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SELP and *SELPLG* Genetic Variation Is Associated with Cell Surface Measures of SELP and SELPLG: The Atherosclerosis Risk in Communities (ARIC) Carotid MRI Study

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Abstract

BACKGROUND—P-selectin (SELP) and its ligand, P-selectin glycoprotein ligand 1 (SELPLG), play key roles in both the inflammatory response and the atherosclerotic process. Previous studies have shown genetic variation in the *SELP* gene [selectin P (granule membrane protein 140kDa, antigen CD62)] to be associated with plasma SELP concentrations; however, the major biological function of SELP (and SELPLG) is at the cell surface. We therefore investigated the association of *SELP* polymorphisms with platelet SELP measures and polymorphisms in the *SELPLG* gene (selectin P ligand) with lymphocyte, granulocyte, and monocyte SELPLG measures among 1870 participants in the Atherosclerosis Risk in Communities (ARIC) Carotid MRI study.

METHODS—Whole-blood flow cytometry was used to analyze leukocyte and platelet markers in the ARIC Carotid MRI study. The allele frequencies for the *SELP* and *SELPLG* polymorphisms of whites and African Americans were markedly different; therefore, all analyses were race specific.

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RESULTS—*SELP* T715P was significantly associated with lower values for platelet SELP measures in whites ($P = 0.0001$), whereas *SELP* N562D was significantly associated with higher values for SELP measures in African Americans ($P = 0.02$). *SELPLG* M62I was significantly associated with lower granulocyte and monocyte SEL-PLG measures in African Americans ($P = 0.003$ and $P = 0.0002$, respectively) and with lower lymphocyte SELPLG measures in whites ($P = 0.01$).

CONCLUSIONS—Specific *SELP* and *SELPLG* polymorphisms were associated with cell surface measures of SELP and SELPLG in both whites and African Americans in the ARIC Carotid MRI study. To our knowledge, this study is the first to examine the association of *SELP* and *SELPLG* genetic variation with measures of cell surface SELP and SELPLG.

The pathogenesis of atherosclerosis is known to contain an important inflammatory component involving the recruitment and adhesion of circulating leukocytes to the vascular endothelium (1,2). P-selectin (SELP),[6] a member of the selectin family of adhesion molecules, initiates leukocyte rolling and mediates interactions of leukocytes with the endothelium, platelets with the endothelium, and leukocytes with platelets (3–5). Leukocyte–endothelium and leukocyte–platelet interactions require the presence of a counterligand on the leukocyte surface, P-selectin glycoprotein ligand 1 (SELPLG) (6–8). Multiple studies have provided evidence supporting a key role for SELP and SELPLG in atherosclerotic lesion formation, thrombosis, and arterial wall changes (1,5,9–14).

Previous studies have shown variation in the *SELP*[7] gene [selectin P (granule membrane protein 140kDa, antigen CD62)] to be associated with the concentration of soluble SELP in the plasma (15–18). However, the studies that have examined the association of SELP and SELPLG concentrations with cardiovascular disease and those that have evaluated associations of *SELP* and *SELPLG* (selectin P ligand) genetic variation with cardiovascular disease have produced inconsistent results (8,19–26). The major biological function of SELP and SELPLG is at the cell surface, where these cell adhesion molecules mediate the interaction of leukocytes, platelets, and the endothelium (3–8). Although the concentration of soluble SELP has been used as an indicator of platelet activation, measures of soluble SELP are affected by multiple mechanisms, including active cleavage from the platelet surface, simple shedding from the platelet surface, and direct secretion from the cell in the form of an alternatively spliced *SELP* mRNA lacking the sequence encoding the protein's transmembrane domain (5,27,28). One can avoid the caveat of not having a clearly identifiable source of SELP by measuring it on the cell surface with flow cytometry (5). To our knowledge, no study to date has examined the relationship of *SELP* and *SELPLG* genetic variation to measures of SELP and SELPLG on the cell surface. We investigated the association of 4 *SELP* polymorphisms with measures of platelet SELP and the association of 2 *SELPLG* polymorphisms with lymphocyte, granulocyte, and monocyte SELPLG measures among 1870 participants in the Atherosclerosis Risk in Communities (ARIC) Carotid MRI study.

Materials and Methods

STUDY DESIGN AND STUDY PARTICIPANTS

The ARIC Carotid MRI study was conducted from 2004–2005 and included 2066 participants. The participants were selected from the ARIC study cohort according to the results of the last ultrasound examinations of the carotid artery (visits 3 and 4, 1993–1998). The participants included those with high values for carotid artery wall thickness and a random sample of

[6]Nonstandard abbreviations: SELP, P-selectin; SELPLG, P-selectin glycoprotein ligand 1; ARIC, Atherosclerosis Risk in Communities; CHD, coronary heart disease.
[7]Human genes: *SELP*, selectin P (granule membrane protein 140kDa, antigen CD62); *SELPLG*, selectin P ligand.

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participants with carotid arteries of typical thickness; equivalent numbers of participants were selected from the 4 ARIC field centers. The ARIC study is a prospective investigation of atherosclerosis and its clinical sequelae in 15 792 African American and white men and women who were 45–64 years of age at the time of recruitment (1987–1989). Participants underwent a baseline evaluation and had up to 3 follow-up visits through 1998. Detailed descriptions of the ARIC study design and methods have been published elsewhere (29). The study was approved by local institutional review boards. For the current analyses, participants were excluded if they prohibited use of their DNA for research purposes or had missing information for all measurement variables; approximately 1870 ARIC Carotid MRI participants were ultimately available for analysis.

EXAMINATION AND LABORATORY MEASURES

All measures used in the described analyses were from the 2004–2005 MRI examination. Seated blood pressure was measured 3 times with a random-zero sphygmomanometer, and the mean of the last 2 measurements was used. Hypertension was defined as a systolic blood pressure ≥140 mmHg, a diastolic blood pressure ≥90 mmHg, or current use of antihypertensive medications. Diabetes was defined by a fasting glucose concentration ≥7.0 mmol/L (126 mg/dL), a nonfasting glucose concentration ≥11.1 mmol/L (200 mg/dL), and/or a history of or treatment for diabetes. Cigarette-smoking status was analyzed by comparing current smokers to individuals who had formerly or never smoked. Body mass index (in kilograms per meter squared) was calculated from height and weight measurements. Details of flow cytometry measurements are provided elsewhere (30). In brief, flow cytometric analysis was performed on a Coulter Epics XL instrument (Beckman Coulter). Strict internal quality-assurance procedures were applied. The proportion of cells expressing the antigen of interest and the relative level of antigen expression were assessed by measuring the median fluorescence intensity. Data were extracted from the acquired flow cytometric list mode data files and transferred into Microsoft Office Excel files.

GENOTYPE DETERMINATION

We selected the following *SELP* and *SELPLG* polymorphisms on the basis of their being missense mutations or having previously been reported to be associated with coronary heart disease (CHD) and/or stroke: *SELP* S290N (rs6131), *SELP* N562D (rs6127), *SELP* V599L (rs6133), *SELP* T715P (rs6136), *SELPLG* 5′UTR (rs8179131), and *SELPLG* M62I (rs2228315). Genotyping was carried out with SNPlex or TaqMan assays (Applied Biosystems). Sequences for the primers and probes are available from the authors upon request. The ARIC study has a rigorous blind duplicate program. The percent agreement for blind duplicate data for these polymorphisms was 96.3% for *SELP* S290N, 94.8% for *SELP* N562D, 96.3% for *SELP* V599L, 97.8% for *SELP* T715P, 99.7% for *SELPLG* 5′UTR, and 97.3% for *SELPLG* M62I.

STATISTICAL ANALYSIS

All analyses were based on methods appropriate for a stratified random sample. In particular, all analyses were weighted by the inverse of the sampling fractions in the 8 sampling strata (4 field centers × 2 intima–media thickness groups). In brief, the ARIC Carotid MRI study reexamined a subset of the original ARIC cohort. The participants were selected as described above. The sampling fractions were based on those persons actually screened for participation. SAS software (version 9.1, SAS Institute) was used for analysis of descriptive statistics (PROC SURVEYMEANS, PROC SURVEYFREQ), and SUDAAN (RTI International) was used for linear and logistic regression. Correction factors for finite populations were not applied. Tests of differences between groups in weighted means or proportions were from weighted linear or logistic regression models that accounted for the sampling. SUDAAN REGRESS was used

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for continuous variables to calculate adjusted means and proportions by subgroup of interest, and LOGISTIC was used for dichotomous variables.

SUDAAN CROSSTAB was used to calculate genotype frequencies as weighted frequencies. Allele frequencies were calculated from genotype frequencies; e.g., the frequency of allele A was calculated as $P(AA) + P(AB)/2$, where $P(AA)$ and $P(AB)$ are the weighted frequencies of genotypes AA and AB, respectively. The test for the Hardy–Weinberg equilibrium used the weighted genotype frequencies and their covariance matrix, with the δ method applied to obtain the test statistic. Variant alleles were identified as the low-frequency allele in white participants, and homozygous nonvariant genotypes were designated as the referent group in the statistical analyses.

Results

All *SELP* and *SELPLG* genotype distributions were in accordance with Hardy–Weinberg equilibrium expectations. Whites and African Americans had markedly different allele frequencies for each of the polymorphisms studied; consequently, we analyzed the racial groups separately. When alleles were observed to be rare, heterozygous and homozygous genotypes carrying the rare allele were combined for further genotype–phenotype analyses. The *SELPLG* 5′UTR polymorphism was not polymorphic in the white participants; therefore, results for this polymorphism are presented for African Americans only.

Table 1 presents SELP and SELPLG flow cytometry data by racial group, along with the results for basic risk factors. White and African American ARIC participants differed significantly with respect to all of the basic risk factors investigated except smoking status. Compared with the white participants, the African American participants were younger, had a higher body mass index, and had a higher proportion of diabetic and hypertensive individuals. African Americans also had significantly higher values for all flow cytometry measures of SELP and SELPLG than white participants (Table 1).

Table 2 presents SELP data for flow cytometry measures according to *SELP* genotype and racial group. In whites only, *SELP* 715TP and 715PP genotypes were associated with significantly lower values for platelet SELP measures compared with the 715TT genotype. In African Americans only, the *SELP* 562ND and 562DD genotypes were associated with significantly higher values for platelet SELP measures, compared with the 562NN genotype. Table 3 presents SELPLG data for flow cytometry measures according to *SELPLG* genotype and racial group. In whites, the *SELPLG* 62MI and 62II genotypes were associated with significantly lower values for lymphocyte SELPLG measures compared with the 62MM genotype. In African Americans, both the *SELPLG* 62MI and 62II genotypes (compared with 62MM) and the *SELPLG* 5′UTR GA and AA genotypes (compared with 5′UTR GG) were associated with lower values for the lymphocyte, granulocyte, and monocyte SELPLG measures.

Noting that we had previously found the *SELPLG* M62I polymorphism to be associated with the risk of both incident CHD and incident ischemic stroke in African American participants in the ARIC study (31), we further investigated whether the data for the SELP and SELPLG flow cytometry measures were associated with disease status in the African American participants of the ARIC Carotid MRI study. All values for SELP and SELPLG flow cytometry measures were higher in the ischemic stroke case group than in the controls, but these findings were not statistically significant (data not shown); however, the number of cases identified in the ARIC Carotid MRI study was small (African American CHD cases, 24; stroke cases, 23). We therefore do not have sufficient power to detect a difference in disease status with regard to the flow cytometry measures.

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Discussion

The major biological function of SELP and SELPLG is at the cell surface, where they mediate the interaction of leukocytes, platelets, and the endothelium (3–8). Because multiple mechanisms can produce changes in the values for soluble SELP, one can avoid the caveat of not having a clearly identifiable source of SELP by measuring SELP, as well as SELPLG, on the cell surface via flow cytometry (5). The current investigation of the ARIC Carotid MRI study used flow cytometry to obtain values for cell surface measures of SELP and SELPLG. We found *SELPLG* M62I to be associated with decreased values for lymphocyte SELPLG measures in both whites and African Americans, as well as decreased values for granulocyte and monocyte SELPLG in African Americans only. In a previous study, we found *SELPLG* M62I to be associated with a decreased risk for both incident CHD and incident ischemic stroke in African American participants in the ARIC study (31).

The *SELPLG* M62I polymorphism is positioned adjacent to the SELP-binding domain. Although the functional consequence of the M62I polymorphism is unknown, the proximity of this variant to the binding domain suggests that it has an effect on SELP binding (8,32). The implication of SELP and SELPLG in the generation of procoagulant microparticles has revealed novel roles for SELP and SELPLG in thrombosis and has led to the use of SELP inhibitors as a therapeutic target for vascular and thrombotic diseases (5,33–36). SELPLG could be considered as an alternative target for intervention. Variation in the *SELPLG* gene that alters SELP/SELPLG binding capacity represents an important target in reducing the effect of SELPLG molecules present on the cell surface, which may lead to fewer leukocyte–endothelium and leukocyte–platelet complexes and a potential reduction in the risk of stroke. The antibody used in the flow cytometry assay (KPL1) maps to a tyrosine-sulfation motif positioned between amino acid residues 5 and 11 of SELPLG (37). Therefore, it is unlikely that the *SELPLG* M62I polymorphism we have investigated has an impact on the affinity of the KPL1 antibody used in the flow cytometry assay.

Although the number of cases within the ARIC Carotid MRI study was not adequate to investigate the association of SELP and SELPLG flow cytometry measures with incident ischemic stroke, all values for SELP and SELPLG flow cytometry measures were higher in the stroke case group than in the controls. In a previous study, we had determined *SELPLG* genetic variation to be associated with a decreased risk of incident ischemic stroke in the ARIC cohort (31). Considering our previous findings along with the results of the current study (that *SELPLG* genetic variation is associated with decreased values for cell surface measures of SELPLG in the ARIC Carotid MRI study), we propose that this combined evidence strongly supports the hypothesis that measures of cell surface SELPLG are related to the risk of incident ischemic stroke. Of course, further studies are warranted to replicate the current findings, to determine the causal relationship between SELPLG cell surface measures and the risk of disease, and to determine the functional consequence of the *SELPLG* M62I polymorphism. Furthermore, we must also consider that although the *SELPLG* M62I polymorphism was associated with measures of SELPLG on the lymphocyte cell surface in both whites and African Americans, the association with disease was statistically significant only for African Americans. Therefore, our results cannot discern whether the *SELPLG* M62I polymorphism itself has a direct causative effect; it may be in linkage disequilibrium with the true functional variant(s). Given that linkage-disequilibrium patterns of whites and African Americans are different, if we are not investigating the true functional variant, we may have detected an association with a “marker” polymorphism in one race and not the other.

Results from the current study demonstrate that the *SELPLG* M62I polymorphism is associated with significantly lower values for SELPLG granulocyte and monocyte measures in African Americans; this polymorphism also exhibits a strong trend of association with lower values

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for SELPLG lymphocyte measures in both whites and African Americans. A possible mechanism by which the *SELPLG* M62I polymorphism might affect disease risk is by altering the affinity of SELP to bind to SELPLG, thereby attenuating the recruitment and adhesion of leukocytes and platelets to the endothelium wall and/or reducing leukocyte–platelet aggregates. To our knowledge, no study to date has examined the association of *SELP* and *SELPLG* genetic variation with SELP and SELPLG cell surface measures. Further studies are warranted to replicate the current findings in other large US populations of whites and African Americans and to determine the functional consequence of the *SELPLG* M62I polymorphism. These results, when considered together with prior research on SELP and SELPLG function and if replicated in future studies, provide preliminary evidence that could lead to new targets for therapeutic intervention.

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Table 1

ARIC Carotid MRI basic risk factors and flow cytometry measures by race.[a]

	Whites (n = 1411)	African Americans (n = 459)	P
Basic risk factors			
Age, years	70.6 (5.5)	69.2 (5.5)	<0.0001
BMI,[b] kg/m^2	28.5 (5.1)	31.4 (6.0)	<.0001
Male sex, %	45.8	35.3	0.0009
Smoker, %	7.2	9.9	0.1
Diabetic, %	21.1	36.9	<.0001
Hypertensive, %	61.3	78.0	<.0001
Flow cytometry measures			
Platelet SELP, %	27.6 (13.1)	30.9 (15.7)	0.0008
Platelet SELP, MFI	21.5 (5.1)	22.8 (6.2)	0.0008
Lymphocyte SELPLG, MFI	51.8 (10.0)	59.6 (10.8)	<.0001
Granulocyte SELPLG, MFI	76.7 (14.1)	88.4 (14.3)	<.0001
Monocyte SELPLG, MFI	111.6 (15.5)	114.6 (14.1)	0.001

[a] Data are presented as the mean (SD) or as a percentage, as indicated.

[b] BMI, body mass index; MFI, median fluorescence intensity.

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Table 2

ARIC Carotid MRI SELP flow cytometry measures by *SELP* genotype and race.[a]

		Flow cytometry measures			
***SELP* genotype**	**n**	**Platelet SELP, %**	***P***	**Platelet SELP, MFI[b]**	***P***
Whites					
SELP S290N					
SS	890	27.6 (0.6)	0.1	21.5 (0.3)	0.4
SN	428	27.0 (0.8)		21.3 (0.3)	
NN	56	32.3 (2.4)		22.3 (0.7)	
SELP N562D					
NN	398	28.1 (0.8)	0.2	21.6 (0.4)	0.08
ND	614	28.0 (0.7)		21.7 (0.3)	
DD	251	26.0 (1.0)		20.8 (0.3)	
SELP V599L					
VV	1070	27.3 (0.5)	0.2	21.2 (0.2)	0.02
VL + LL	300	28.6 (0.9)		22.1 (0.3)	
SELP T715P					
TT	1100	28.1 (0.5)	0.01	21.6 (0.2)	0.0001
TP	266	25.7 (1.0)		20.8 (0.3)	
PP	20	20.7 (3.3)		18.6 (0.7)	
African Americans					
SELP S290N					
SS	197	31.1 (1.4)	0.1	22.8 (0.7)	0.2
SN	192	30.2 (1.2)		22.4 (0.4)	
NN	52	35.7 (2.6)		25.0 (1.3)	
SELP N562D					
NN	283	29.8 (1.0)	0.02	22.2 (0.3)	0.02
ND + DD	125	34.6 (1.8)		24.7 (1.1)	
SELP V599L					
VV	66	34.1 (2.2)	0.3	23.6 (0.9)	0.4
VL	233	31.0 (1.2)		23.0 (0.6)	

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	Flow cytometry measures				
***SELP* genotype**	**n**	**Platelet SELP, %**	***P***	**Platelet SELP, MFI[b]**	***P***
LL	140	29.6 (1.5)		22.2 (0.5)	
SELP T715P					
TT	404	30.6 (0.9)	0.4	22.9 (0.4)	0.1
TP + PP	19	33.5 (3.7)		21.3 (1.0)	

[a] Data are presented as the mean (SE) and adjusted for age and sex.

[b] MFI, median fluorescence intensity.

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Table 3

ARIC Carotid MRI SELPLG flow cytometry measures by *SELPLG* genotype and race.[a]

		Flow cytometry measures					
***SELPLG* genotype**	**n**	**Lymphocyte SELPLG, MFI[b]**	***P***	**Granulocyte SELPLG, MFI**	***P***	**Monocyte SELPLG, MFI**	***P***
Whites							
SELPLG M62I							
MM	1195	52.2 (0.4)	0.01	76.7 (0.5)	0.4	111.4 (0.6)	0.4
MI + II	171	50.0 (0.8)		75.5 (1.2)		112.7 (1.5)	
African Americans							
SELPLG 5′UTR							
GG	365	60.1 (0.7)	0.009	89.1 (0.9)	0.05	115.5 (0.8)	0.007
GA + AA	82	56.5 (1.2)		84.8 (1.9)		110.0 (1.8)	
SELPLG M62I							
MM	242	61.0 (0.9)	0.06	89.5 (1.1)	0.003	117.3 (1.0)	0.0002
MI	160	58.8 (0.9)		88.8 (1.3)		112.8 (1.3)	
II	33	57.0 (1.8)		82.0 (1.9)		107.3 (2.5)	

[a] Data are presented as the mean (SE) and adjusted for age and sex.

[b] MFI, median fluorescence intensity.