Antibodies to Endothelial Cells in Borderline Hypertension

Johan Frostegård, MD, PhD; Ruihua Wu, MD, PhD; Caroline Gillis-Haegerstrand, MD, PhD; Carola Lemne, MD, PhD; Ulf de Faire, MD, PhD

Background—Antibodies to endothelial cells (aECs) and to cardiolipin (aCLs) are implicated in autoimmune diseases like systemic lupus erythematosus vasculitis. β2-Glycoprotein 1 (β2GP1) is a cofactor for aCLs. The present study investigated the possible role of aECs, aCLs, and aβ2GP1 in borderline hypertension.

Methods and Results—Seventy-three men with borderline hypertension (BHT) and 73 age-matched normotensive (NT) men were recruited from a population screening program. Antibody levels were determined by ELISA. Presence of carotid atherosclerosis was determined by B-mode ultrasonography, and 29 individuals had atherosclerotic plaques. BHT men had significantly higher aEC and aβ2GP1 levels of IgG class than NT control subjects (P=0.029 and P=0.0001, respectively). aEC levels of IgM class were higher in BHT (P=0.012), but not aβ2GP1 levels. There was no correlation between aCL levels and BHT. Individuals with atherosclerotic plaques had significantly higher aEC levels of both IgG (P=0.042) and IgM subclasses (P=0.018) than those without plaques, but no difference was found in aCL and aβ2GP1 levels. Endothelin and aECs of IgM class were significantly associated.

Conclusions—We demonstrate the first evidence of a significant elevation of aEC and aβ2GP1 levels in borderline hypertension. These findings provide a new link between hypertension and atherosclerosis and indicate that humoral immune reactions to the endothelium may play an important role in both conditions. (Circulation. 1998;98:1092-1098.)

Key Words: endothelium ■ antibodies ■ glycoproteins ■ atherosclerosis ■ hypertension

According to the response-to-injury hypothesis, an initial event leading to atherosclerosis is damage and/or activation of the endothelium. The endothelium has also been implicated in hypertension, a leading risk factor for the development of atherosclerosis, and several studies have demonstrated signs of endothelial dysfunction in hypertension. Furthermore, vascular tone is regulated by the endothelium, as exemplified by nitric oxide and endothelin-1, both secretory products of endothelial cells (ECs). Atherosclerosis is a chronic inflammation in the vascular wall, in which T cells and monocytes/macrophages, possibly activated by oxidized LDL (oxLDL), play an important role. The humoral immune system may also be of great importance in atherosclerosis, and antibodies to oxLDL have been demonstrated to be related to the degree of atherosclerosis. In hypertension, alterations of immune function, including decreased T-cell responses and abnormalities in complement function, have been reported, although available data are comparatively scarce. We recently demonstrated that antibody levels to immunogenic heat shock proteins (HSPs) are enhanced in borderline hypertension (BHT), which may provide a link between enhanced mechanical stress to the endothelium at lesion-prone sites and atherosclerosis. Enhanced levels of antibodies to ECs (aECs) have been demonstrated in autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis with systemic manifestations, and Wegener’s granulomatosis, and also Crohn’s disease and vasculitis. A direct pathogenic role of the antibodies has been suggested, but the knowledge of the exact nature of antigens involved is limited. Antibodies to cardiolipin (aCLs) have been related to myocardial infarction, and β2-glycoprotein 1 (β2GP1) is an important cofactor for aCLs. To investigate the role of aECs, antibodies to β2GP1 (aβ2GP1s), and aCLs in BHT and early atherosclerosis, we studied a group of 146 middle-aged men in which BHT patients were compared with age-matched control subjects. We report here that serum aEC and aβ2GP1 levels are enhanced in patients with BHT.

Methods

Study Groups
In 1985, a blood pressure screening program was started in Åkersberga, a small community 35 km north of Stockholm. All men 35 to 55 years old were asked by mail to visit the primary health care center and have their blood pressure measured. BHT was defined as diastolic blood pressure (DBP) of 85 to 94 mm Hg, and by this criterion, 193 patients with BHT were identified. These individuals were followed up with yearly visits for 3 years. At these follow-ups, 20% of the subjects became hypertensive and 20% normotensive.
(NT), with the major change (13% to 15%) occurring already at the 1-year follow-up visit. After 3 years, 81 men were still within the range for BHT on the basis of repeated measurements over the entire time period.

These 81 individuals with BHT were invited to participate in the present investigation, together with 80 age-matched male control subjects from the original population who had a DBP ≤80 mm Hg at the initial measurement. To obtain 80 age-matched control subjects, 105 NT men were asked to participate, of whom 23 declined to participate and 2 had a DBP >80 mm Hg. The blood pressure of the control subjects was measured on 2 occasions a few weeks apart. For the subjects to participate in the study, their DBP had to be ≤80 mm Hg on both occasions. All blood pressure measurements during the entire recruitment procedure and study period were performed by 1 person, a specially trained nurse.

The study was approved by the local ethics committee of Karolinska Hospital and was conducted in accordance with the Helsinki Declaration. All subjects gave informed consent before entering the program of which this study was a part. Of the 81 men with BHT and the 80 NT control subjects who agreed to participate in the program, 73 in the BHT and 73 in the NT group completed all procedures of the present study. None of the subjects had any other illnesses or were regularly using any drugs known to influence blood pressure, metabolic variables, or inflammatory variables.

**Study Program**

All subjects were investigated according to the same schedule. Men with BHT and their control subjects were investigated simultaneously when possible and never more than 4 weeks apart. Blood samples were taken between 8 and 9:30AM, after 8 to 12 hours of fasting. All samples were drawn after 15 minutes of rest in the supine position.

**Analysis of Total Serum Immunoglobulin Levels**

Serum immunoglobulins, IgG, IgM, and IgA, were determined by immunoturbidimetry. Specific anti-IgG, anti-IgM, and anti-IgA reagents and calibrators were obtained from Dako. The turbidimetric reaction was quantified in a Hitachi 911 analyzer by measurement of light transmission at 340-nm wavelength.

**Cell Culture**

ECs were isolated and cultured from 3- to 5-cm-long segments of the saphenous vein derived from patients undergoing coronary bypass surgery, as described in detail previously. Briefly, the vein was rinsed and then filled with a collagenase solution (0.1%, Worthington). Harvested cells were routinely cultured in MEM (Gibco BRL) with the addition of 40% pooled heat-inactivated (56°C, 30 minutes) human serum, antibiotics, and CAMP-elevating compounds. Two days before the experiments, the ECs were gently detached with a 0.1% trypsin/0.02% EDTA (1:1) solution. The cells were seeded on gelatin-coated plastic wells (24-well plates, Costar) at a density corresponding to 100,000 cells/cm² in MEM containing only 30% human serum and antibiotics. The ECs were characterized as endothelial by immunohistochemical staining of von Willebrand factor–related antigen, PG1 production, and their typical cobblestone appearance. In each experiment, cells from a single donor were used. The use of human great saphenous veins was approved by the ethics committee at the Karolinska Hospital.

**Detection of Antibody Levels**

Antibodies to ECs were detected essentially as described earlier. The ECs were suspended in the RPMI 1640 medium containing 20% heat-inactivated FCS and seeded on the 96-well flat-bottom tissue culture plates at a density of 1 × 10⁴ cells/well. After the ECs were incubated for 2 days, the plates were washed 3 times with PBS, pH 7.4. The ECs were fixed for 15 minutes at room temperature with 0.2% glutaraldehyde. The fixed cells were washed 4 times in the washing buffer (PBS/0.2% BSA). The plates were blocked by 200 μL of blocking buffer (PBS/1% BSA and 0.1 mol/L glycine) for 1 hour at room temperature. The serum samples were diluted 1:50 in washing buffer, and 100 μL of this dilution was added to each well and incubated at 37°C for 2 hours. IgG and IgM antibodies to cardiolipin (CL) were determined by ELISA essentially as described.

Antibody reactivity to β2GP1 was detected by coating irradiated Titertek 96-well polyvinylchloride microplates (Flow Laboratories) with 50 μg/well of 30 μg/mL β2GP1 (Calbiochem B 18278) dissolved in 10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4 (HEPES buffer), at 4°C overnight. The plates were blocked with 0.3% gelatin for 1 hour. After washing, the wells were incubated with 50 μL of 50-times-diluted samples for 1 hour at room temperature (2 mmol/L of EDTA was included in buffer). Control assays were performed in the absence of β2GP1.

After 3 washings with PBS, the plates were incubated with 50 μL/mL of alkaline phosphatase–conjugated goat anti-human IgG (Sigma A-3150) diluted 1:9000 or IgM (Sigma A-3275) diluted 1:7000 with PBS at 37°C for 2 hours. After 3 washings, 100 μL of substrate (phosphatase substrate tablets, Sigma 104; 5 mg in 5 mL diethanolamine buffer, pH 9.8) was added. The plates were incubated at room temperature for 30 minutes and read in an ELISA Multiskant Plus spectrophotometer at 405 nm. Each determination was done in triplicate. The coefficient of variation between triplicate tests was <5%. Investigators were blinded, and patient and control samples were mixed.

**Cross-Reactivity Between Antibodies**

To investigate whether there was an immunological cross-reactivity between antibodies tested, competition assays were performed. Sera at a dilution giving 50% of maximal binding to the compound coated were preincubated with ECs as indicated. The sera were incubated overnight with the different competitors at 4°C, and inhibition of binding to ECs was tested. The percentage of inhibition was calculated as follows: Percent inhibition = (OD control − OD with competitor × 100)/OD control, where OD is optical density.

**Analysis of Plasma Lipoprotein, Insulin, IGFbp-1, and Endothelin-1 Levels**

VLDL, LDL, and HDL were determined as previously described. The insulin resistance was calculated by the formula IR=fasting insulin/22.5 e−h merged glucose. References 27 and 28. Insulin-like growth factor–binding protein–1 (IGFbp-1) was analyzed by radioimmunoassay. Endothelin-1 in plasma was analyzed by a competitive immunoassay as described in detail earlier.

**Blood Pressure Measurements**

An identical procedure was followed at each occasion during the entire recruitment period. All blood pressure measurements were performed with a mercury sphygmomanometer. The cuff was adjusted according to the circumference of the arm and placed at the level of the heart. Blood pressure was recorded as the mean of 2 measurements taken after 5 minutes of rest in the supine position. Systolic blood pressure (SBP) and DBP were defined according to the auscultatory Del Mar Avionics P-IV (P-IV, model 1990, Del Mar Avionics) every 15 minutes for the complete 24-hour period. Patients completed a diary during the period, noting body posture, going to bed, waking up, and so forth. Data were transferred to a computer unit at the end of the period. Artifacts were defined as any of the following: SBP<50 mm Hg, SBP>250 mm Hg, DBP>SBP, DBP<30 mm Hg, and DBP>150 mm Hg. No other editing was performed.

**Carotid Ultrasound**

The right and left carotid arteries were examined with a duplex scanner (Acuson 128XP/5) and a 7.0-MHz linear array transducer. The subjects were investigated in the supine position with the head turned slightly away from the sonographer, as described earlier. Plaque was defined as a localized intimal-medial thickening of
Blood pressure, mm Hg 125/75 (±11/±4) mm Hg compared with 141/89 (±10/±2) mm Hg in the BHT group, which indicates a significant difference. The BHT group also had a significantly higher BMI and waist-to-hip ratio. The 2 groups were well matched for age.

The BHT men had significantly altered metabolic profiles, with fasting hyperinsulinemia and dyslipoproteinemia, as previously presented (Table 1; Reference 3). In the BHT group, 26% of the subjects had plaque on one or both sides; the corresponding figure for the NT group was 14% (10 versus 10 subjects, P=NS). The basal level of endothelin-1 was significantly increased in the BHT group.

**Antibody Levels**

In the population as a whole, the aEC levels of both IgM and IgG types were significantly higher in the BHT group than in the NT group (Table 2). The aβ2GP1 levels of IgG type were significantly higher in the BHT group than in the NT group (P<0.0001), whereas there was no significant difference in IgM antibody levels (Table 2).

If individuals with carotid atherosclerotic plaques were excluded, the aEC levels of IgM were significantly higher in BHT men than in the NT group (0.24 versus 0.20; P=0.01). Likewise, the aβ2GP1s of IgG classes were significantly higher in BHT men than in the NT group (0.22 versus 0.18; P<0.0001). If individuals with BHT were excluded, the aEC levels of IgM were nonsignificantly higher in individuals with carotid atherosclerotic plaques than in those without (0.24 versus 0.20; P=0.09), whereas the aEC or aβ2GP1 levels of IgG type did not differ (data not shown).

Individuals with plaque (n=29) had higher aEC levels of both IgG and IgM types compared with individuals without (n=117). However, the aβ2GP1 levels of both IgG and IgM types did not differ between individuals with plaque and those without (Table 3).

### Results

**Statistical Methods**

Variables were tested for skewness. For skewed variables, nonparametric tests were used for comparisons between the groups (Mann-Whitney U test), whereas Student’s t test was used for normally distributed variables. Categorical variables were compared by the χ² test. Spearman rank correlation coefficients were calculated to estimate interrelations between aECs, metabolic variables, and blood pressure levels. The significance level was set at P<0.05. Values in the text are given as mean±SD.

**Results**

**Characteristics of Case and Control Subjects**

Basic characteristics of the 2 study groups are presented in Table 1. The mean blood pressure level in the NT group was 125/75 (±11/±5) mm Hg compared with 141/89 (±10/±2) mm Hg in the BHT group, which indicates a significant difference.
Antibody levels to CL did not differ between the BHT and NT groups or between individuals with plaque and those without. There was no difference in antibody levels to ECs, β2GP1, or CL between smokers and nonsmokers (data not shown).

To exclude the possibility that differences in antibody levels simply reflected enhanced total antibody levels, IgA, IgG, and IgM were determined. There was no difference between the BHT group and control subjects (IgG, 9.71 ± 1.86 versus 9.76 ± 2.31 mg/mL and IgM, 2.25 ± 0.81 versus 2.1 ± 0.88 mg/mL, respectively).

**Correlations Between Antibody Levels**
The correlation between aECs, aCLs, and aβ2GP1s are shown in Table 4. There were significant correlations between aEC, aCL, and aβ2GP1 levels against both the IgG and IgM isotypes. Furthermore, antibodies to HSP65, which we recently found to be elevated in BHT, correlated significantly with antibodies to ECs (P < 0.02) but not with antibodies to β2GP1 or CL.

**Cross-Reactivity Between Antibodies**
To study possible cross-reactivity between the antibodies, we performed competition experiments, with CL, β2GP1, and ECs and as a control an unrelated antigen, PPD. The sera were tested at a dilution that gave 50% of maximal binding to ECs. To test whether antibodies to ECs could be outcompeted by ECs themselves, serum was added to wells for 24 hours, and then the serum was moved to another plate coated with ECs. When inhibition >25% was considered positive, β2GP1 inhibited serum binding to ECs in 6 of 7 subjects tested but CL in only 1 individual. ECs could outcompete binding to ECs in all individuals tested. In the Figure, the inhibitory capacity of β2GP1 compared with an unrelated antigen, PPD, was tested. β2GP1 inhibited binding to ECs, but PPD had no effect.

**Correlations to Metabolic Variables**
In the population as a whole and the 2 groups separately, there were no significant correlations between aECs, aβ2GP1s, or aCLs and lipoproteins, BMI, waist-to-hip ratio, or intimal-medial thickness (data not shown).

However, there were interesting correlations between aβ2GP1 but not aEC or aCL levels and other indicators of the metabolic syndrome, as shown in Table 5. aβ2GP1 levels of IgG type correlated with insulin, IGFbp1, and insulin resistance, and aβ2GP1 levels of IgM type correlated with IGFbp1. An intriguing finding was the correlation between aECs of IgM type and endothelin in the BHT group (R = 0.25, P = 0.039).

There was a significant association between 24-ABP determinations and aEC levels of IgG class in the BHT group (P = 0.037) and the 2 groups together (P = 0.0042) but not in the NT group alone. Smoking was not correlated to the antibodies tested (data not shown).

### TABLE 4. Correlations Between Antibody Levels in BHT and NT Groups (All Individuals)

<table>
<thead>
<tr>
<th>Regression, R</th>
<th>aEC/aCL</th>
<th>aEC/aβ2GP1</th>
<th>aβ2GP1/aCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM R</td>
<td>0.289</td>
<td>0.441</td>
<td>0.298</td>
</tr>
<tr>
<td>P</td>
<td>0.0003</td>
<td>0.0001</td>
<td>0.0003</td>
</tr>
<tr>
<td>IgG R</td>
<td>0.196</td>
<td>0.18</td>
<td>0.176</td>
</tr>
<tr>
<td>P</td>
<td>0.016</td>
<td>0.034</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Values are given as mean±SD. Group differences were determined by Student’s t test.

### TABLE 5. Association Between Antibody Levels to β2GP1 and Metabolic Factors

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression, R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>IgM R</td>
<td>0.01</td>
</tr>
<tr>
<td>P</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG R</td>
<td>0.175</td>
</tr>
<tr>
<td>P</td>
<td>0.037</td>
<td></td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>IgM R</td>
<td>0.01</td>
</tr>
<tr>
<td>P</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG R</td>
<td>0.215</td>
</tr>
<tr>
<td>P</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>IGFbp1</td>
<td>IgM R</td>
<td>0.185</td>
</tr>
<tr>
<td>P</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG R</td>
<td>0.176</td>
</tr>
<tr>
<td>P</td>
<td>0.035</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as mean±SD. Group differences were determined by Student’s t test.
Age was not associated with αβ2GP1 or aCL levels; however, there was a correlation with aECs of IgM type \((P=0.039)\) but not IgG type.

### Discussion

The main finding in this report is that BHT is significantly associated with aEC antibody levels of both IgM and IgG types and with αβ2GP1 levels of IgG type. Total antibody concentrations showed no difference between control subjects and BHT patients, indicating that the results do not simply reflect total Ig levels. Individuals with the presence of carotid atherosclerosis as determined by carotid ultrasound had significantly enhanced aEC levels compared with individuals without carotid atherosclerosis. This is in line with recent findings indicating that aEC levels are enhanced in individuals without carotid atherosclerosis. These findings indicate that both BHT and atherosclerosis may be related to endothelial changes leading to B-cell activation and thus antibody formation, but the relative contribution of BHT and atherosclerosis remains to be elucidated.

BHT is a condition with only relatively minor cardiovascular alterations compared with normal individuals. The enhanced aEC level is therefore likely to reflect a humoral immune response associated with very early changes in the endothelium. Endothelial dysfunction has been described in hypertension, e.g., as an impaired vasodilatation due to defective NO production, and changes in the endothelium including loss of heparan sulfate and sialic acid have been reported. It is thus possible that aECs react with neoepitopes formed or exposed on the endothelium, thus being secondary to changes induced by metabolic factors. aEC levels may therefore be a marker for endothelial dysfunction in apparently healthy individuals like those in the present study. These antibodies may initiate atherosclerotic lesions and also aggravate changes induced by metabolic factors, by activation of complement, deposition of immune complexes, induction of adhesion molecules, and attraction of phagocytic cells, which taken together may lead to aggravation and/or induction of atherosclerosis.

An intriguing finding was the strong association of aECs of IgM type with endothelin, the most potent vasoconstrictor. Antihypertensive therapy with calcium channel blockers prevents and improves endothelial dysfunction. 20 Several different mechanisms, including direct effects of the elevated blood pressure levels on the arterial wall, have been suggested. Conversely, however, atherosclerosis may also be causally related to hypertension by means of an impaired endothelial function leading to a defective secretion of nitric oxide. Clearly, the interaction between atherosclerosis and hypertension is complex, and the different possibilities are not mutually exclusive.

During recent years, the role of the immune system in atherosclerosis has attracted increasing attention. Activated T
cells and monocytes are present in the lesions, and oxLDL has been identified as a possible factor inducing the inflammatory component of atherosclerosis, because oxLDL activates lymphocytes and monocytes to antibody formation and secretion of proinflammatory cytokines.\textsuperscript{1-7} Furthermore, aβ2GP1 has been suggested to be involved in oxLDL-uptake by macrophages antibodies\textsuperscript{48} and may therefore also play a role in early atherosclerosis. Comparatively little is known about the role of the immune system in hypertension.\textsuperscript{10,11}\textsuperscript{10} One possibility is that immunogenic HSPs are induced at lesion-prone sites by mechanical stress, which may be enhanced in hypertension, and thus elicit an immune response in the artery wall; this hypothesis may provide an explanation of how mechanical stress, as in hypertension, may induce atherosclerosis.\textsuperscript{12} An intriguing finding was therefore the strong correlation between aEC and anti-HSP60 antibody levels, which suggest that HSP60 may be involved in the antigenicity of ECs, and it is possible that increased stress to the vascular wall, as may be present in BHT, enhances the HSP60 expression in the endothelium, which triggers an immune reaction directed at HSP60 in endothelial cells.

Taken together, our results indicate that antibodies to endothelial cells and to an associated plasma protein, β2GP1, may play an important role in the early stages of both atherosclerosis and hypertension.

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References

9. Bergmark C, Wu R, de Faire U, Lefvert AK, Swedenborg J. Patients with early-onset peripheral vascular disease have increased levels of autoan-


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