Carotid artery atherosclerosis

Polymorphisms in endothelial nitric oxide synthase and carotid artery atherosclerosis

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The development of vascular diseases generally depends on both environmental and genetic factors.[1] The precise mechanism or mechanisms by which genetic factors promote atherosclerotic lesion formation is still under investigation. The natural history of atherosclerosis-related diseases is further complicated by a plethora of oxidation-sensitive mechanisms and pathogenic events that occur in the earliest stages of life.[2] Nitric oxide (NO), a potent vasodilator produced by endothelial cells, plays an important part in the regulation of blood pressure and regional blood flow, inhibits platelet aggregation and leucocyte adhesion to vascular endothelium, and inhibits vascular smooth-muscle-cell proliferation.[3]

NO is generated by the endothelial nitric oxide synthase (eNOS) gene (*NOS3*), which is highly polymorphic. Specifically, NO is constitutively generated from the conversion of L-arginine to L-citrulline by the enzymatic action of eNOS. Historically, eNOS was classified as a constitutively expressed enzyme regulated by calcium and calmodulin. In the past 5 years, it has become clear, however, that eNOS activity and NO release can be regulated by post-translational control mechanisms (fatty-acid modification and phosphorylation) and protein–protein interactions (with caveolin-1 and heat shock protein 90), which directly impinge on the duration and magnitude of NO release.[4 5] eNOS is a membrane-associated NOS isoform that is modified by co-translational N-myristoylation at glycine 2 and post-translational cysteine palmitoylation at positions 15 and 26, and these fatty acids are important for its targeting in the Golgi region and plasmalemmal caveolae. The proper localisation of eNOS is necessary for its interactions with other regulatory proteins (scaffolds, chaperones, kinases) that fine-tune the cycles of eNOS activation and inactivation. The major negative regulatory protein for eNOS is caveolin-1. Caveolin-1 is the major coat protein of caveolae, and has several faces that may influence the biology of proteins that localise to cholesterol-rich plasmalemma caveolae. Caveolin-1 can serve as a cholesterol binding protein, and traffic cholesterol from the endoplasmic reticulum through the Golgi to the plasma membrane. The primary binding region of caveolin-1 for eNOS is within amino acids 60–101, and to a lesser extent within amino acids 135–178. Furthermore, the caveolin–eNOS immunocomplex is disrupted in the presence of caveolin scaffolding peptides (amino acids 82–101). NO is known to have a multitude of beneficial effects against atherosclerosis.[4 5] This concept promoted an intensive investigation to understand whether *NOS3* gene polymorphisms influence predisposal to atherosclerosis, atherosclerotic lesion progression or both. Here, we discuss the current status of the science and clinical evidence in this area. A search for original articles focusing on polymorphisms, and eNOS and carotid artery atherosclerosis was done in Medline and PubMed, which were published between 1990 and 2005. The search terms used were eNOS, atherosclerosis and polymorphism. All papers identified were in English, and were full-text papers. We also searched the reference lists of identified articles for further papers.

HERITABILITY

The metric commonly used to summarise the familial and genetic nature of a trait is heritability (H, h2 or 2G/2P). The heritability of a trait is the proportion of variation in the trait between individuals (2P) attributable to genetic variation (2G). Heritability of a trait is a population-specific and environment-specific parameter, and its value, high or low, does not indicate the role of genes in any specific individual or patient. Heritability does, however, allow prediction of the expected degree of familial aggregation of a trait; traits with high heritability values should prove useful in the identification of trait-related genes.

CAROTID ARTERY LESION PROGRESSION

Non-invasive evaluation of intimal–medial thickening (IMT) is easy in the carotid artery by use of B-mode ultrasonography that has a graded, predictive relationship to overt clinical disease.[6 7] Carotid plaque is a focal thickening of the carotid wall caused by atherosclerosis. Plaque definitions vary, and include focal thickening >50% of the surrounding wall,[8] focal widening with protrusion into the lumen,[9] localised IMT of at least 0.75–1.5 mm[10 11] and focal acceleration of flow.[12] Carotid atherosclerosis is also typically defined as thickening greater than a given threshold, whether focal or not.[13] Other phenotypes related to carotid atherosclerosis might include plaque echogenicity (fibrotic plaques) or echolucency (lipid deposition, intraplaque haemorrhage and vulnerability to rupture).[14]

Plaque as a carotid atherosclerosis phenotype is not studied as frequently as carotid IMT. The heritability of plaque determined by localised IMT of 1.5 mm was estimated at 23–28%.[10] Carotid plaque has also been reported to be more strongly related to parental death from early parental coronary heart disease than to IMT.[15] Similar candidate genes probably influence atherosclerotic lesion formation and IMT.

ENOS POLYMORPHISMS AND CAROTID ATHEROSCLEROSIS

Several genetic variants have been examined in relation to carotid atherosclerosis, more often by association than by linkage analysis.[16 17] Most have genetic variants related to IMT as a continuous trait, although many have reported dichotomous carotid plaque measures derived from arbitrary threshold levels of IMT.

NOS exists in three forms: inducible, neuronal and endothelial.[3 4] eNOS is presumed to be responsible for most of the endothelial and vascular effects of NO. Several *NOS3* polymorphisms have been identified, one of which, 894T of exon 7, produces the substitution Glu298→Asp.[18] This variant induces a conformational change that is thought to reduce *NOS3* activity and has been associated with several effects: increased vasoconstrictive response to phenylephrine; increased blood pressure response to endurance training; and hypertension, coronary heart disease and myocardial infarction.[19 20]

A second polymorphism in the 5′ flanking region (786T→C) reduces *NOS3* gene promoter activity and has been associated with coronary spasm.[21] No relationship between the 894G→T variant and carotid atherosclerosis has been found in independent studies,[18 20–22] although one study did

show T homozygosity to be more frequent in individuals with plaque than in those without.[19] The 786T→C polymorphism was more frequent in carotid stenosis cases in the only study in which it has been investigated.[21] This is important since the same variant is involved in vasospasm and in carotid artery stenosis.

The intronic polymorphisms have not been widely studied,[19] although loss of heterozygosity for the intron 13 CA repeat has been detected in excised carotid plaques.[23] In patients undergoing haemodialysis, increased plaque frequency was noted in people with the 786T→C polymorphism in combination with *APOE*4*; the same interaction was found for a *NOS3* intron 4 polymorphism tightly linked to the 786T→C locus.[22] It has been investigated whether the 786T→C, 894G→T and 4a/4b variants of *NOS3* increase susceptibility to carotid atherosclerosis.[24] The study group was comprised of 304 (≥70%) patients with severe carotid stenosis and 544 controls. Results indicated that the genotype distribution and allele frequency of 4a/4b but not the 786T→C or 894G→T polymorphisms were significantly different between patients and controls. Logistic regression with adjustment for other risk factors showed that the 4a allele and the combined genotypes 4a4a+4a4b/894TT+GT and –786CC+TC/894TT+GT were associated with carotid stenosis (odds ratio (OR) 1.5, $p = 0.02$; OR 1.8, $p = 0.01$; OR 1.5, $p = 0.04$, respectively). The researchers concluded that the 4a allele and the eNOS-combined genotypes were independent predisposing factors to carotid atherosclerosis.

In another study, the possible role of *NOS3* variants as risk factors for early atherosclerosis was assessed, specifically, whether two polymorphisms located in exon 7, 298E→D and 786T→C, in the promoter region are associated with functional changes in the endothelium and carotid IMT.[25] Carotid IMT was assessed by high-resolution ultrasonography in 118 genotyped healthy young nonsmokers. With respect to 298E→D carriers, those with the Asp/Asp genotype displayed a significantly greater carotid IMT than those with Glu/Glu or Glu/Asp genotypes (Asp/Asp 0.45 (SD 0.03) mm; Glu/Glu 0.37 (SD 0.01) mm; Glu/Asp 0.35 (SD 0.01) mm, $p = 0.0002$). No difference in IMT was found across the 786T→C genotypes; by multivariate regression analysis, Asp/Asp genotype was found to be the only significant and independent predictor of IMT ($p = 0.006$). Thus, these data suggest that the amino acid substitution Glu298→Asp in carriers of the 298E→D polymorphism might be related to early carotid atherogenesis.

A study carried out among 2448 participants of the Study of Health in Pomerania and measuring the eNOS E298D polymorphism by 5-exonuclease assay was published recently.[26] The D/D298 genotype was associated with an increased risk of atherosclerotic plaques at the level of the common carotid arteries (multivariate OR 1.57, 95% CI 1.05 to 2.34, $p = 0.025$), yet neither were in the carotid bifurcations nor in the internal or external carotid arteries.[26] The D/D298 genotype was independently associated with both higher mean (adjusted increase by 0.046 mm, 95% CI 0.013 to 0.078, $p = 0.006$) and, more importantly, higher maximum carotid IMT (0.137 mm, 95% CI 0.064 to 0.209, $p>0.001$) in the low-risk control group of subjects without carotid lesions.[26] This finding strongly indicates that the association of the E298D genotype with atherosclerosis in the carotid arteries is site-specific, and is possibly modified by cardiovascular risk.

Taken together, the findings suggest that some eNOS polymorphisms might have independent roles in the pathogenesis or predisposal to atherosclerosis. Table 1 summarises studies implicating a possible role of eNOS gene variant in the development of vascular damage in the whole context of cardiovascular and cerebrovascular diseases.[27–38] Some studies depict a pathophysiological scenario, however, in which *NOS3* is just one of the players in a very complex disease.

PROBLEMS WITH INTERPRETATION AND APPLICATION OF DATA

Several issues, such as the following, could account for the discordance between the clinical studies evaluating *NOS3* polymorphisms in carotid atherosclerosis: sampling or random type I errors in positive studies; lack of power in negative studies; genetic heterogeneity; population stratification or confounding; gene–environment interactions modulating gene regulation and expression; and differences in the technical methods employed. By contrast, stimulation of inducible nitric oxide synthase and NO overproduction causes metabolic insulin resistance and characterises atherosclerosis, heart failure and cardiogenic shock in humans, suggesting a ''Yin–Yang'' effect of NO in the cardiovascular homeostasis.[39–43] Moreover, recent studies have demonstrated that neuronal NOS (nNOS) also exerts important vasculoprotective effects in vivo.[44] Indeed, in apolipoprotein E-knockout mice, deficiency of nNOS induced progression of aortic vascular lesion formation. In these models, nNOS was upregulated in vascular lesions, and was predominantly expressed in the neointima and medial smooth muscle cells.

Clinical evaluations of single-nucleotide polymorphisms are correlative and cannot show causal relationships. Novel activities in the large-scale purification of new, hitherto unknown proteins, and in the investigation of their structure and function will be initiated.[45] This approach will deepen our insights into vascular cell biology and biological evolution of a healthy vessel into a damaged artery. Furthermore, only a small portion of the total RNA transcribed in human cells becomes mature mRNA and constitutes the human transcriptome, which is context-dependent, and varies with development, physiology and pathology.[46] A small proportion of different repetitive sequences, which make up more than half of the human genome, is retained in mature transcripts and shapes their function.

Microarrays provide the opportunity to measure transcription from regions of the genome without bias towards the location of known genes.[47 48] The so-called tiling microarray experiments that assay transcription at regular intervals throughout the genome have shown evidence of large amounts of transcription outside the boundaries of known genes.[48] This transcription is observed in polyadenylated RNA samples and appears to be derived from intergenic regions, from introns of known genes and from sequences antisense to known transcripts.[48] The better understanding of these phenomena and the necessary multidisciplinary efforts will lead to new protein markers for medical diagnostics, to the identification of proteins as novel drug targets in the early treatment of vascular damage and atherosclerosis-related diseases.

Sequencing of the human genome has increased the potential for genetic information to aid in the prevention, diagnosis and treatment of common chronic diseases such as atherosclerosis-related diseases. Overall, progress in the recognition of genetic factors implicated in vascular diseases is now such that it poses the question of how to integrate these data into a clinical perspective. To be able to give the most relevant information to the patient and their family, and to use this information to optimise the medical management, have become new objectives. This can only be achieved with the tandem collaboration between the physician and the geneticist, who knows the legislative framework which governs the performance of genetic tests. The systematic collection and interpretation of family history information is currently the most appropriate screening approach to identify individuals with genetic susceptibility to atherosclerosis-related

Table 1 A selection of relevant studies showing the correlation between nitric oxide synthase (NOS) polymorphisms and cardiovascular events

Polymorphism	Clinical feature	Reference
G894T	Premature myocardial infarction	Antoniades *et al*,[28] 2006
G894T	Abdominal aortic aneurysm	Fatini *et al*,[27] 2005
T(−786)C	More advanced imbalance of autonomic activity in patients with congestive heart failure	Binkley *et al*,[29] 2005
Asp298	Coronary spastic angina	Ogimoto *et al*,[34] 2005
894 G→T	No evidence of a significant role in the development of CHD	Spence *et al*,[35] 2004
Glu298→Asp T(786)→C	Early atherosclerosis No correlation with early atherosclerosis	Paradossi *et al*,[25] 2004
4a/4a 4a4a/−786CC	ACS, higher predisposition to ACS	Fatini *et al*,[24] 2004
Glu298Asp T(786)→C intron-4	Increased risk of CHD, no significant association with CHD	Casas *et al*,[30] 2004
Glu298Asp	Coronary spasm	Chang *et al*,[31] 2003
Glu(298)→Asp T(786)→C Glu(298)→Asp/T(786) →C	Severe CHD higher risk	Colombo *et al*,[32] 2003
T(786)→C	Severe ICA	Ghilardi *et al*,[21] 2002
Glu298→Asp	Carotid atherosclerosis	Karvonen *et al*,[20] 2002
Asp298	Carotid atherosclerosis	Lembo *et al*,[19] 2001
[(CA), polymorphism] in intron 13	CHD	Stangl *et al*,[33] 2000
894 G→T	CHD and recent MI	Hingorani *et al*,[37] 1999
Glu(298) →Asp	Susceptibility to AMI	Hibi *et al*,[36] 1998
Glu298Asp	AMI	Shimasaki *et al*,[38] 1998
Glu298asp E298D genotype	Carotid atherosclerosis	Wolff *et al*,[26] 2005

ACS, acute coronary syndrome; AMI, acute myocardial infarction; CHD, coronary heart disease; ICA, internal carotid artery; MI, myocardial infarction.

diseases. During the genetic counselling consultation, and after compiling information on the disease and the family, the geneticist gives the most suitable information on the genetic components of the disease. Depending on the illness, instrumental investigation for the relatives could be advocated. The performance of a molecular test is then discussed, as a function of its feasibility, relevance and the wishes of the patient. The complexity of the medical and psychological issues varies according to the situation (presymptomatic diagnosis, prenatal diagnosis, diagnostic test, prognostic test) and the conditions. In all cases, strict rules clearly laid down by legislation must be respected, in such a way as to protect patients from possible unfavourable repercussions, and to assure them of better medical strategic management and a greater well-being. It has been hypothesised that the degree of atherosclerosis in the carotid artery is correlated to the polymorphism related to eNOS phenotypes (reviewed in Manolio *et al*[49]). To address this issue in detail, it was recently investigated whether the eNOS G/T polymorphism (Glu298Asp variant) is linked to the severity of carotid atherosclerosis in patients with end-stage renal disease.[50] The relationship between eNOS genotypes and carotid wall-to-lumen ratio was further analysed by a categorical approach, and in a multiple logistic regression analysis, the OR of increased carotid wall-to-lumen ratio was found to be strongly associated to the T allele. Thus, the T allele of eNOS gene seems to be an independent predictor of intimal lesions and vascular remodelling, and is associated with the severity of carotid atherosclerosis.

Another beneficial application of genetic information is the identification of variants that influence response to cardiovascular pharmaceutical agents. For example, knowledge of genetic variants that influence blood pressure response to antihypertensive drugs (most of them possess vasculoprotective properties) may allow more individualised tailoring of antihypertensive drug treatment, and provide greater insight into the molecular mechanisms regulating blood pressure levels and causing hypertension and atherosclerosis. Progress towards tailoring treatment for vascular diseases and individualising target genetic background has just started. Guidelines for the management of atherosclerosis including the consideration of individual genetic background will be published in the future.

CONCLUSIONS

The automation of sequencing methods has allowed improved investigation of the human genome and the human proteome. Yet, the sheer array of factors that have been uncovered, such as polymorphisms, is frightening. These methods will be mitigated, however, when biochemists start to translate the information of thousands of hitherto unknown protein-coding genes accommodated in the human and in other genomes into the structure and function of the encoded proteins. The impending co-operation of clinical biochemistry with molecular medicine will mainly be based on two fields: the achievements of modern protein biochemistry, and the results of genome and proteome research.

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