Interaction Between Smoking and Polymorphism in the Promoter Region of the VEGFA Gene Is Associated with Ischemic Heart Disease and Myocardial Infarction in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To determine whether variants in the vascular endothelial growth factor A (VEGFA) gene are associated with ischemic heart disease (IHD) and/or myocardial infarction (MI) in patients with rheumatoid arthritis (RA), and whether there is evidence of a gene-smoking interaction.

Methods. PCR-RFLP assays were used to determine the genotypes of VEGFA single-nucleotide polymorphisms (SNP) including VEGFA–2578A/C (rs699947), –460C/T (rs833061), +405C/G (rs2010963), and +936C/T (rs3025039) in 418 subjects with RA. Smoking history was obtained on each patient, and IHD and MI status was recorded. Associations with IHD/MI were assessed using contingency tables and logistic regression analyses.

Results. Strong linkage disequilibrium was detected among VEGFA–2578, –460, and +405. SNP located in the VEGFA promoter region (–2578, –460) were found to be associated with IHD and MI, whereas +405 and +936, in the 5'-untranslated region (UTR) and 3'-UTR, respectively, were not. Haplotype analysis suggested that the A/C/G haplotype was associated with increased risk of IHD (OR 2.37, 95% CI 1.22–4.62) and MI (OR 4.10, 95% CI 1.45–11.49). Smoking was also independently associated with IHD and MI, and evidence of interaction between smoking and the VEGFA promoter SNP was found. Multivariate analyses indicated that the strongest associations with IHD and MI were due to the combined effect of the VEGFA–2578 A allele and smoking (OR 3.52 and 7.11, respectively), independent of risk factors such as age, sex, diabetes, C-reactive protein, hypercholesterolemia, and hypertension.

Conclusion. Interaction between smoking and polymorphism in the VEGFA gene is associated with IHD and MI in patients with RA. (First Release March 1 2011; J Rheumatol 2011;38:802–9; doi:10.3899/jrheum.101095)

Key Indexing Terms:
RHEUMATOID ARTHRITIS SMOKING POLYMORPHISM CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a major comorbid condition in rheumatoid arthritis (RA), with increased prevalence compared with that in the general population[1,2]. Studies have shown that the clinical presentation of ischemic heart disease (IHD) appears to be different to that in the normal population, with RA patients more likely to experience unrecognized myocardial infarction (MI) and sudden death[3]. Apart from classical risk factors such as smoking, hypertension, dyslipidemia, and diabetes, a number of characteristics of RA may contribute to the increased risk of developing CVD. These include autoantibodies, extraarticular disease, increased inflammatory burden, and medications such as nonsteroidal antiinflammatory drugs and steroids[4,5,6,7]. Few data about the genetic predisposition to CVD in RA have been published. We and others have shown that endothelial dysfunction and the risk of cardiovascular events and/or cardiovascular mortality is increased in RA patients carrying certain HLA-DRB1 shared-epitope alleles[8,9,10,11]. A study from Sweden has suggested an association of plasminogen activator inhibitor type-1 (PAI-1 4G/5G) with IHD[12] and an association has been found between polymorphism in the lymphotoxin-A gene and risk of MI in RA[13]. A promoter polymorphism in the interleukin 6 (IL-6) gene, and a polymorphism in the methylene tetrahydrofolate reductase (MTHFR) gene have been associated with CVD in RA[14,15].

Polymorphism within the vascular endothelial growth factor A (VEGFA) gene (+936C/T) has been reported to be

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Supported by the Medical Research Council and the Haywood Rheumatism Research and Development Foundation.

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Accepted for publication January 7, 2011.

involved in the development of RA[16]. As a fundamental promoter of normal and abnormal angiogenesis, VEGF plays an important role in the pathogenesis and progression of RA[17,18,19]. The role of VEGF in the development of CVD in RA has not been investigated, although studies have provided evidence that polymorphisms in the VEGFA gene are associated with development of atherosclerosis, increased risk of acute MI, and poor clinical outcome in chronic heart failure[20,21,22,23,24,25,26]. It has been suggested that these associations may be explained by the regulation of VEGF expression, with increased levels providing a protective effect[20,21]. Interestingly, the +936 T allele associated with RA susceptibility[16] has been associated with lower plasma levels of VEGF[27].

The human VEGFA gene is localized to chromosome 6p12, and is organized in 8 exons. At least 6 different isoforms of VEGF are generated by alternative splicing of the VEGFA gene[28]. VEGF is regulated by a variety of growth factors, cytokines, hormones, and hypoxia[29,30,31]. Single-nucleotide polymorphisms (SNP) in the promoter region (–2578 and –460), 5'-untranslated region (+405) and 3'-UTR (+936) have been associated with differential expression of VEGF protein, although there is inconsistency between studies[27,32,33,34,35,36,37]. In order to investigate the possible role of the VEGFA gene in the development of CVD in patients with RA we examined the association of these SNP with the presence of IHD or previous MI in a cohort of patients recruited into a study of comorbid disease in RA. Since smoking has a causal role in the development of atherosclerotic changes in CVD[38] we also looked for any evidence of interaction between smoking and the VEGFA gene.

MATERIALS AND METHODS

Patients. We studied a cohort (n = 418) of consecutively recruited RA patients of Caucasian origin, resident in North Staffordshire, England. All patients had a diagnosis of RA, and met the 1987 American College of Rheumatology criteria[39]. Most patients (93%) had been treated with one or more disease-modifying antirheumatic drugs. The majority were being treated with methotrexate, sulfasalazine, or hydroxychloroquine. The commonest combination therapy was methotrexate and sulfasalazine. A small number of patients were being treated with steroids (~10%) or cytotoxic drugs such as azathioprine or cyclophosphamide (< 5%). A number of the patients were receiving biologic agents (mainly etanercept and infliximab) at the time of recruitment (14%). Ethical approval was obtained from the North Staffordshire local research ethics committee, and all patients provided written informed consent.

A core set of demographic and clinical variables was recorded at recruitment. These included the Disease Activity Score (DAS28), Disability Index of the Health Assessment Questionnaire (HAQ)[40], presence of erosions, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), visual analog pain score, IgM rheumatoid factor (RF), anti-cyclic citrullinated peptide antibody (anti-CCP), and presence or absence of rheumatoid nodules. Evidence of cardiovascular disease (IHD, previous MI, heart failure, etc.) was obtained from a structured interview, review of the medical notes, and current and cumulative medication. All patients also underwent resting 12-lead electrocardiography (ECG). A diagnosis of IHD was based on presence of angina pectoris, previous MI (physician diagnosed), or evidence of coronary artery disease based on angiography, functional testing or previous revascularization procedures such as coronary artery bypass grafting. Hypertension was considered to be present if there was a physician's diagnosis in the medical notes or the patient was taking antihypertensive medication. Hypercholesterolemia was considered to be present if a diagnosis had been recorded in the medical notes, or if the patient had ever been prescribed lipid-lowering medication. Evidence of diabetes (type 1 and 2) was based on a physician's diagnosis in the medical notes, or use of antidiabetic medications. Other comorbid conditions (e.g., cancer, renal disease, liver disease, respiratory disease) were also recorded, as well as current drug therapy for any comorbid conditions. All patients underwent a physical and medical examination (weight, height, pulse, blood pressure, etc.). A history of current or past cigarette smoking was obtained from a questionnaire completed by each patient at recruitment. Patients with a smoking history (ever-smokers) were those who had smoked at least 1 cigarette/day for at least 1 year.

VEGFA SNP typing. SNP rs699947, rs833061, rs2010963, and rs3025039 are commonly known as VEGFA–2578(A/C), –460(C/T), +405(C/G), and +936(C/T), respectively. VEGFA–2578 is named after its position relative to the translation start site of the VEGFA gene (–1540 relative to the transcription start site), whereas the others are based on the positions relative to the transcription start (–460, +405) or end (+936) site.

Leukocyte DNA was isolated from peripheral blood samples using a Nucleon DNA extraction kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer's instructions. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods were used to determine the VEGFA genotypes. The primers and restriction enzymes for –2578C/A, –460T/C, +405C/G, and +936C/T were 5'-GGC CTT AGG ACA CCA TAC C-3' (forward) and 5'-CAC AGC TTC TCC CCT ATC C-3' (reverse) (BstYI); 5'-TGT GCG TGT GGG GTT GAG CG-3' (forward) and 5'-TAC GTG CGG ACA GGG CCT GA-3' (reverse) (BstU I); 5'-ATT TAT TTT TGC TTG CCA TT-3' (forward) and 5'-GTC TGT CTG TCT GTC CGT CA-3' (reverse) (BsmFI); and 5'-AGG GTT TCG GGA ACC AGA TC-3' (forward) and 5'-CTC GGT GAT TTA GCA GCA AG-3' (reverse) (Nla III), respectively.

All primers were obtained from Sigma-Genosys (Haverhill, Suffolk, UK) and restriction enzymes from New England Biolabs (Hitchin, Herts, UK) or Promega UK Ltd. (Southampton, Hampshire, UK). The reaction mixtures and conditions were as described[16,32]. All amplification reactions were performed in a Flexigene thermal cycler [Techne (Cambridge) Limited, Cambridge, UK] using a 96 well heating block. Following DNA amplification, the products were digested with the appropriate restriction enzyme followed by electrophoresis on 2% agarose gels.

Statistical analysis. The relationship between VEGFA genotypes and IHD/MI was initially assessed using contingency tables. Multivariate logistic regression analysis was used to investigate the association between IHD/MI and VEGFA polymorphisms when adjusting for other possible confounders such as age, sex, smoking, etc. Evidence of interaction between smoking and VEGFA polymorphisms was assessed by examining for evidence of departure from additivity using the methods of Rothman and Greenland[41]. Using this approach the attributable proportion due to interaction (AP) was calculated, together with 95% confidence intervals, as detailed by Andersson, *et al*[42]. The AP refers to the attributable proportion of disease that is due to interaction among persons with both exposures. In the case of no biological interaction, AP = 0, while an AP = 1.0 corresponds to complete additive interaction. This method has been suggested to be the most robust when using odds ratios in place of relative risks[43].

Software used for analysis included the Number Cruncher Statistical System for Windows (NCSS 2000), the Linkage Disequilibrium Analyser (LDA version 1.0), and Hapstat (version 3.0, Department of Biostatistics, University of North Carolina, Chapel Hill, NC, USA). Pair-wise linkage disequilibrium was analyzed by LDA, and haplotype analysis was conducted in Hapstat. Dominant/recessive or additive genetic models were applied to analyze the association of VEGFA SNP and haplotypes with

IHD/MI. Power calculations were performed using an online power calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). As an example, for SNP VEGFA–2578 (rs699947) and +460 (rs833061), the power of the study for finding a difference between RA patients with and without IHD with an estimated OR between 2.0 and 2.5, a type I error rate of 0.05, a dominant inheritance mode, and an 18.6% frequency in this RA population was between 67% and 85%.

RESULTS

Characteristics of the RA patients. Patient characteristics are displayed in Table 1. Evidence of IHD and previous MI was found in 18.7% and 11.0% of patients, respectively. Of the 46 patients with previous MI, 13 had no medical history of MI and were identified on the basis of their ECG (Q-wave development in absence of any conduction defect, suggesting full-thickness MI). Patients with IHD or MI were older than those without IHD or MI (68.0 vs 61.0 yrs, respectively, $p < 0.0001$; and 68.5 vs 61.0 yrs, $p = 0.0002$) and more likely to be male (30.1% vs 13.1%, respectively, $p < 0.0001$; and 21.3% vs 6.0%, $p < 0.0001$). There was no significant difference in disease duration between patients with and those without IHD/MI.

Distributions of the VEGFA SNP. Genotypes of the 4 VEGFA polymorphisms were determined in 418 subjects. The frequencies of VEGFA genotypes were: –2578 AA 26.3%, AC 48.1%, CC 25.6%; –460 CC 24.9%, CT 50.2%, TT 24.9%; +405 GG 47.9%, CG 42.6%, CC 9.6%; and +936 CC 72.0%, CT 24.6%, TT 3.4%. Genotypes of –2578, –460, +405, and +936 were all distributed in accord with a close fit to Hardy-Weinberg equilibrium. Pair-wise linkage disequilibrium coefficients (*D'*)[44] between SNP are shown in Table 2. Strong linkage disequilibrium was detected in the following pairs, (–2578, –460), (–2578, +405), and (–460, +405). No linkage disequilibrium was observed between +936 and any other SNP within the upstream region of the VEGFA gene.

Association of VEGFA SNP with IHD and MI. Associations between VEGFA genotypes and the presence of IHD/MI, without adjustment for confounders, are shown in Table 3. The associations of VEGFA–2578, –460, and +405 with IHD/MI were best explained by dominant models in which the A, C, and G alleles, respectively, provided an increased risk, although for +405 it was not statistically significant. The VEGFA+936 polymorphism was not associated with IHD or MI. Adjustment for age and sex in logistic regression analyses made little or no difference to the associations found (data not shown).

Since there is strong linkage disequilibrium between VEGFA–2578, –460, and +405, the associations involving –460 and +405 may be due to the greater association of –2578 with IHD and MI. This was supported by logistic

Table 1. Demographic and clinical characteristics of RA patients stratified by the presence of ischemic heart disease (IHD). Values are n (%) or median (interquartile range).

Variable	All Patients	Patients without IHD	Patients with IHD	p†
Age, yrs	62.0 (54.0–69.0)	61.0 (54.0–68.0)	68.0 (58.8–74.3)	0.001
Age of onset, yrs	50.0 (41.0–58.0)	49.0 (40.8–57.0)	55.0 (45.0–65.0)	0.001
Duration, yrs	9.0 (4.0–18.0)	10.0 (3.0–18.0)	8.0 (3.9–19.3)	NS
Male:female	136:282	95:245	41:37	< 0.0001
Body mass index	27.3 (24.5–30.5)	27.3 (24.4–30.5)	27.3 (25.2–30.6)	NS
Rheumatoid factor	239/418 (57.2)	185/340 (54.4)	54/78 (69.2)	0.02
Anti-CCP	306/406 (75.4)	251/332 (75.6)	55/74 (74.3)	NS
ESR	19 (10–35)	18 (10–34)	26 (10–43)	NS
CRP (≥ 10 mg/l)	224/417 (53.7)	169/339 (49.9)	55/78 (70.5)	0.001
Nodules	54/418 (12.9)	40/340 (11.8)	14/78 (18.0)	NS
Erosions	303/411 (73.7)	251/334 (75.1)	52/77 (67.5)	NS
DAS28*	4.2 (1.4)	4.2 (1.4)	4.4 (1.4)	NS
HAQ score	1.6 (1.0–2.0)	1.6 (0.9–2.0)	1.8 (1.3–2.3)	0.01
Ever smoker	278/418 (66.5)	211/340 (62.1)	67/78 (85.9)	0.0001
Current smoker	73/418 (17.5)	60/340 (17.6)	13/78 (16.7)	NS
Previous myocardial infarction	46/418 (11.0)	—	46/78 (59.0)	—
Hypertension	163/418 (39.1)	115/340 (33.8)	48/78 (61.5)	< 0.0001
Hypercholesterolemia	70/418 (17.2)	41/340 (12.1)	29/78 (37.2)	< 0.0001
Diabetes (1 and 2)	31/418 (7.4)	14/340 (4.1)	17/78 (21.8)	< 0.0001
DMARD use	389/418 (93.1)	317/340 (93.2)	72/78 (92.3)	NS
Methotrexate use	247/418 (59.1)	212/340 (62.3)	35/78 (44.9)	0.007
Steroid use	40/418 (9.6)	29/340 (8.5)	11/78 (14.1)	NS
Biologic agent use	60/418 (14.4)	53/340 (15.6)	7/78 (9.0)	NS

* Mean (standard deviation). † p values show significant differences between patients with and without IHD. CCP: cyclic citrullinated peptide; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DAS: Disease Activity Score; HAQ: Health Assessment Questionnaire; DMARD: disease-modifying antirheumatic drug; NS: nonsignificant.

Table 2. Pair-wise linkage disequilibrium of VEGFA single-nucleotide polymorphisms measured by *D′*.

Pair Locus	*D′* value	Standard Error
pair (–2578, –460)	0.933	0.016
pair (–2578, +405)	0.985	0.022
pair (–2578, –936)	0.110	—
pair (–460, +405)	0.931	0.018
pair (–460, +936)	0.041	—
pair (+405, +936)	0.070	—

$D' = D/D_{max}$ (Lewontin, *et al*[44]).

regression analysis that included the –2578 A allele, –460 C allele, and +405 G allele together as independent variables, and used forward stepwise selection to determine which factor(s) was most strongly associated with IHD and MI. The VEGFA–2578 A allele maintained significance in relation to both IHD (OR 2.96, 95% CI 1.28–6.83) and MI (OR 8.39, 95% CI 1.97–35.77), while the other 2 SNP lost significance in these models (adjusted for age and sex).

Association of VEGFA haplotypes with IHD and MI. Common haplotypes of VEGFA–2578, –460, and +405, and their relationship with the presence of IHD/MI are presented in Table 4. The frequencies of rare haplotypes are not shown (frequency threshold = 0.05). The A/C/G haplotype displayed a significant difference in distribution between patients with and those without IHD/MI, suggesting an increased risk associated with this haplotype. No significant difference in the frequency of C/T/C or C/T/G was found between patients with and those without IHD/MI.

Association of VEGFA-smoking interaction with IHD and MI. Cigarette smoking is a well known risk factor for CVD in the normal population and in RA. In this study, we found that patients who ever smoked had an increased likelihood of IHD (OR 2.53, 95% CI 1.28–4.97, p = 0.007, adjusted for age and sex) and MI (OR 4.20, 95% CI 1.44–12.26, p = 0.009, adjusted for age and sex), compared to patients who had never smoked.

We also found evidence of a strong interaction between smoking and the SNP within the VEGFA promoter with regard to both IHD and MI (Table 5). A strongly enhanced risk was observed only in patients with both a VEGFA risk allele and a history of smoking. The attributable proportion (AP) due to interaction for the VEGFA–2578 A allele plus smoking, and the –460 C allele plus smoking, were significant for both IHD and MI, although the AP for the +405 G allele plus smoking did not reach significance. Patients who had ever smoked but who did not carry a VEGFA risk allele, or those who carried a risk allele but did not smoke, did not have a significantly increased risk of having IHD or MI compared to the patients who neither smoked nor carried risk alleles.

Multivariate associations with IHD and MI. We carried out multivariate logistic regression analysis using models containing the VEGFA risk allele/smoking interaction terms as well as other known clinical risk factors such as hypertension, hypercholesterolemia, diabetes, ESR, CRP, and risk factors associated with RA (RF, anti-CCP, nodular disease). The "best" models obtained from forward selection analyses are shown in Table 6. For both IHD and MI, independent associations were found with the VEGFA–2578 A/smoking interaction term, hypercholesterolemia, and male sex. The

Table 3. Frequency of ischemic heart disease and myocardial infarction in RA patients stratified by VEGFA SNP genotypes. Values are n (%).

	Ischemic Heart Disease			Myocardial Infarction		
	Negative	Positive (%)	OR (95% CI)	Negative	Positive (%)	OR (95% CI)
VEGFA–2578 (A/C)						
CC	98	9 (8.4)	1.0 (referent)	105	2 (1.9)	1.0 (referent)
AC	154	47 (23.4)	3.19 (1.52–6.69)	167	34 (16.9)	8.69 (2.35–32.10)
AA	88	22 (20.0)	2.63 (1.17–5.93)	100	10 (9.1)	4.41 (1.08–17.99)
A allele	242	69 (22.2)	2.97 (1.45–6.09)	267	44 (14.1)	7.02 (1.93–25.60)
VEGFA–460 (T/C)						
TT	93	11 (10.6)	1.0 (referent)	100	4 (3.8)	1.0 (referent)
CT	165	45 (21.4)	2.23 (1.15–4.48)	179	31 (14.8)	3.91 (1.42–10.84)
CC	82	22 (21.2)	2.22 (1.03–4.79)	93	11 (10.6)	2.75 (0.89–8.47)
C allele	247	67 (21.3)	2.22 (1.14–4.33)	272	42 (13.4)	3.48 (1.28–9.45)
VEGFA+405 (C/G)						
CC	36	4 (10.0)	1.0 (referent)	39	1 (2.5)	1.0 (referent)
CG	146	32 (18.0)	1.80 (0.63–5.14)	159	19 (10.7)	3.22 (0.59–17.57)
GG	158	42 (21.0)	2.17 (0.77–6.13)	174	26 (13.0)	3.99 (0.74–21.47)
G allele	304	74 (19.6)	1.98 (0.72–5.46)	333	45 (11.9)	3.59 (0.68–18.89)
VEGFA+936 (T/C)						
TT	11	3 (21.4)	1.0 (referent)	13	1 (7.1)	1.0 (referent)
CT	89	14 (13.6)	0.53 (0.14–1.99)	95	8 (7.8)	0.80 (0.13–4.98)
CC	240	61 (20.3)	0.84 (0.24–2.87)	264	37 (12.3)	1.28 (0.23–7.13)
—						

Table 4. Frequency of VEGFA haplotypes in RA patients with and without ischemic heart disease (IHD) or myocardial infarction (MI).

VEGFA Haplotype (–2578/–460/+405)	All Patients EM Frequency††	IHD Negative EM Frequency††	IHD Positive EM Frequency††	IHD OR (95% CI)	MI Negative EM Frequency††	MI Positive EM Frequency††	MI OR (95% CI)
A/C/G	0.483	0.469	0.544	2.37 (1.22–4.62)†	0.476	0.541	4.10 (1.45–11.59)†
C/T/C	0.297	0.309	0.242	0.73 (0.49–1.09)*	0.307	0.217	0.62 (0.37–1.04)*
C/T/G	0.182	0.190	0.147	0.74 (0.46–1.20)*	0.185	0.161	0.85 (0.47–1.53)*
Rare	0.038						

†† Frequency estimated by the expectation/maximization algorithm. Maximum EM iteration = 500, EM convergence tolerance = 0.0001, frequency threshold = 0.05. Best odds ratio achieved in † dominant model, * additive model.

Table 5. Association of VEGFA polymorphisms and ever having smoked with ischemic heart disease (IHD) and myocardial infarction (MI) in patients with RA. Values are n (%).

	IHD Negative	IHD Positive (%)	IHD OR (95% CI)	MI Negative	MI Positive (%)	MI OR (95% CI)
Smoke/VEGFA–2578 A						
–/–	38	2 (5.0)	1.0 (referent)	40	0 (0.0)	1.0 (referent)
–/+	91	9 (9.0)	1.59 (0.38–6.77)	96	4 (4.0)	3.77 (0.26–17.15)
+/–	60	7 (10.4)	1.91 (0.43–8.45)	65	2 (3.0)	3.09 (0.14–11.84)
+/+	151	60 (28.4)	6.15 (1.65–22.87)	171	40 (19.0)	19.13 (1.84–101.7)
			AP 0.63, 95% CI 0.27–0.98			AP 0.76, 95% CI 0.52–0.99
Smoke/VEGFA–460 C						
–/–	36	2 (5.3)	1.0 (referent)	38	0 (0.0)	1.0 (referent)
–/+	93	9 (8.8)	1.48 (0.35–6.29)	98	4 (3.9)	3.52 (0.24–15.96)
+/–	57	9 (13.6)	2.41 (0.56–10.32)	62	4 (6.1)	5.54 (0.37–25.19)
+/+	154	58 (27.4)	5.53 (1.52–20.61)	174	38 (17.9)	16.99 (1.63–90.12)
			AP 0.54, 95% CI 0.07–1.00			AP 0.52, 95% CI 0.13–0.91
Smoke/VEGFA+405 G						
–/–	18	1 (5.3)	1.0 (referent)	19	0 (0.0)	1.0 (referent)
–/+	111	10 (8.3)	1.16 (0.20–6.89)	117	4 (3.3)	1.49 (0.07–6.64)
+/–	18	3 (14.3)	2.33 (0.31–17.56)	20	1 (4.8)	2.85 (0.05–7.61)
+/+	193	64 (24.9)	4.11 (0.76–22.23)	216	41 (16.0)	7.48 (0.69–38.96)
			AP 0.57, 95% CI –0.28 to 1.43			AP 0.56, 95% CI –0.02 to 1.14

AP: the attributable proportion due to interaction.

Table 6. Multivariate stepwise logistic regression analysis of variables associated with ischemic heart disease (IHD) and myocardial infarction (MI).

Variable	IHD Regression Coefficient	IHD OR (95% CI)	IHD p	Variable	MI Regression Coefficient	MI OR (95% CI)	MI p
Smoke* + VEGFA–2578 A (+/–)	1.259	3.52 (1.84–6.72)	0.0001	Smoke* + VEGFA–2578 A (+/–)	1.962	7.11 (2.57–19.72)	0.0002
Diabetes† (+/–)	1.511	4.53 (1.84–11.19)	0.001	Hypercholesterolemia (+/–)	1.160	3.19 (1.43–7.10)	0.004
CRP ≥ 10 mg/l (+/–)	1.084	2.96 (1.58–5.55)	0.0007	Male (+/–)	1.086	2.96 (1.41–6.21)	0.004
Hypercholesterolemia (+/–)	1.043	2.84 (1.44–5.58)	0.002	Hypertension (+/–)	1.036	2.82 (1.30–6.11)	0.009
Male (+/–)	0.600	1.82 (1.00–3.30)	0.048	Methotrexate use (+/–)	–0.764	0.47 (0.22–0.97)	0.040
Age, per year	0.029	1.03 (0.99–1.06)	0.062	Rheumatoid factor (+/–)	0.844	2.33 (1.002–5.40)	0.049
Hypertension (+/–)	0.544	1.72 (0.93–3.18)	0.082	CRP ≥ 10 mg/l (+/–)	0.672	1.96 (0.90–4.27)	0.090
Methotrexate use (+/–)	–0.575	0.56 (0.32–0.99)	0.096				

* Patients who have ever smoked and carry a VEGFA–2578 A allele, compared with all remaining patients. † Type 1 or type 2 diabetes.

presence of diabetes and CRP (≥ 10 mg/l) was associated with IHD, while hypertension was associated with MI. RF was also associated with MI, but not with IHD. Methotrexate use was negatively associated with MI, and showed a trend towards negative association with IHD. Variables not significant in these models were the VEGFA–460 C/smoking and VEGFA+405 G/smoking interaction terms, anti-CCP status, ESR, and nodular disease.

DISCUSSION

The results of our study indicate that SNP in the VEGFA promoter region (–2578 and –460) are associated with an increased risk of IHD, and particularly MI, in patients with RA. Data also suggested that another SNP (+936), in the 3'-UTR of the VEGFA gene, was not involved in this association. Strong linkage disequilibrium across the SNP including –2578, –460, and +405, but excluding +936, was observed in this study.

The VEGFA–2578 polymorphism showed the strongest association with IHD and MI. This suggests that the –2578 SNP may provide the primary association in which the A allele increased the risk and/or the C allele played a protective role. These results are consistent with the findings of Howell, *et al*[20], who showed that the –2578 AA genotype was a risk factor, while the CC genotype was protective in the development of atherosclerosis. Similarly, an association between the –2578 AA genotype and the severity of coronary artery disease has been shown in a Brazilian population[25]. Other studies have indicated that the –2578 CC genotype is associated with higher production of VEGF than –2578 AA, and that haplotypes including the –2578 A allele are associated with lower circulating levels than those that include the –2578 C allele[45,46]. It is interesting that in the study by Lambrechts, *et al*[46] the association between VEGFA haplotypes and plasma level of VEGF was only significant in patients with amyotrophic lateral sclerosis, and not in healthy spouses of these patients. Similarly, a study by Prior, *et al*[36] in healthy individuals found no association between plasma levels of VEGF and a haplotype containing the –2578 and +405 polymorphisms. These findings raise the possibility that conditions that arise during the disease state (e.g., inflammation, hypoxia) may have a differential effect on gene expression of particular VEGFA alleles. Our own preliminary findings on serum levels in RA patients with different genotypes are consistent with this (unpublished observations). We find that patients carrying the –2578 CC genotype have the highest serum levels of VEGF, which adds weight to the suggestion that this genotype is associated with a protective effect due to greater production of VEGF[20].

It is particularly notable that the A allele of VEGFA–2578 has an insertion of 18 nucleotides with the sequence 5'-TCC CAC TCT TCC CAC AGG-3' (accession number AF098331) starting from –2549 relative to the translation start site[47]. This unusual insertion could be responsible for the increased risk associated with this allele, so it would be of great interest to investigate the functional importance of this insertion further.

The upstream region of VEGFA is highly polymorphic. Numerous SNP have been discovered across the promoter and 5'-untranslated regions[33,47]. Epidemiological research has focused mainly on VEGFA SNP with good minor/major allele proportions such as VEGFA–2578, –460, and +405. All these SNP have been suggested as functional polymorphisms associated with protein expression and/or certain disease conditions[32,33,34,35,36,37]. From our results, it appears that the VEGFA–2578 polymorphism, or the associated 18-nucleotide insertion, plays a crucial role in relation to the risk of IHD/MI in RA. The association of other polymorphisms described here may be due to the linkage with the –2578 SNP. Alternatively, a number of polymorphisms within the promoter and 5'-UTR may be involved, including rare polymorphisms that have not been studied.

We further observed a strong interaction between VEGFA SNP (–2578, –460) and smoking relative to IHD and especially MI. Our data suggested that the strongest interaction was between the VEGFA–2578 A allele and smoking, and it is particularly noteworthy that the risk of IHD or MI was increased only in patients carrying this combination. Further, > 60% of the "excess" risk for IHD and > 75% of the "excess" risk for MI were directly attributable to gene-smoking interaction. In the absence of the –2578 A allele there was no significant association between smoking and IHD or MI, suggesting a possible protective effect of the –2578 CC genotype.

The kind of interaction demonstrated here has been described before in relation to the increased risk of esophageal adenocarcinoma[48]. There is evidence that the combination of VEGF and certain chemical constituents in cigarette smoke may be important in particular biological pathways related to angiogenesis, atherosclerosis, and inflammation. Studies on nicotine have demonstrated that it can induce VEGF expression via several pathways involving nicotinic acetylcholine receptors[49,50], αvß3 integrin[50], and angiotensin-converting enzyme[51]. In contrast, cigarette smoke exposure has been shown to inhibit VEGF expression through decreased expression of hypoxia-inducible factor-1α (HIF-1α), resulting in impaired angiogenesis[52]. Further, it has been hypothesized that the ability of the coronary artery collateral circulation to protect against myocardial ischemia is strongly associated with the ability to induce VEGF in response to hypoxia[53]. More recently it has been demonstrated that haplotypes that include the –2578 and +405 SNP influence VEGF expression in human myoblasts under hypoxic conditions[36]. Given the importance of the VEGFA promoter region in the hypoxia response[31,32,54], we speculate that the association of IHD/MI with the VEGFA gene-smoking interaction in our study might be explained by a mechanism involving differential responses to hypoxia.

There are some limitations to this study. First, the number of patients with IHD and/or MI was relatively small, so further studies, preferably with a larger number of cases, will be needed to confirm these results. Second, the associations of VEGFA polymorphisms with IHD and MI were examined only in an RA population. We do not know therefore whether similar associations exist in the general popu-

lation or whether this is somehow related to the disease process in RA. Several publications have suggested an association of VEGFA polymorphisms with certain cardiovascular disorders[20,21,22,23,24,25,26,55], but as far as we aware none have looked at interaction between smoking and VEGFA polymorphisms. Further studies in other RA and non-RA patients are therefore needed. Another limitation is the possibility that some patients with silent, non-full-thickness, or atypical MI were missed. However, we were able to identify 13 patients with previously unrecognized MI who had ECG evidence of a full-thickness MI. In a small number of patients (n = 8) there was a bundle branch block severe enough to prevent assessment. In the analyses these were classed as not having IHD. However, exclusion of these patients made no significant difference to the associations found.

Finally, the study was cross-sectional in design, so it was possible to assess only patients who had survived previous cardiac events. However, the patients recruited to this study are being followed over a 5-year period, so future studies will be able to assess both fatal and nonfatal cardiac events, as well as further development of IHD in this population.

ACKNOWLEDGMENT

We thank Michelle Kirwan, Ann Barcroft (research nurses), and Janet Turner (research assistant) for their help with data collection and entry, and Dr. John Glossop for critical review of the manuscript.

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