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Anti-cardiolipin from periodontitis patients induces MCP-1 production by human umbilical vein endothelial cells

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Abstract

Aim—Periodontal diseases are associated with a variety of systemic diseases, including cardiovascular disease and stroke, and patients with periodontitis demonstrate elevated levels of anticardiolipin antibodies. We sought to determine if anticardiolipin antibodies from periodontitis patients induced monocyte chemotactic protein-1 production by human vascular endothelial cells.

Materials and Methods—IgG was purified from sera from 53 subjects, including chronic and aggressive periodontitis patients and periodontally healthy controls, with elevated or normal IgG anticardiolipin levels. In addition, anticardiolipin antibodies were specifically removed from some sera by immunoabsorption.

Results—We found that, irrespective of diagnostic category, IgG from subjects with elevated anticardiolipin induced significantly greater monocyte chemotactic protein-1 production by human vascular endothelial cells than IgG from those subjects with normal anticardiolipin titers. Removal of anticardiolipin from IgG preparations from periodontitis patients significantly reduced their ability to induce monocyte chemotactic protein-1.

Conclusions—Since elevated titers of anticardiolipin are found in a significantly greater proportion of patients with periodontitis than in periodontally healthy individuals, and these antibodies activate endothelial cells to produce monocyte chemotactic protein-1, they may explain some of the associations noted between periodontal infections and systemic conditions.

Keywords

anticardiolipin; periodontal disease; endothelial cells; antibodies; monocyte chemotactic protein-1

Introduction

Anticardiolipin antibodies are most commonly found in patients with autoimmune diseases such as systemic lupus erythematosus (SLE) or the antiphospholipid syndrome (APS) (Tripodi et al., 2011). Such patients are known to have increased incidence of thrombotic events as well as adverse pregnancy outcome (fetal involution) and are also thought to demonstrate early atherosclerosis (Kobayashi et al., 2005, Matsuura and Lopez, 2008). It is hypothesized that some of these manifestations of disease are in part a consequence of the interaction of autoantibodies, including β 2-glycoprotein 1-dependent anticardiolipin antibodies (aCl), with the endothelium, leading to vascular inflammation and thrombosis.

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this paper.

For example, aCI can interact directly with endothelial cells to upregulate adhesion molecules and induce production of cytokines such as MCP-1 and molecules such as tissue factor (Cho et al., 2002), and can behave as opsonins by interacting with modified low-density lipoproteins to enhance uptake of cholesterol into macrophages (Matsuura and Lopez, 2008). Additionally, it has been demonstrated that a variety of microbial pathogens can induce the production of cross-reactive aCI-like antibodies that can induce antiphospholipid syndrome-like symptoms in animal models (Blank et al., 2002a), leading to the hypothesis that some infectious diseases can promote APS-like symptoms, including accelerated atherosclerotic cardiovascular disease, in the absence of autoimmune disease (Artenjak et al., 2012).

There is substantial evidence that periodontitis contributes to the magnitude of systemic inflammation, and it has been hypothesized that this may constitute a mechanism whereby periodontal diseases could contribute to the development or progression of a variety of systemic conditions (Teles and Wang, 2011). Many studies demonstrate associations of periodontitis with cardiovascular disease (CVD), though data unequivocally demonstrating a cause and effect relationship between these conditions are lacking (Lockhart et al., 2012). Upregulation of the endothelium promoting egress of inflammatory cells from the bloodstream and into atheromatous lesions is a fundamental pathologic characteristic of CVD (Libby et al., 2009). Thus, assessment of endothelial function has been one approach that investigators have used to study associations of periodontitis with CVD and to assess the impact of periodontal therapy on CVD measures (Amar et al., 2003, Higashi et al., 2008, Pischon et al., 2007, Tonetti et al., 2007). These indirect approaches to assessment of CVD risk indicate that there is endothelial dysfunction associated with periodontitis that may be improved by therapy aimed at decreasing periodontal inflammation.

Our previous studies have indicated that a significant proportion of individuals with periodontitis demonstrate elevated serum concentrations of aCI (Schenkein et al., 2003). It has recently been demonstrated that oral bacterial pathogens may be cross-reactive with β 2GPI, the target antigen of aCI (Wang et al., 2008) and that periodontal therapy can decrease systemic antibody levels of aCI (Gunupati et al., 2011). Our observation that periodontitis patients' sera can contain elevated concentrations of aCI (Schenkein et al., 2003), and studies indicating that aCI can be associated with cardiovascular diseases even in patients without autoimmune disease (Artenjak et al., 2012) raise the question of whether the aCI found in periodontitis has pathogenic properties. The intent of this study was to evaluate the potential pathogenicity of aCI found in periodontitis patients by determining their ability to induce production of the key cytokine MCP-1 by endothelial cells.

Methods

Clinical Methods and Serum Samples

This study was approved by the Internal Review Board of Virginia Commonwealth University. Serum samples used for this study were from subjects described in previous publications (Schenkein et al., 2003, Schenkein et al., 2007). The subjects were identified at Virginia Commonwealth University School of Dentistry and included patients with generalized aggressive and chronic periodontitis as well as subjects without periodontitis. All participants were determined to be systemically healthy by medical history. Each participant received a comprehensive periodontal evaluation that included determination of pocket depth (PD), attachment loss (AL), plaque index (PI), (Silness and Le, 1964) and gingival index (GI)(Le and Silness, 1963) at 6 sites per tooth. At the time of examination a blood sample was taken and processed for serum which was stored at -70°C . Serum samples chosen for inclusion in this study were selected from those previously assayed for aCI content so as to provide sufficient numbers of sera within each diagnostic category to

test hypotheses regarding the impact of diagnosis on aCI function. Sera testing positive for aCI from periodontally healthy subjects was very limited due to the low prevalence of positive tests within this group (Schenkein et al., 2003).

The diagnostic criteria were as follows. Subjects with normal periodontium (NP) included those of any age with no evidence of AL or pockets greater than 3 mm, other than in buccal or lingual areas of gingival recession. Subjects with Chronic Periodontitis (CP) were of age >25 years with AL 2 mm or greater on more than one tooth in any extent or severity pattern that is consistent with plaque level and age. The definition of Generalized Aggressive Periodontitis (GAgP) for the subjects in this report conform to current diagnostic guidelines for these diseases (Armitage, 1999) with the additional requirement that such patients had a history of disease onset prior to age 35. They presented with at least 8 teeth with 5 mm or more attachment loss at interproximal sites, at least 3 of the affected teeth were not first molars and incisors. The total initial sample number included 23 from subjects with CP, 15 samples from subjects with GAgP, and 21 NP subjects.

Purification of IgG and Depletion of aCI from IgG preparations

Purification of IgG from patients samples was accomplished using two methods, which proved to be equivalent with respect to IgG yield and purity. First, after samples were adjusted to 1M NaCl and the pH was adjusted to 7.8 by addition of .5M Na₂HPO₄, one mL of each sample was applied to a one mL column of Protein G Sepharose that was equilibrated in 1M NaCl, .05M PO₄, pH 7.8. Elution was accomplished by addition of .1M Glycine .2M NaCl, pH 2.00, and eluted samples were immediately neutralized by addition of 1M NaOH. Subsequently, samples were dialyzed against PBS. In the second method, samples were dialyzed against .005M PO₄, pH 7.8. and one mL of each sample was applied to a one mL column of DEAE Sephacel, equilibrated in .005M PO₄, pH 7.8. Pass through fractions were collected and the column was washed until A₂₈₀ was less than .02.

Following both isolation procedures fractions were assayed for anti CL using the Varelisa Cardiolipin IgG kit. (Phadia, Thermo Fisher Scientific, US). Positive fractions were pooled and concentrated, where necessary, using Amicon Ultra centrifugal filters (Millipore, Billerica, MA), then reassayed for anti CL. Total IgG was determined by ELISA using Nunc-Immuno Plate MaxiSorp Surface (NUNC, Thermo Fisher Scientific, US) coated with affinity purified goat anti human IgG (Jackson Immuno Research, West Grove, PA) diluted to 2 µg /mL in .005M PO₄ pH 7.8. Plates were washed, and samples and standards were added. Following incubation plates were again washed and F(ab)² HRPO-tagged affinity purified goat anti-human IgG (Jackson Immuno Research) was added. Following further incubation, plates were again washed and developed using chromagen TMB. All incubations were for 30 minutes at room temperature.

Generation of an aCI immunoabsorbant was accomplished using the method of Pengo and Biasiolo (Pengo and Biasiolo, 1993). The aCI immunoabsorbant was placed in wells of MultiScreen-HTS Filter Plates (Millipore, Billerica, MA). Patient IgG samples were added to the wells and then were incubated with shaking for 60 minutes at room temperature. Filter plates were then mounted onto sterile tissue culture plates and centrifuged (500 × g) for 10 minutes. The resulting supernatants were assayed for IgG aCI and total IgG.

Following purification of the IgG samples it was noted that 6 samples, 3 each from the NP and CP group, contained contaminating LPS determined as described by Cho et al (Cho et al., 2002). Thus, the final sample number included 53 subjects (18 NP, 20 CP, and 15 GAgP).

Culturing of HUVEC cells

HUVEC cells (American Type Culture Collection, ATCC, Manassas, VA) at 1E6 in Vascular Cell Basal Medium (ATCC) supplemented with Endothelial Cell Growth Kit – VEGF (ATCC), were incubated at 37°C in 5% CO₂ in Biocoat Gelatin Cellware (Becton Dickinson, Franklin Lakes, NJ) in 75 cm² vented flasks and allowed to grow until confluent. Media were changed twice a week. When cells were confluent, plates were washed with Basal Medium to remove protein and replaced with Trypsin-EDTA 1X (Mediatech, Inc., Manassas, VA). Plates were incubated at 37°C for 10 minutes, and eluted cells were then washed in Basal Medium – VEGF, resuspended to 40 ml, and 200 µl of the suspension was added to each well on Biocoat 96 well plates (Becton Dickinson, Franklin Lakes, NJ). Plates were incubated at 37°C, 5% CO₂ until HUVECs were confluent.

Experiments were carried out using a single HUVEC cell line utilizing second or third passage cells. 20 µg of IgG were added from each sample per well and incubated at 37°C in 5% CO₂ for 18 h (Cho et al., 2002). Supernatants were removed and assayed for MCP-1 using a Quantikine MCP-1 ELISA (R & D Systems, Minneapolis, MN). Triplicate cultures were established for each condition and mean results from the 3 cultures were determined.

Statistical methods

Comparisons of demographic characteristics and periodontal measures between groups and comparison of MCP-1 levels between subjects with elevated and normal aCI concentrations were accomplished by analysis of variance (ANOVA). Pre- and post-absorption levels of MCP-1 stimulation was analyzed by repeated measures ANOVA. Associations of IgG aCI and MCP-1 production were analyzed by linear regression analysis, and the impact of demographic factors was determined by multiple regression analysis.

Results

Sera from 59 subjects were chosen for analysis based upon periodontal diagnosis and previously measured concentrations of IgG aCI. Six IgG samples were found to contain contaminating LPS following purification and were not further analyzed. Table 1 illustrates the demographic and periodontal characteristics of the 53 subjects. 18 periodontally healthy subjects (NP), 20 CP subjects, and 15 GAgP subjects were studied. CP subjects were older than the NP and GAgP subjects. The composition of the groups by both race and sex were not significantly different. Periodontal measures differed significantly between the groups for PD and ALOSS, and NP subjects had more teeth than the AP subjects.

IgG was purified from subjects' sera and added to cultures of HUVEC for assessment of stimulation of MCP-1 production. Figure 1 demonstrates that there is a significant association between the IgG aCI serum titer and MCP-1 production by HUVEC in response to IgG purified from those samples ($p < .0001$, $r^2 = .48$). We further determined the association between clinically elevated levels of aCI and stimulation of MCP-1 by comparing preparations from samples with >15 Units/ml to those with < 15 Units/ml. As seen in Figure 2, MCP-1 produced by HUVEC was significantly greater for IgG from sera containing elevated aCI titers than for those with normal aCI titers (420.7 pg/ml versus 152.1 pg/ml, $p < .0001$). We further noted that there was no influence of the demographic characteristics of the serum donors (age, race, sex, or smoking history) on the relationships between IgG aCI titer and MCP-1 production.

We then assessed MCP-1 production by HUVEC within each diagnostic category for patients with normal and elevated IgG aCI. As shown in Table 2, for each diagnostic group, including the NP subjects, IgG from subjects with elevated aCI levels stimulated greater levels of MCP-1 than IgG from subjects with normal IgG aCI titers.

Finally, to confirm that MCP-1 stimulation was due to IgG aCl content, IgG preparations from 4 CP subjects with varying aCl IgG titers were immunoabsorbed on a Sephadex column containing β 2GP1 bound to cardiolipin. Table 3 illustrates the results of these studies. Removal of antibodies reactive with cardiolipin/ β 2GP1 resulted in significant depletion of IgG aCL content of the samples. Consequent stimulation of HUVEC cells with pre-absorption and post-absorption IgG preparations resulted in a significant decrease of MCP-1 production in the presence of the absorbed samples.

Discussion

Our previous data indicated that aCl serum concentrations are elevated in a higher proportion of patients with either CP or GAgP than in NP subjects (Schenkein et al., 2003). In addition, our data have shown that elevation of aCl, especially in GAgP patients, is associated with elevated levels of soluble adhesion molecules indicative of endothelial cell activation and elevated C-reactive protein (Schenkein et al., 2007). Since these previous studies excluded, by history, patients with known autoimmune diseases or infections known to stimulate systemic production of aCl, these results raised the question of whether the antibodies detected in the immunoassay for aCl in periodontal disease patients had characteristic biological functions of genuine autoimmune antibodies as might be found in SLE or APS, or if they were simply non-functional cross-reactive antibodies. To begin to answer this question, we chose sera from subjects with normal and elevated IgG aCl and assessed the ability of IgG purified from these sera to stimulate endothelial cells to produce MCP-1. The results indicated a clear association of IgG from sera containing elevated titers of aCl with stimulation of endothelial cells to produce MCP-1. This association is found within the two periodontitis subject groups as well as within the periodontally healthy subjects. However, it should be noted that the prevalence of elevated aCl in the NP subject group is nearly identical to that found in the general population (approximately 5%), while the prevalence of elevated aCl in periodontitis subjects is significantly higher (15–20%) (Schenkein et al., 2003). Thus, periodontitis patients appear to be more prone to develop these antibodies and the antibodies likely have pathogenic properties.

Several studies have shown that patients with SLE, especially those demonstrating elevated antiphospholipid antibodies, are prone to accelerated atherosclerosis, and it has been hypothesized that the interaction of aCl and with the endothelium may play a critical mechanistic role explaining this association (Narshi et al., 2011). IgG from SLE and APS patients with elevated aCl titers have been shown to increase monocyte adhesion to endothelial cells (Simantov et al., 1995), to upregulate endothelial cell adhesion molecules (Gharavi et al., 2002, Pierangeli and Harris, 1994, Pierangeli et al., 1995), and to stimulate MCP-1 production (Cho et al., 2002). It is noteworthy that the association of antiphospholipids with accelerated atherosclerosis is a controversial concept and that several studies have failed to demonstrate this association (Artenjak et al., 2012).

There are significant differences between the clinical manifestations of APS and the systemic clinical conditions associated with periodontal infections. Patients with APS are prone to develop arterial and venous thrombi and women with APS frequently experience recurrent spontaneous abortion (Tripodi et al., 2011). In addition, there is some evidence for early atherosclerosis in these patients. Such patients usually have serum aCl levels in the very high range. On the other hand, periodontal infections are associated with preterm labor, low birth weight, atherosclerosis, endothelial dysfunction, and myocardial infarction, though cause and effect relationships are yet to be proven (Lockhart et al., 2012). The concentrations of aCl in sera from these patients, though clinically elevated (i.e. greater than levels found in 95 % of the general population), are largely in the moderate range. Thus, there are similarities between the sequelae of APS and conditions with increased risk in

periodontitis patients but the severity of the associated clinical manifestations are quite different, as are the serum concentrations of these antibodies. Furthermore, APS appears to involve additional functional thrombotic defects and is frequently characterized by the appearance of additional autoantibodies that contribute to the onset of prothrombotic events. Nevertheless, it is difficult to ignore the similarities between these conditions, especially in view of data indicating that the antibodies found in periodontitis patients appear to have some proinflammatory effects on endothelial cells. The results are consistent with an interpretation that aCI in periodontitis patients could contribute to systemic disease in a manner consistent with the concentrations of antibodies found in these patients in comparison to those with true autoimmune disease.

We consider it very likely that aCI antibodies in periodontitis patients can be induced by oral microbial pathogens, and have preliminarily observed in our laboratory that *P. gingivalis*, a pathogen in periodontitis, can induce production of antibodies reactive with cardiolipin/ β 2GP1 complexes in both mice and rabbits (unpublished observations, 2012). It is known that aCI and APS-like symptoms can be induced by a variety of microbial pathogens (Avcin et al., 2008, Blank et al., 2002b, Garcia-Carrasco et al., 2009). This can occur by molecular mimicry due to the presence of microbial peptide sequences homologous to the target antigenic peptide for pathogenic aCI present on β 2GP1 (Blank et al., 2002b). It has in fact been demonstrated that some periodontal microbial pathogens, including *P. gingivalis*, *A. actinomycetemcomitans*, and *T. denticola* have peptide sequences with sufficient homology to a key hexapeptide in β 2GP1 to induce mutually cross-reactive antibodies with the potential to be pathogenic (Wang et al., 2008). There are additional studies showing that infections may trigger APS in other rheumatic diseases, suggesting a microbial origin for both pathogenic and non-pathogenic aCI (Amital et al., 2008). In periodontitis, this type of antibacterial immune response could be in part responsible for associations of periodontal infections with adverse cardiovascular and pregnancy outcomes in some patients, or they merely may be markers of events that co-occur with these conditions. We consider it unlikely that aCI in periodontitis patients are true autoantibodies in that the presence of autoimmune disease was an exclusion criterion for this study.

In summary, IgG from patients with periodontitis who also have elevated serum concentrations of aCI stimulates increased production of the key cytokine MCP-1 from HUVEC cultures. Since periodontitis patients with no evidence of systemic disease demonstrate a significantly higher prevalence of elevated serum levels of aCI compared to periodontally healthy subjects, and since these potentially pathogenic antibodies may be induced by a variety of periodontal pathogens, it is proposed that molecular mimicry with the production of aCI could be one pathogenic link between periodontal infection and systemic disease.

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Clinical Relevance

Scientific Rationale for the Study

Anticardiolipin antibodies, frequently found in periodontitis patients, are of unknown pathogenicity but can be associated with thrombosis, stroke, myocardial infarction, and adverse pregnancy outcomes.

Principal findings

We found that anticardiolipin from periodontitis patients stimulate endothelial cells to produce a cytokine, MCP-1, that is central to the development of atherosclerotic lesions.

Practical implications

The data indicate that it is possible that these antibodies are pathogenic and could explain some of the associations between periodontitis and systemic diseases. Tests for anticardiolipin are routine clinical assays, and could be incorporated into a risk profile for these patients.

Figure 1.

MCP-1 production by HUVEC following stimulation by IgG from systemically healthy subjects with or without periodontitis. 20 ug of IgG were added from each sample per well and incubated at 37°C in 5% CO₂ for 24h. Experiments were carried out in triplicate and the mean concentration of MCP-1 in culture supernatants was determined. There was a significant association between the IgG aCI serum titer and MCP-1 production for all samples ($p < .0001$, $r^2 = .48$).

Figure 2.

MCP-1 production by HUVEC following stimulation with IgG from subjects with elevated (IgG aCI >15 U/ml) or normal (IgG aCI <15 U/ml) aCI titers. 20 ug of IgG were added from each sample per well and incubated at 37°C in 5% CO₂ for 24h. Experiments were carried out in triplicate and the mean concentration of MCP-1 in culture supernatants was determined. MCP-1 produced by HUVEC was significantly greater for IgG from sera containing elevated aCI titers than for those with normal aCI titers (420.7 ug/ml versus 152.1 ug/ml, $p < .0001$). Diamonds illustrate mean values and confidence intervals.

Table 1

Demographic and clinical characteristics of the subject population

	NP ¹ (n=18)	CP ² (n=20)	GAgP ³ (n=15)	p-value
Age (SEM)	38.2 (2.4)	47.4 (2.3)	32.0 (2.7)	P=.0002
Race: % A, B, W ⁴	0, 22.2, 77.8	0, 35.0, 65.0	6.7, 53.3, 62.3	P=.15
Sex: %Female	72.2	55.0	46.7	P=.30
Mean PD: mm (SEM) ⁵	2.00 (0.17)	2.62 (0.16)	3.90 (0.19)	P=.0001
Mean ALOSS (mm) ⁵	0.37 (0.41)	2.29 (0.38)	3.70 (0.44)	P=.0001
N teeth	27.4 (1.41)	22.9 (1.34)	24.0 (1.55)	P=.07

¹ NP: Normal Periodontium² CP: Chronic Periodontitis³ GAgP: Generalized Aggressive Periodontitis⁴ A, B, W: Asian, Black, White⁵ PD: pocket depth; ALOSS: attachment loss

Table 2

Stimulation of HUVEC production of MCP-1 by IgG from periodontal diagnostic groups according to IgG aCI serum levels

Diagnosis ^I	IgG aCI concentration	N	MCP-1 (pg/ml) (SEM)	p-value
NP	< 15 U/ml	14	168.40 (39.6)	P=0.0167
	>15 U/ml	4	393.26 (74.2)	
CP	< 15 U/ml	10	101.37 (89.2)	P=.0079
	>15 U/ml	10	478.58 (89.2)	
GAgP	< 15 U/ml	8	187.16 (48.6)	P=.0175
	>15 U/ml	7	393.26 (74.2)	

^INP = Normal Periodontium; CP = Chronic Periodontitis; GAgP = Generalized Aggressive Periodontitis

Table 3

Effect of removal of aCl from IgG preparations on stimulation of MCP-1 production by HUVEC

IgG aCl (U/mg IgG)		MCP-1 (pg/ml)±SEM		
Pre-absorption	Post-absorption	Pre-absorption	Post-absorption	
1.00	0.31	520.86 ± 54.55	84.76 ± 20.72	P=.004
3.72	0.35	749.88 ± 94.76	93.07 ± 20.67	P=.002
22.00	13.90	1384.39 ± 142.92	717.00 ± 167.89	P=.03
3.12	0.41	766.50 ± 108.66	202.29 ± 5.49	P=.007