously shown that in humans most autoantibodies against MDA-p45 are of IgM type and that the plasma levels of these IgM decrease with age. Among subjects 61 years or younger high levels of MDA-p45 IgM was associated with increased carotid IMT and risk for development of acute coronary events. [13]. The finding that IgG and IgM against MDA-p45 have opposite associations with the severity of carotid disease suggests the interesting possibility that switching antibody expression from IgM to IgG may be part of an endogenous defence mechanism against atherosclerosis. Several other studies have provided support for the existence of an antibody-mediated protection against atherosclerosis. Using a bone marrow transplantation strategy, Major et al [20]. demonstrated that B cell deficiency in LDL receptor KO mice was associated with a reduced level of anti-oxidized LDL antibody and increased atherosclerosis. Increased atherosclerosis has also been observed in apo E KO mice following removal of the spleen and this effect is completely inhibited by transfer of isolated

splenic B cells [21]. The increased atherosclerosis observed in Rag-1 mice in response to carotid cuff-injury is diminished by transfer of spleen B cells from wild type mice [22]. In apo E KO mice immunized with MDA-LDL there is a significant association between the increase in specific IgG and inhibition of atherosclerosis [11]. Moreover, the development of atherosclerosis in these mice has been found to be reduced by repeated injections of polyclonal human IgG [23].

Although there was a substantial correlation between the levels of IgG against the native and MDA-modified form of p45 in the present study, they differed to some extent in their pattern of association with cardiovascular disease. While the level of IgG against native p45 was higher among controls there was no association with the severity of carotid stenosis. In contrast, the levels of IgG against MDA-p45 showed significant inverse correlation with the severity of carotid stenosis among both cases and controls. The reason for the different associations for IgG against native and MDA-p45 remains to be fully understood, but may reflect that they are generated at different stages of LDL oxidation. However, the close association between autoantibodies against the native and MDA-modified sequences suggests that they reflect the same biological process

The generation of oxidized LDL is believed to be a crucial factor in the initiation as well as the progression of atherosclerosis [24]. It is generally believed that oxidation of LDL primarily takes place in the arterial wall but some of this oxidized LDL appears to subsequently leak back out into the circulation. Tsimikas et al [25] have recently reported that circulating levels of oxidized LDL are strongly related to the severity of coronary artery disease. We have also observed a correlation between high levels of oxidized LDL in plasma and metabolic factors characteristic for the metabolic syndrome, including low HDL

cholesterol, high triglycerides, high insulin and increased BMI. Additionally, a weak association was observed between high levels of MDA-p45 IgG and decreased levels of oxidized LDL in plasma, suggesting that these antibodies may help to clear circulating oxidized LDL from plasma.

There are some limitations to the present study that need to be considered. The patient population studied is relatively small and there is a considerable overlap in p45 IgG levels between cases and controls. Accordingly, these studies do not provide sufficient support for a role of p45 IgG measurements in individual risk stratification. The importance of the present findings is given by the clinical support they provide to previous experimental studies demonstrating an atheroprotective effect of immune responses against the p45 apo B sequence as expressed in oxidized LDL. In conclusion, our findings provide the first clinical support to the notion that the p45 epitope is a feasible target for active immunization and/or treatment with recombinant humanized antibodies in humans.

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TABLE. Baseline characteristics of subjects with coronary events (myocardial infarction or deaths due to CHD), and controls matched for age, sex, smoking, hypertension and examination period. Values are expressed as median (range) or as proportions.

	Subjects	
	Cases	**Controls**
Number	75	148
Age (years)	61 (49 – 67)	61 (49 – 67)
Male sex (%)	70	69
Life style factors		
Never smoked (%)	29	26
Former smokers (%)	44	48
Current smokers (%)	27	26
Anthropomorphic and blood glucose status		
BMI (kg/m^2)	26.4 (18.6 – 36.5)	26.3 (16.0 – 40.6)
Blood glucose (mM)	5.0 (3.6 – 21.4)	4.9 (3.8 – 12.0)
Diabetes mellitus (%)	18	11
Anti-diabetic medication (%)	13	3
Blood pressure status		
Diastolic blood pressure (mm Hg)	90 (74 –126)	90 (70 –130)
Systolic blood pressure (mm Hg)	150 (108 –200)	154 (112 –210)
Hypertension (%)	64	64
BP lowering medication (%)	30	23
Blood lipid status		
Total cholesterol (mM)	6.28 (3.47 – 8.24)	6.00 (4.08 – 9.90)
LDL-cholesterol (mM)	4.4 (1.7 – 6.2)	4.0 (1.6 – 7.6)
HDL-cholesterol (mM)	1.1 (0.6 – 2.5)	1.2 (0.6 –2.9)
Oxidized LDL (U/L)	86.6 (34.9 - 184.0)	85.9 (32.7 – 163.4)
Triglycerides (mM)	1.5 (0.5 – 10.0)*	1.2 (0.4 – 7.3)
Lipid-lowering medication (%)	12	6
IgG against p45		
IgG against native p45 (abs units)	0.09 (0-1.75)*	0.16 (0-1.87)
IgG against MDA-p45 (abs units)	0.14 (0-1.10)	0.16 (0-1.12)
Carotid ultrasonography		
Carotid stenosis, (%)	12.5 (0 - 60)*	5.0 (0 - 60)

BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
*$P<0.05$

Figure legends

Figure1. Scatter plot demonstrating the association between plasma levels of autoantibodies against the native and MDA-modified p45 sequence of apo B-100.

Figure 2. Tertiles of IgG levels against MDA-p45 and severity of carotid stenosis. *P*=0.006 for trend, after adjusting for age, sex, systolic blood pressure, LDL and HDL cholesterol.

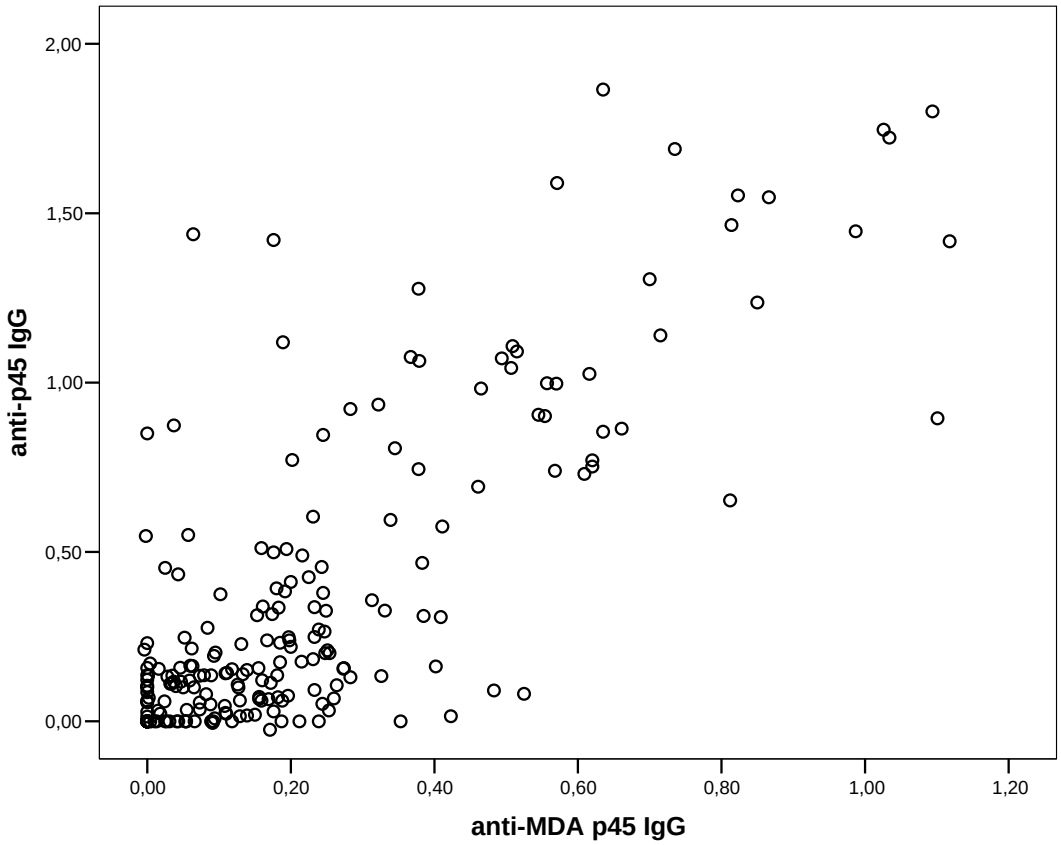
2,00
1,50
1,00
0,50
0,00
anti-p45 IgG
0,00
0,20
0,40
0,60
0,80
1,00
1,20
anti-MDA p45 IgG

Figure 1

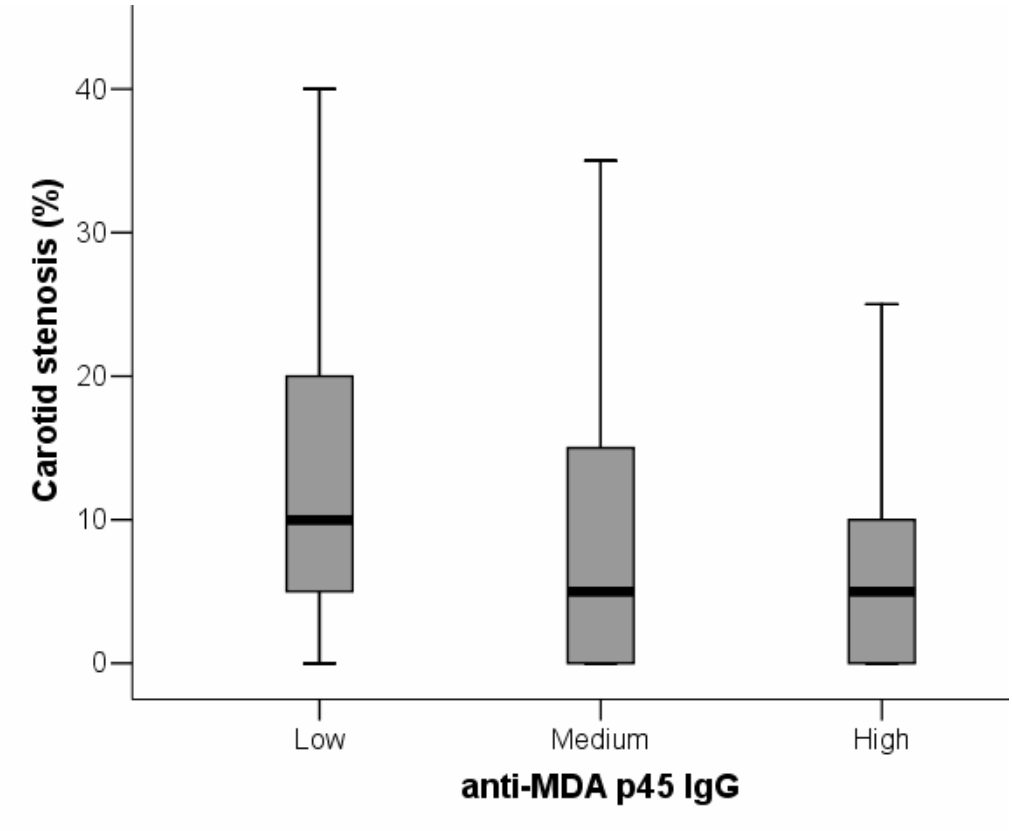
Carotid stenosis (%)
40
30
20
10
0
Low
Medium
High
anti-MDA p45 IgG

Figure 2