original paper

The IL-6 G-174C polymorphism may be associated with ischaemic stroke in patients without a history of hypertension

J Balding[1], WJ Livingstone[1], SJ Pittock[2], L Mynett-Johnson[3], T Ahern[1], A Hodgson[1], OP Smith[4]
Thrombosis and Haemostasis Laboratory[1], Institute of Molecular Medicine, Trinity Centre for Health Sciences, St James's Hospital, Department of Neurology[2], Beaumont Hospital, Department of Genetics, Trinity College, Department of Haematology[4], Our Lady's Hospital for Sick Children, Dublin, Ireland

Abstract

Background Recent data suggest that inflammatory reactions are involved in the pathogenesis of cerebral ischaemia.

Aim To investigate whether certain inflammatory genetic polymorphisms are associated with the occurrence of ischaemic stroke.

Methods We investigated the prevalence of six polymorphisms in cytokine genes (IL-6, TNF-α, TNF-β, IL-1β, IL-10, and IL-1Rα) in a group of ischaemic stroke patients (n=105) and in a control population (n=389). We analysed the prevalence of these polymorphisms in different stroke subtypes and in relation to outcome six months post-stroke.

Results There was no significant variation in cytokine gene polymorphism frequencies between control and stroke populations or for different stroke subtypes. Subgroup analysis demonstrated that the prevalence of the IL-6 -174 CC genotype was significantly lower in stroke patients without a history of hypertension compared to controls.

Conclusion The IL6 -174 CC genotype may be protective against stroke in those patients who have no history of hypertension. Further studies are required to verify these findings.

Introduction

Ischaemic stroke is a major complication of atherosclerotic cardiovascular disease, resulting in considerable morbidity and mortality.[1] Traditional risk factors such as hypertension, diabetes, smoking and obesity do not fully account for individual risk.[2,3] Inflammatory reactions have been implicated in increasing the risk of both stroke and myocardial infarction (MI), and the expression levels of the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β have been found to be increased following acute stroke.[4-8] This is possibly linked to the loss of anti-inflammatory and anti-coagulant action caused by low levels of circulating activated protien C (APC) that have been detected in stroke patients compared to both neurological controls and community control subjects.[4] Lower circulating APC levels have also been associated with positive infection/inflammation status in both stroke and control groups.[4] Inflammatory mediators such as IL-1 and TNF are known to reduce thrombomodulin from the endothelial cell surface, resulting in downregulation of the thrombomodulin-dependent protein C activating capacity; a process that most likely contributes to inflammation-mediated disseminated intravascular coagulation.[9,10] Therefore, it appears that increased pro-inflammatory cytokine levels and inflammation may contribute to lowered APC levels and a predisposition to stroke.

Given the observations that levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 are elevated in ischaemic stroke,[6-8] we investigated whether polymorphisms in genes encoding cytokines play a role in the pathophysiology of ischaemic stroke. A number of the polymorphisms studied have been previously reported to have functional effects on the levels of the protein that they encode and have also been associated with various inflammatory and/or autoimmune diseases.[11]

Materials and methods

Patients and controls

EDTA anti-coagulated whole blood was prospectively collected from a consecutive cohort of ischaemic stroke patients (n=105; male 60%, female 40%; mean age of stroke 69 years; range (35-99 years) admitted to a Dublin City teaching hospital over a nine-month period. The study was approved by the hospital ethical committee and consent obtained from all participating subjects and controls. Stroke was defined as acute onset of neurological deficit lasting more than 24 hours or leading to death with no apparent cause other than cerebrovascular disease.

The diagnosis of stroke was based on history and examination. Transfers from other hospitals and patients with acute intracerebral haemorrhage, subdural, extradural or subarachnoid haemorrhage were excluded on the basis of cranial imaging. Patients were allocated to one of four stroke subtypes (Oxfordshire Community Stroke Project [OCSP]) and level of handicap assessed by a study neurologist at three time points within 48 hours of admission and at two weeks and six months using the Oxford handicap scale also known as the modified Rankin Disability Scale, an observer-rated, global measure of handicap assessing any limitation in the patients social role. It is rated from 0 (no symptoms) to 5 (severe handicap, totally dependent, requiring constant attention night and day) and has been shown to have good interrater reliability.[12,13]

EDTA anti-coagulated whole blood was obtained from 389 healthy blood donors (male 58%, female 42%, mean age 37.1, range 18-65 years) collected from The Northern Ireland Blood Transfusion Service (n=60) and The Blood Transfusion Service Board (Dublin and Cork and mobile units throughout the Republic of Ireland) (n=329).

DNA isolation

DNA was extracted from whole blood using overnight proteinase K (1mg/ml) cell lysis at 37°C in the presence of 0.5% sodium dodecyl sulphate followed by phenol/chloroform extraction and ethanol precipitation.

PCR, restriction enzyme digestion and agarose gel electrophoresis

PCR amplification of all polymorphic sites was performed in a 50µl total volume. The standard reaction mix consisted of Taq DNA Polymerase buffer with $MgCl_2$ (Promega, Madison, WI, USA) (50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 1.5mM $MgCl_2$), 0.4 units of DNA Taq polymerase, 2µl of genomic DNA, 4% dimethyl sulphoxide (DMSO), 30µM each of deoxyribonucleoside triphosphates, and 0.2µM each of sense primer and antisense primer. Primer sequences, cycling parameters for each assay, changes to the standard PCR reaction mix, restriction digestion conditons, and agarose gel electrophoresis conditions were as previously published.[11]

Statistical analysis

Allele and genotype frequencies were compared between patient and control groups, and between patient sub-populations, by means of the chi-squared test and Fisher's exact test when appropriate. A χ^2 p value of <0.05 was considered statistically significant. Statistics were performed using Statview software (Statview version 4.5, Abacus Concepts Inc, Berkeley, CA, USA).

Results

Of 117 consecutive ischaemic stroke patients enrolled in the study, 105 consented to give blood for genetic analysis. The demographic and clinical characteristics of this study population are shown in Table 1. The mean age of the cohort was 69 years (SD 12.6) and the male to female ratio was 1.5:1. There were no statistical differences in respect of gender for any of the clinical characteristics studied.

Table 1. Patient demographics, pre-stroke function, risk factors, and stroke subtyping

Risk factors	n	(%)
Any prior cerebrovascular event	38	(36)
Previous cerebrovascular accident (CVA)	25	(24)
Previous transient ischaemic attack (TIA)	27	(26)
Smokers (at any time)	66	(63)
Hypertension	48	(46)
Previous arterial event 33 (31)		
Pre-stroke function		
Oxford handicap scale (OHS) <2	82	(78)
Oxford handicap scale (OHS) ≥ 2	23	(22)
Stroke subtype		
Total anterior circulation infarcts (TACI)	29	(28)
Lucunar circulation infarcts (LACI)	40	(38)
Partial anterior circulation infarcts (PACI)	23	(22)
Posterior circulation infarct (POCI)	13	(12)
Rankin disability scale (6 months post event)		
0 No symptoms	15	(12)
1 Minor symptoms	16	(13)
2 Some restriction	24	(20)
3 Significant restriction	14	(11)
4 Severe handicap	10	(8)
5 Severe handicap – totally dependent	5	(4)
6 Dead	20	(16)

Genotype frequencies for all six polymorphisms in the control group, stroke population, and subgroups of the stroke population (previous arterial event, smoker, ex-smoker, non-smoker, and those suffering from hypertension) are listed in Table 2. No significant variation between the control and stoke populations was identified. Moreover, there was no significant difference found for any of these polymorphisms and stroke subtypes as defined by the OCSP. The subgroups of level of handicap (Rankin Disability Scale) were too numerous and thus contained too few individuals to determine any association with genotype. The prevalence of IL-6 -174 G allele carriers was higher in the group of stroke patients with no history of hypertension compared to those with hypertension (98% vs 77%, p=0.0009 'no hypertension' vs 'hypertension'), and compared to the control group (98% vs 83%, p=0.0027 'no hypertension' vs 'controls'). No other significant associations were observed amongst the different groups within the stroke population, although the A allele of the TNF-α 308 polymorphism was increased in the group of stroke patients who have suffered a previous arterial event (p=0.07).

Discussion

Stroke is a complex disease with multiple risk factors. These include advanced age, smoking, hypertension, diabetes, coagulation disorders, low apo E levels, and abnormal cholesterol levels.[14] Several of the risk factors for stroke such as diabetes, hypertension, and coagulation disorders, are likely to be under genetic influence. Moreover, twin and family studies give evidence for a genetic background to stroke, and it has been found that significantly more patients who suffer a stroke have a family history of stroke or heart disease compared to age- and sex-matched controls.[15-17] However, many subjects with one or more of the risk factors mentioned above do not develop stroke, and some patients who suffer a stroke do not have these risk factors, indicating that other factors may be involved in the pathophysiology of ischaemic stroke. With this in mind, and with an increasing amount of evidence for the involvement of pro-inflammatory reactions in the pathophysiology of ischaemic stroke,[4-8] the prevalence of a range of polymorphisms in cytokine genes in a stroke population and in a large control population was investigated.

No substantial link between any polymorphism in this study and the occurrence of stroke could be established. Of note, however, a significant increase in the frequency of the IL-6 –174 G allele carriers in the group of ischaemic stroke patients with no hypertension was detected compared to those with hypertension and compared to controls. Thus the IL-6 -174 polymorphism may be associated with stroke when patients with hypertension, a known stroke risk factor, are removed from the study group. This G to C transition in the IL-6 gene is in an area of the promoter region thought to have a negative effect on gene transcription. Fishman et al found that the IL-6 -174 CC genotype is associated with low levels of IL-6 in normal controls, and is at a reduced frequency in young systemic-onset juvenile chronic arthritis patients, indicating a protective role in this disease, whilst the G allele was associated with increased IL-6 plasma level.[18]

Reports on the functionality of this polymorphism since the study by Fishman et al have been varied. Nauck et al found no difference in plasma IL-6 concentration between groups with different IL-6 genotypes in 942 individuals with coronary artery disease.[19] Schluter et al found no association between serum IL-6 levels and genotype in sepsis patients.[20] Bonafe et al looked at serum levels in 700 individuals aged 60 to 110 years of age and

Table 2. Genotype counts (%) for cytokine polymorphisms in a control population and a stroke patient population

Polymorphism	Genotype	Control group (n=389)	Stroke (n=105)	Previous arterial event (n=33)	Smoker (n=38)	Ex-smoker/ non-smoker (n=66)	Hypertension (n=48)	No hyperten -sion (n=55)
IL-6-174	GG	123 (32)	33 (32)	13 (39)	13 (34)	20 (30)	15 (31)	18 (33)
	GC	198 (51)	60 (57)	14 (43)	21 (55)	38 (58)	22 (46)	36 (65)
	CC	68 (17)	12 (11)	6 (18)	4 (11)	8 (12)	11 (23)[a]	1 (2)[a]
TNFB	B1B1	52 (13)	16 (15)	6 (18)	6 (16)	10 (15)	5 (11)	11 (20)
	B1B2	205 (53)	53 (51)	14 (43)	20 (53)	32 (49)	28 (58)	24 (44)
	B2B2	132 (34)	36 (34)	13 (39)	12 (31)	24 (36)	15 (31)	20 (36)
TNF-α-308	GG	233 (60)	59 (56)	15 (45)	18 (47)	41 (62)	25 (52)	33 (60)
	GA	140 (36)	40 (38)	15 (45)	17 (45)	22 (33)	21 (44)	18 (33)
	AA	16 (4)	6 (6)	3 (10)	3 (8)	3 (5)	2 (4)	4 (7)
IL-10-592	CC	235 (60)	65 (62)	21 (64)	22 (58)	43 (65)	27 (56)	38 (69)
	CA	139 (36)	35 (33)	11 (33)	13 (34)	21 (32)	21 (44)	12 (22)
	AA	15 (4)	5 (5)	1 (3)	3 (8)	2 (3)	0 (0)	5 (9)
IL-1β+3953	CC	240 (62)	66 (63)	24 (73)	22 (58)	43 (65)	32 (67)	32 (58)
	CT	125 (32)	35 (33)	7 (21)	16 (42)	19 (29)	15 (31)	20 (36)
	TT	24 (6)	4 (4)	2 (6)	0 (0)	4 (6)	1 (2)	3 (6)
IL-1RN	A1A1	183 (47)	49 (47)	14 (43)	17 (45)	32 (48)	22 (46)	27 (49)
VNTR	A1A2	159 (41)	41 (39)	15 (45)	16 (42)	25 (38)	19 (40)	21 (38)
	A1A3	15 (4)	6 (6)	2 (6)	4 (10)	1 (2)	2 (4)	3 (6)
	A2A2	31 (8)	9 (9)	2 (6)	1 (3)	8 (12)	5 (10)	4 (7)

n=number of individuals.

found an association between levels and genotype only in males.[21] Burzotta et al looked at 111 patients with coronary artery disease and found an association between the GG genotype and higher plasma IL-6.[22] GG homozygosity was associated with longer stays in the intensive care unit and in the hospital in patients with multivessel coronary artery disease undergoing elective coronary artery bypass graft surgery, with rates of postoperative death, myocardial infarction and stroke at 8% in GG carriers and 2% in C allele carriers, whilst the GG genotype is significantly more common in multi-infarct dementia patients compared to controls.[22,23]

The expression levels of circulating IL-6 have been found to be increased following acute stroke[6], and thus the putative lower levels of IL-6 associated with the C allele may be protective in individuals who do not have the known stroke risk factor hypertension, whilst the higher levels thought to be associated with the GG genotype may be a risk factor for stroke. Alternatively, this polymorphism may be in linkage disequilibrium with a functional disease marker located nearby in the genome, rather than directly causing a change in IL-6 levels.

To conclude, the incidence of stroke was not significantly associated with any of the cytokine polymorphisms studied, although the IL-6 polymorphism may be associated with stroke when patients with the known risk factor hypertension are removed from the study group. A larger study is needed to confirm this association. A larger patient cohort is also required to investigate further the association of inflammatory polymorphisms and stroke subtype in order to overcome the lack of statistical power when the patient population is split into smaller subtypes. Despite the evidence for the involvement of inflammation in the pathophysiology of stroke, there is little evidence as yet that inflammatory polymorphisms are a risk factor. Other genetic risk factors, such as ones in the coagulation and haemostasis systems are being investigated,[24] and it is possible that polymorphisms in this area could be of greater importance in the occurrence of stroke than polymorphisms of the inflammatory system.

References

1. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362: 801-9.
2. Anderson KM, Wilson PW, Odell PM et al. An updated coronary risk profile. A statement for health professionals. *Circulation* 1991; 83: 356-62.
3. Heller RF, Chinn S, Pedoe HD et al. How well can we predict coronary heart disease? Findings in the United Kingdom Heart Disease Prevention Project. *Br Med J* (Clin Res Ed) 1984; 288: 1409-11.
4. Macko RF, Ameriso SF, Gruber A et al. Impairments of the protein C system and fibrinolysis in infection-associated stroke. *Stroke* 1996; 27: 2005-11.
5. Roivainen M, Viik-Kajander M, Palosuo T et al. Infections, inflammation, and the risk of coronary heart disease. *Circulation* 2000; 101: 252-7.
6. Kim JS, Yoon SS, Kim YH et al. Serial measurement of interleukin-6, transforming growth factor-beta, and S-100 protein in patients with acute stroke. *Stroke* 1996; 27: 1553-7.
7. Liu T, McDonnell PC, Young PR et al. Interleukin-1 beta mRNA expression in ischemic rat cortex. *Stroke* 1993; 24: 1746-50.
8. Liu T, Clark RK, McDonnell PC et al. Tumor necrosis factor-alpha expression in ischemic neurons. *Stroke* 1994; 25: 1481-8.
9. Esmon CT, Taylor FB, Jr, Snow TR. Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C. *Thromb Haemost* 1991;

66: 160-5.
10. Taylor FB, Jr., Chang A, Esmon CT et al. Protein C prevents the coagulopathic and lethal effects of *Escherichia coli* infusion in the baboon. *J Clin Invest* 1987; 79:918-25.
11. Balding J, Livingstone WJ, Conroy J et al. Inflammatory bowel disease: the role of inflammatory cytokine gene polymorphisms. *Mediators Inflamm* 2004; 13: 181-7.
12. Bamford JM, Sandercock PA, Warlow CP et al. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke* 1989; 20: 828.
13. Bamford J, Sandercock P, Dennis M et al. Classification and natural history of clinically identifiable subtypes of cerebral infarction. *The Lancet* 1991; 337: 1521-6.
14. Rastenyte D, Tuomilehto J, Sarti C. Genetics of stroke — a review. *J Neurol Sci* 1998; 153: 132-45.
15. Brass LM, Isaacsohn JL, Merikangas KR et al. A study of twins and stroke. *Stroke* 1992; 23: 221-3.
16. Kiely DK, Wolf PA, Cupples LA et al. Familial aggregation of stroke. The Framingham Study. *Stroke* 1993; 24: 1366-71.
17. Graffagnino C, Herbstreith MH, Roses AD et al. A molecular genetic study of intracerebral hemorrhage. *Arch Neurol* 1994; 51: 981-4.
18. Fishman D, Faulds G, Jeffery R et al. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene onIL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *J Clin Invest* 1998; 102: 1369-76.
19. Nauck M, Winkelmann BR, Hoffman MM et al. The interleukin-6 G(-174)C promoter polymorphism in the LURIC cohort: no association with plasma interleukin-6, coronary artery disease, and myocardial infarction. *J Mol Med* 2002; 80: 507-13.
20. Schluter B, Raufhake C, Erren M et al. Effect of the interleukin-6 promoter polymorphism (-174 G/C) on the incidence and outcome of sepsis. *Crit Care Med* 2002; 30: 32-7.
21. Bonafe M, Olivieri F, Cavallone L et al. A gender-dependent genetic predisposition to produce high levels of IL-6 is detrimental for longevity. *Eur J Immunol* 2001; 31: 2357-61.
22. Burzotta F, Iacoviello L, Di Castelnuovo A et al. Relation of the -174 G/C polymorphism of interleukin-6 to interleukin-6 plasma levels and to length of hospitalisation after surgical coronary revascularization. *Am J Cardiol* 2001; 88: 1125-8.
23. Pola R, Gaetani E, Flex A et al. -174 G/C interleukin-6 gene polymorphism and increased risk of multi-infarct dementia: a case-control study. *Exp Gerontol* 2002; 37: 949-55.
24. Tatlisumak T, Fisher M. Hematologic disorders associated with ischemic stroke. *J Neurol Sci* 1996; 140: 1-11.

Correspondence to: Professor OP Smith, Department of Haematology, Our Lady's Hospital for Sick Children, Crumlin,
Dublin 12.Tel: 01 4096055; email: owen.smith@olhsc.ie