Review

Angiotensin I-Converting Enzyme

Genotype and Disease Associations

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Cardiovascular diseases (CVD) represent a paradigm for the complex interplay of environmental risk factors and multiple genetic risk factors. Genetic abnormalities that are disease-causing, however, are less frequent than genetic factors that confer increased risk for CVD. Genetic predisposition for CVD appears to be the end result of cumulative effects of common genetic polymorphisms, which would confer only modestly increased risk when present as a single genetic risk factor.¹ Such cumulative effects may be further modulated by environmental factors. Furthermore, in patients with already diagnosed CVD, the genetic risk factors may influence the patients' response to therapy and diet.^{1,2}

Polymorphisms of the genes encoding components of the renin-angiotensin system (RAS) represent an area of intense research for cardiovascular disease associations, with promising, although sometimes contradictory reported findings.

Angiotensin I-Converting Enzyme: A Key Component of the RAS

The renin-angiotensin system not only is essential in cardiovascular hemodynamics, but plays an important role in the development of CVD. Angiotensin I-converting enzyme (ACE) is a key component of both the RAS and the Kinin-Kallikrein system (Figure 1).

The Genes of the RAS

The RAS gene system comprises the renin, angiotensinogen (AGT), angiotensin I-converting enzyme (ACE), and angiotensin II receptor types 1 and 2 (AGTR₁, AGTR₂) genes.

The renin gene maps to chromosome 1q32, spans approximately 12 kb, and comprises 10 exons and nine introns.^{3,4} The angiotensinogen gene maps to chromosome 1q42-43, spans approximately 13 kb, and com-

prises five exons and four introns^{5,6}; exons 1 and 5 encode for the 5' and 3' untranslated regions of mRNA, respectively. The ACE gene maps to chromosome 17q23, spans 21 kb, and comprises 26 exons and 25 introns.^{7,8} The two major species of ACE mRNA are a 4.3-kb endothelial-type mRNA (transcription encompassing exons 1 to 26, excluding exon 13) and a 3-kb testicular type ACE mRNA (transcription encompassing exons 13 to 26). Exon 26 encodes for the functionally important membrane-anchoring domain of the ACE protein. The endothelial type of ACE mRNA is found not only in endothelial cells, but also in epithelial cells. The angiotensin II receptor type 1 gene maps to chromosome 3, and the angiotensin II receptor type 2 gene maps to chromosome X.^{9,10}

Circulating RAS Components

RAS functions as an endocrine system. The renin gene is expressed primarily in the juxtaglomerular cells of the kidney, where renin is synthesized, stored, and released into the circulation. Prorenin is cleaved to form renin, which is stored in tissue granules until it is released in response to specific secretagogues. Secretion of renin from the kidneys is controlled by several factors. The macula densa are a specialized group of distal convoluted tubular cells that act as chemoreceptors for sodium and chloride levels in the distal tubule. Sodium retention increases blood volume, which is followed by an increase in blood pressure. This increase in blood pressure activates a negative feedback regulation of the juxtaglomerular cells in the kidney, which sense renal perfusion pressure and renin production are inhibited. Renin secretion is autonomically modulated via sympathetic innervation of the renal tubules and arterioles.

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Figure 1. Role of ACE in the RAS and Kinin-Kallikrein systems.

Circulating renin catalyzes the angiotensinogen-to-angiotensin I conversion. The angiotensinogen gene is expressed in the liver, the site of AGT synthesis and release into the circulation. The angiotensin I (Ang I) generated by renin activity is a vasoinactive decapeptide. Conversion of angiotensin I to angiotensin II (Ang II) is the key reaction in the RAS pathway, generating the effector of the system, Ang II, a potent vasoconstrictor. The reaction is catalyzed by ACE (kininase II; EC 3.4.15.1), a zinc metalloproteinase member of the Alu family that functions as a dipeptidyl carboxypeptidase (DCP1). The mechanisms controlling the circulating ACE levels are less clear than those for renin. The most likely genetic control is at the level of transcription and would involve linkage disequilibrium with regulatory elements of the ACE gene. Once the protein is translated and bound to the cell membrane, release would require cleavage of the hydrophobic bonds that anchor the protein to the membrane.

ACE cleaves the C-terminal His-Leu dipeptide from Ang I, generating the vasoactive octapeptide Ang II.¹¹ Further conversion of Ang II to Ang III is possible by cleavage of the aspartic acid from position 1 of the octapeptide; however, the generated Ang III is less potent as a vasoconstrictor, compared to Ang II.¹¹ Circulating ACE is found in biological fluids, such as plasma, amniotic and seminal fluids, and originates from endothelial cells.

ACE also acts as a protease on bradykinin, cleaving the C-terminal Phe-Arg dipeptide, with the net effect of inactivating this vasodilator. Therefore, ACE enzymatic activity will result in a double effect: activation of a vasoconstrictor/pressor (Ang II) agent and inactivation of a vasodilator agent (bradykinin). Ang II is also an aldosterone-stimulating peptide. Aldosterone promotes depletion of potassium while promoting the retention of sodium and water; therefore Ang II exerts a negative feedback on renin production due to volume expansion and/or to a direct effect on juxtaglomerular cells.

Tissue RAS Components

RAS also functions as a paracrine system. Ang II is demonstrated to be produced in multiple target organs by local RAS pathways. All components of the RAS, for example, are present in cardiac tissue^{12,13}; transcripts

for all RAS components are found in both atrial and ventricular tissue.^{13,14} However, under normal conditions the renin responsible for local/cardiac Ang I generation appears to derive from circulation, being of renal origin. Under pathological conditions, renin can be also produced in the heart.¹⁵ The key component of the tissue RAS, as in circulating RAS, is ACE. At the cellular level, the ACE molecule projects into the extracellular space and is anchored to the plasma membrane by the C-terminal hydrophobic region that spans the membrane and ends in a short cytoplasmic tail. Ang II generated by ACE activity exerts its effects by binding to angiotensin II receptors, type 1 and type 2; AGTR₁ is the major mediator of physiological effects of Ang II (vasoconstriction, hypertrophy, catecholamine liberation at sympathetic nerve endings). Both AGTR₁ and AGTR₂ are transmembrane receptors, comprising seven membrane-spanning domains, and are coupled to G-proteins. Both AGTR₁ and AGTR₂ mRNAs are expressed in the heart. However, AGTR₁ is the principal receptor mediating Ang II cardiac and circulatory effects. Cardiac effects include direct inotropic activity resulting in increased myocardial contraction, as well as cell growth and proliferation, resulting in cardiac remodeling, hypertrophy, and ventricular dilatation.¹¹ AGTR₂ appears to be the dominant receptor in both atrial and ventricular myocardium, as well as in the adrenal medulla and uterus.^{11,16} Functionally, AGTR₂ is an antagonist of AGTR₁, inasmuch as it has an antiproliferative effect.¹⁷

Ang II can also be generated in the tissues, including myocardium, by pathways other than RAS; non-RAS pathways involve nonspecific carboxypeptidases and chymotrypsin-like proteinases. An example of one of these is chymase (serine-proteinase), which catalyzes an efficient Ang II generation at tissue levels. Production of Ang II by these non-RAS alternative pathways is not inhibited by therapy with ACE inhibitors. The chymase pathway has been demonstrated in various cell types, including myocardium, endothelial cells, and mast cells.^{18,19} Chymase levels have been found higher in the ventricles than in the atria, and ventricle levels do not appear to change significantly in heart failure.¹⁹

ACE Gene Polymorphism

Several polymorphisms have been reported in genes of the RAS and represent genetic factors that affect both circulating and tissue RASs. These include polymorphisms in the angiotensinogen, ACE, and AGTR₁ genes.

The ACE gene polymorphism was first reported by Rigat et al in a study that addressed the role of the ACE gene in the genetic control of plasma ACE levels.²⁰ Normally, plasma ACE levels show marked interindividual variation but appear to be remarkably stable when measured repeatedly in the same subject. The normal ranges for plasma ACE levels and the units of measurement depend on the detection method used. Rigat et al used direct radioimmunoassay measurement of the enzyme (in $\mu\text{g/L}$); subsequently, functional assays using spectrophotometric measurements (in U/L) have been used.

Reference ranges for each method must be established in the testing laboratory. A current and widely used method is a spectrophotometric method using the synthetic tripeptide substrate *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG). The normal ranges are age-dependent and vary widely in adults (8–52 U/L).

A previous study of healthy families had shown intrafamilial similarities of ACE levels, suggesting they are controlled by a major gene.²¹ The polymorphism discovered by Rigat et al is of the insertion/deletion type; the two ACE alleles differ in size because of the insertion of a 287-bp DNA sequence in intron 16 of the ACE gene.²⁰

The allele frequencies reported in this study were 0.406 for the insertion (I) allele and 0.594 for the deletion (D) allele.²⁰ The distribution of the three genotypes was in agreement with Hardy-Weinberg equilibrium, with frequencies of 0.18 for the II genotype, 0.46 for the ID genotype, and 0.36 for the DD genotype. The correlation between genotype and plasma ACE levels showed a significant relationship between D allele dose and ACE concentration, with the highest ACE levels found for the DD genotype.²⁰ The I/D polymorphism accounted for approximately half of the observed variance in ACE levels in this study group represented by 80 healthy Caucasians.

The ACE polymorphism was initially detected by restriction fragment length polymorphism (RFLP) analysis and Southern hybridization with a human ACE cDNA probe.²⁰ Subsequent studies of ACE polymorphism and disease associations used polymerase chain reaction (PCR) for genomic DNA amplification. The first PCR-based detection of the I/D ACE polymorphism was reported by Rigat et al,²² who used a set of primers flanking the insertion sequence; the generated amplicons corresponding to the I and D alleles differ in size by the length of insertion sequence (ie, 287 bp) and allow discrimination between the three genotypes: II, ID, and DD. This standard PCR method was used by Rigat et al to establish allele frequencies in 199 unrelated individuals; the obtained values (D allele 0.573 and I allele 0.427) were almost identical to those obtained previously with Southern hybridization.^{20,22} However, family studies performed by Shanmugam et al²³ showed the possibility of mistyping ID heterozygotes with this PCR method. Genotyping a large pedigree in which one of the parents was an II homozygote showed several DD genotypes among the offspring; the possibility of mistaken paternity was excluded.²³ Changing reaction conditions and the inclusion of 5% dimethylsulfoxide in the PCR reaction mixture improved genotyping significantly and showed ID heterozygosity in the offspring. The cause of the amplification of ID genotypes as DD is unclear; it is possible that amplification of the I allele is suppressed in some heterozygotes, or there is preferential amplification of the shorter, D allele; a similar phenomenon has been observed by Perna et al,²⁴ in the PCR amplification of another *Alu*/D polymorphism. To safeguard against any ID to DD mistyping, an additional PCR amplification reaction was devised for the confirmation of all DD genotypes obtained in the first standard PCR²³; this confirmation PCR method includes a new sense primer that is insertion-specific (5' end of

insertion sequence) and the standard antisense primer, generating a 408-bp amplicon if the I allele is present.²³ A true DD genotype would show no amplification, because of the lack of an annealing site for the new sense primer. Using this method, the authors reported 100% accuracy of ACE polymorphisms.²³ This combination of standard and confirmatory PCR reaction was subsequently used in multiple studies addressing the issue of disease associations of ACE DD genotype.

Functional Effects of ACE Polymorphism

Effect of Genotype on Enzymatic Levels

The ACE DD genotype is associated with increased circulating ACE levels, which are generally two times as high as those found for II genotypes; ID heterozygotes are associated with intermediate ACE levels.²⁰ This relationship of D allele dose and enzymatic levels, originally reported by Rigat et al,²⁰ was repeatedly confirmed by other studies, for both circulating and cellular ACE.^{25–34} However, because the ACE I/D polymorphism is intronic, the mechanism of ACE overexpression in subjects with DD genotype is unclear; it is possible that this relationship is the result of tight linkage to another locus involved in the regulation of ACE gene expression.¹¹

Physiological Effects of ACE Genotype: Elite Population Studies

Elite population studies represent an alternative way of assessing the significance of genetic polymorphisms via their physiological effects; such studies are useful complements to the more traditional approach of disease association studies.

The role of RAS genes in the regulation of both cardiac and vascular physiology suggests that they are potential candidate genes for cardiovascular performance. Genes that are involved in angiotensin II production or function, such as ACE, AGTR₁, and AGTR₂, have been studied in elite normal populations to evaluate the role of genetic polymorphisms in determining cardiovascular performance, particularly arterial compliance and cardiac hypertrophy. One such study was made of elite endurance athletes by Gayagay et al.³⁵ The study group was a cohort of 64 Australian national rowers attending preselection trials for the 1996 Olympic Games in Atlanta, Georgia. The control group was represented by 114 age- and sex-matched subjects (healthy volunteers and blood donors). All subjects in both the study and control groups were Caucasian. ACE genotyping showed a frequency of the I allele that was significantly higher in the rowers compared to the controls (0.57 versus 0.43).³⁵ The genotype frequencies also showed a significant increase in II genotype in the athlete group at 0.30 versus 0.18 in the controls; the frequency of the DD genotype was significantly lower in the athlete group at 0.16 compared to 0.32 in the control group. Genotypic frequencies in both athletes and controls were in Hardy-Weinberg equilibrium, making selection bias unlikely. These findings suggest

that the down-regulation of the ACE gene associated with the II genotype would produce less systemic ACE activity, and consequently reduced cardiac afterload, whereas lowered cardiac ACE levels would reduce the risk for pathological ventricular hypertrophy. Together, the systemic and cardiac effects would lead to more efficient ventricular-vascular coupling during exercise.³⁵ Another possible mechanism by which the ACE polymorphism could be involved is through linkage disequilibrium of the I allele with another gene or regulatory element, which is still unknown. Unlike the ACE genotype, polymorphisms of AGTR₁ and AGTR₂ genes did not show significantly different frequencies between the elite athlete group and the controls.³⁵ Interestingly, some population studies also showed low frequencies of the D allele associated with a reduced prevalence of cardiovascular disease in Japanese, American Indians (Pima and Yanomami), and Samoans.^{36,37}

Another elite population study performed by Schachter et al³⁸ on a cohort of 338 French centenarians, with a mean age of 100.71 years, reports some intriguing findings; an increased frequency of the DD genotype was found in the centenarian group at 39.6%, compared to 25.6% in the control group (160 French adults). There was no sex-related difference in the ACE allele frequencies, and populations were in Hardy-Weinberg equilibrium.³⁸ The overrepresentation of the DD genotype in this study group is surprising, in view of the reported associations of this genotype and coronary artery disease and myocardial infarction. The authors advance the hypothesis that the cardiovascular risk conferred by the D allele is offset by a possible long-term protective effect; such an effect may give some early selective advantage and/or a late reversal of its negative survival influence.³⁸ A protective effect of DD genotype may be related to other biological functions of ACE, besides its role in RAS and Kinin-Kallikrein systems.³⁸ In this respect, neuroendocrine functions of ACE, such as its ability to cleave neuropeptides,³⁹ its regional distribution in the brain,⁴⁰ and immunomodulator functions related to ACE levels in cytotoxic T lymphocytes,²⁹ may contribute to the influence of ACE polymorphism on overall survival and longevity.³⁸ The association of DD genotype with longevity may also derive from linkage disequilibrium to a closely mapping gene, still to be identified. In this context, it is interesting to mention that the gene encoding for the human growth hormone also maps to chromosome 17q23, shows strong linkage to ACE, and appears to have an important role in senescence.^{38,41}

Positive Disease Associations of ACE Polymorphism

Coronary Artery Disease and Myocardial Infarction

The D allele was found to be associated with increased risk of coronary artery disease (CAD), including premature CAD, and myocardial infarction (MI), including premature MI. One of the first studies showing the DD genotype association with increased risk of MI was re-

ported by Cambien et al in 1992;⁴² in this multicenter case-controlled study of 1300 subjects, the DD genotype was found at significantly higher frequency in subjects with MI compared to controls. This positive association was found to be particularly significant in the low-risk patient group, for which the relative risk of MI attributable to the DD genotype was 35% compared to 8% observed in the entire ECTIM (Etude Cas-Témoin de l'Infarctus du Myocarde) study group. Subsequently, in another arm of the ECTIM study, Cambien et al⁴³ showed the association of increased plasma ACE levels due to DD genotype with MI in patients younger than 65 years of age, compared to controls. Other case-controlled studies confirmed these findings^{44–47} and even found that the DD genotype was an independent risk factor for MI.^{44,47} Nakai et al⁴⁴ also evaluated the relationship between frequency of D allele and severity of CAD and found a significantly higher frequency at 0.71 in patients with triple-vessel disease compared to 0.54 in patients with single-vessel disease. A study that evaluated ACE genotype in patients with premature CAD was reported by Alvarez et al⁴⁸; a total of 181 male Caucasian patients who had suffered an episode of MI or had unstable angina before age 50 (average age 43 years) were genotyped for ACE and AGTR₁, compared to an age-matched group of healthy controls. Both study and control groups were remarkably homogeneous, having been drawn from the Asturias region of northern Spain and therefore excluding the effect of geographical/ethnicity bias. The DD genotype was found at a higher frequency of 0.50 in the patient group, compared to 0.41 in the control group.

Furthermore, the hypothesis that patients with DD genotype and MI inherit the risk from their parents secondary to allele dominance was tested in the ECTIM study by assessing the prevalence of myocardial events in their parents; a threefold increase in the chance of having parents with history of MI was found for DD patients compared to controls.⁴⁹ A similar approach was used by Badenhop et al,⁵⁰ who studied genotype frequencies in over 400 children and their grandparents; an overrepresentation of the DD genotype was found in children having two or more grandparents with a history of a coronary event (MI, fatal MI, coronary bypass, graft surgery), with an odds ratio (OR) of 2.8. Additional evidence for the D allele dose being a risk factor for MI came from a study of 213 fatal cases of definite and suspected MI that came to autopsy in the Belfast MONICA project⁵¹; the hypothesis tested was that an increase in ACE gene expression due to the D allele would lead to increased risk of death in patients developing coronary heart disease. In comparison to controls from the same population (the Belfast arm of the ECTIM study), the autopsy cases had an increased frequency of the D allele; ORs were 2.2 for DD *versus* II, and 1.8 for ID *versus* II.⁵¹ The authors conclude that the ACE I/D polymorphism is a risk factor for fatal MI and sudden cardiac death; a possible explanation may reside in the role of ACE in the regulatory mechanisms of the RAS and adrenergic systems, which may confer increased myocardial susceptibility to arrhythmias secondary to vasoconstriction.⁵¹

Indirect evidence for the role of ACE overexpression is provided by therapeutic ACE inhibition studies, such as SAVE (Survival and Ventricular Enlargement Trial) and SOLVD (Studies of Left Ventricular Dysfunction), in which therapy with an ACE inhibitor was demonstrated to reduce the incidence of MI, recurrent MI, and fatal MI, including prehospital coronary death.^{52,53} The beneficial, anti-ischemic effects of various ACE inhibitors have been repeatedly reported in patients with CAD, MI, left ventricular dysfunction, or stroke.^{32,52-57}

Left Ventricular Hypertrophy and Dysfunction

Left ventricular mass is a powerful independent predictor of cardiovascular morbidity and mortality.^{11,27,58} Left ventricular hypertrophy (LVH) is primarily caused by chronic hypertension, but other nonhemodynamic phenomena, including genetic factors, appear to play a role. The association of the ACE polymorphism with increased risk of MI in patients with few other risk factors for coronary events has raised the question of a possible role of this polymorphism in the pathogenesis of LVH. Schunkert et al⁵⁹ studied the association of ACE genotype with LVH in a Caucasian population and reported that the DD genotype is an independent risk factor for development of LVH in normotensive men (OR 2.6) but not in women. The positive association of DD genotype and LVH was confirmed by Iwai et al,⁶⁰ but with no sex difference. Prasad et al⁶¹ suggested that the effect of hypertension on left ventricular mass is achieved only in the presence of the D allele. In this context, it is important that patients with the DD genotype have increased cardiac ACE and Ang II concentrations, and the effects of local RAS activity may be more important than circulating RAS. There are multiple recognized mechanisms for deleterious effects of Ang II, such as induction of hypertrophy in non-infarcted areas, direct toxic effect on myocardial cells, ventricular dilatation and remodeling, stimulation of fibroblast proliferation, promotion of smooth muscle hyperplasia, endothelial dysfunction, increase of left ventricular afterload, and impairment of diastolic relaxation, in addition to the main effects of vasoconstriction, coronary artery constriction, and activation of the sympathetic nervous system.^{11,27,54,62,63} Indirect evidence is again provided by ACE inhibition studies; left ventricular systolic function is improved in postinfarct patients by ACE inhibitors.⁶³ Regression of LVH after therapy with ACE inhibitors is far more significant than that seen in conjunction with a comparable reduction in blood pressure by other antihypertensive agents.²⁷ The DD genotype was shown to be associated with a 5% lower ejection fraction in postinfarct patients, but not in noninfarct patients, leading to the conclusion that the influence of the ACE polymorphism on left ventricular function is modulated by infarction status and coronary anatomy.⁶³

ACE Polymorphism and Hypertension

The association between ACE polymorphism and essential hypertension is controversial. No correlation has

been found between plasma ACE levels and hypertension or between ACE DD genotype and hypertension, in multiple studies.^{27,33,42,43,59,60,64,65} Response to ACE inhibitors in hypertensive patients appears to be determined at least in part by the ACE genotype in the study of Ohmichi et al,³³ but this was not confirmed by Sasaki et al³² or Nagano et al.⁶⁶ A singular study of Zee et al⁶⁷ showed a positive association between ACE polymorphism and hypertension, with a higher frequency of the I allele in hypertensive patients with a family history of hypertension, compared to normotensive controls.

ACE Polymorphism and Venous Thrombosis

The DD genotype was found as a potent risk factor for thrombosis in patients undergoing total hip arthroplasty.²⁸ In this case-controlled study, Philipp et al investigated the association of ACE polymorphism, as well as factor V Leiden mutation and 5,10 methylenetetrahydrofolate reductase (MTHFR) polymorphism with postoperative venous thrombosis. The OR of a thrombotic event after hip surgery was 11.7 for patients with the DD genotype and 5.0 for patients with the ID genotype, compared with the II genotype. The factor V Leiden mutation and MTHFR polymorphism did not increase the risk of thrombosis after hip arthroplasty.²⁸ The plasma ACE levels in this study showed the same pattern previously reported by others, with the highest values in DD patients, intermediate values in ID heterozygotes, and the lowest values in II patients.²⁸ Another study that showed an association between the DD genotype and an increased risk of venous thrombosis was reported by Dilley et al⁶⁸ for an African-American population, with a threefold relative risk in men but not in women. These findings of significant increases in risk for thrombosis in DD homozygotes raise the question of clinical and possibly therapeutic implications and are a strong rationale for further studies of the role of ACE polymorphism in venous thrombosis.²⁸

ACE Polymorphism and Nephropathy

Numerous studies have addressed the role of RAS gene polymorphisms in the development and progression of renal disease. Because of its central role in RAS, the ACE polymorphism has been extensively investigated, again with conflicting results in renal disease, as recently reviewed by Schmidt and Ritz.⁶⁹ There is increasing evidence that the progression of diabetic nephropathy is more rapid in patients with DD genotype.^{30,69,70} Yoshida et al,⁷⁰ for example, studied 139 patients with non-insulin-dependent diabetes mellitus (NIDDM) with a history of diabetes over 10 years, classified into two groups based on serum creatinine, ie, a group with normal renal function and a second group with declining renal function. The two groups were comparable in length of history of NIDDM, blood pressure, and hemoglobin A1c levels. These authors report an OR for loss of renal function in diabetics with a DD genotype of 3.42 and consider ACE polymorphism to be a genetic marker for the progression to chronic renal failure in

diabetics. They also showed a similar association in IgA nephropathy.⁷⁰ Administration of ACE inhibitors has been shown to lead to a significant decrease of proteinuria in chronic renal diseases, including nondiabetic renal diseases. The ACE genotype appears to predict the therapeutic efficacy of ACE inhibition of proteinuria; DD genotype patients are resistant to this renoprotective therapy, whereas ID and II genotype patients have a significant reduction in the degree of proteinuria.^{31,70–75} However, there is still no consensus on using ACE genotyping in the setting of diabetic and nondiabetic nephropathy, because reported studies to date are on limited size patient groups, and large prospective studies would be necessary to assess the impact of the ACE polymorphism on the response to renoprotective treatment.

ACE Polymorphism and Coronary Restenosis after Stent Implantation

Intracoronary stent implantation has been shown to reduce the rate of coronary restenosis, a major postinterventional complication after balloon angioplasty.^{76,77} However, a minority of patients still develop coronary restenosis poststenting. Baseline preintervention patient parameters, however, are not good predictors of restenosis. The role of diabetes and unstable angina, which are demonstrated to increase the risk of restenosis after balloon angioplasty, is controversial in poststent restenosis. Unlike restenosis postangioplasty, which is primarily due to vessel constriction and remodeling, restenosis after stenting is primarily due to neointimal hyperplasia.^{26,78–80} This makes stent implantation a good model for the study of factors that may cause, promote, or block neointimal hyperplasia.

Because RAS has been implicated in the development of neointimal hyperplasia,⁸¹ ACE activity is a crucial step for the RAS pathway, and the resulting increased generation of Ang II is a potent growth factor for smooth muscle cells,⁸² the hypothesis has been advanced that genetic factors affecting RAS and particularly ACE gene expression may be important in pathogenesis of coronary restenosis after stenting. Indirect experimental evidence was obtained by demonstrating that ACE inhibitors block neointimal thickening after arterial balloon denudation in rats, guinea pigs, and rabbits.^{83,84} The clinical relation between restenosis after coronary stenting and ACE polymorphism was investigated by Amant et al⁷⁸ in 146 patients who underwent successful stent implantation and had 6 months' follow-up angiography. The minimal lumen loss and late luminal loss had a significant inverse relationship to the D allele dose; the OR for restenosis was 2.0 per number of D alleles; the association of the number of D alleles and poststent restenosis was independent of other risk factors.⁷⁸ The increased ACE activity due to the presence of the D allele, mainly in the homozygous state, may account for the higher degree of coronary neointimal thickening found in these patients.⁷⁸ These results are in keeping with previous observations that high levels of plasma ACE correlate with structural changes in the arterial wall, such as the increase in carotid intima-media thickness⁸⁵ and its extent.⁸⁶

A significantly increased rate of binary restenosis ($\geq 50\%$ diameter stenosis at 6-month follow-up angiography) after stent implantation was also found in a series of 593 patients by Ribichini et al^{26,87}; the rate was 33.9% in patients with the DD genotype, compared with 16.3% in ID patients and only 2.9% in II patients. The correlation between ACE genotype and the degree of intimal proliferation was also found in this series; the loss in minimal lumen diameter resulting from intrastent restenosis measured angiographically was 0.98 mm in patients with the DD genotype, 0.81 mm in ID patients, and 0.57 mm in II patients.^{26,87} Furthermore, the DD genotype appears to be associated not only with a markedly increased risk of intrastent restenosis, but also with poor long-term clinical outcome, because patients with the DD genotype more frequently have a diffuse type of neointimal proliferation that is more aggressive and more difficult to treat.^{80,87} The frequency of diffuse restenosis was found to be significantly associated with the D allele dose; 66% of all cases with diffuse restenosis were seen in patients with the DD genotype, compared with 32% in ID patients and 2% in II patients.^{26,87} This is a marked overrepresentation of the DD genotype in the patient group with diffuse poststent restenosis. The relative risk for diffuse restenosis is 2.5 (95% confidence interval 1.66–3.77) versus nonstenotic patients and 1.65 (95% confidence interval 1.19–2.27) versus patients with focal intrastent restenosis.^{26,87}

These findings suggest that determination of ACE genotype may identify a subset of patients who are at higher risk for poststent restenosis, particularly diffuse restenosis, and poor long-term prognosis after coronary stenting.^{26,78,87}

Negative Disease Association Studies of ACE Polymorphism

In contrast to the numerous studies reporting positive disease associations of ACE polymorphism with cardiovascular disease (over 300 studies to date), several large-scale studies and a recent meta-analysis did not confirm these findings.^{88–93} Lindpaintner et al⁸⁸ investigated the association between ACE genotype and the incidence of MI and other manifestations of ischemic heart disease, in a large, prospective cohort of U.S. male physicians. At the 10-year follow-up, ischemic heart disease (as defined by angina, coronary revascularization, or MI) had developed in 1250 men of the total of 22,071 subjects enrolled in the Physicians Health Study since 1982. Men with a history of angina, MI, stroke, transient ischemic attacks, or cancer were excluded from enrollment; the subjects were predominantly Caucasian. The study group of 1250 men who had developed ischemic heart disease was genotyped for ACE polymorphism and compared to 2340 matched controls, according to age and smoking history. The presence of the D allele in this study group conferred no appreciable increase in the risk for ischemic heart disease or MI in the overall group or in the low-risk subgroups.⁸⁸ The authors address the discrepancy between the negative findings of this nested

case-controlled study within a prospective study and the positive association findings of earlier studies; the most probable reasons are considered to be differences in the criteria for enrolling or excluding patients and controls, the possibility of differences in genetic background in this more heterogeneous North American population compared with more homogeneous European populations, and low informativeness of the ACE polymorphism.⁸⁸ Moreover, the studied population of U.S. male physicians may not be representative of a standard population sample, not only in terms of gender restriction, but also because physicians are more aware of other risk factors and their control, and therapies with antihypertensive drugs, particularly ACE inhibitors and aspirin, are known to reduce the risk for ischemic heart disease. A subsequent investigation was reported by Jeunemaitre et al⁸⁹ in the CORGENE study, a cross-sectional study involving 463 Caucasians who underwent coronary angiography for established or suspected CAD (156 patients with previous MI, 307 patients without MI). Genotypes were determined for ACE, AGT, and AGTR₁. No significant association was found between these polymorphisms and the clinical characteristics of MI and non-MI patients, with the exception of the AGT 235T allele, which correlated with the extent of coronary lesions.⁸⁹ Two other large cohort studies performed retrospectively on a Danish patient population, based on the Copenhagen City Heart Study, were reported by Agerholm-Larsen et al^{90,91}; these failed to confirm an association between ACE polymorphism and CAD. Subsequently, Agerholm-Larsen et al⁹² performed a meta-analysis of 46 studies published before April 1998, including a total of 32,715 Caucasian subjects. Both small and large studies were included, and the objective was to assess the association of ACE polymorphism with plasma ACE levels, blood pressure, and risk for MI, ischemic heart disease, and ischemic cerebrovascular disease. A positive association was found between the D allele dose and plasma ACE levels, confirming numerous previous reports. For example, in the small studies, plasma ACE levels were increased by 71% and 40% for the DD and ID genotypes, respectively, *versus* the II genotype. In the large studies, plasma ACE levels were also significantly increased by 48% and 21% in the DD and ID genotypes, respectively, compared to the II genotype.⁹² No correlation between ACE genotype and blood pressure was found in this study,⁹² which is consistent with previous negative studies.^{27,33,42,43,59,60,64,65} Similarly, no association was found between ACE genotype and ischemic cerebrovascular disease in either the small or the large studies.⁹² The risk of MI and ischemic heart disease was increased by 47% for the DD *versus* the II + ID genotype in small studies (OR 1.47 with 95% CI 1.30–1.66), but not in large studies (OR 0.99 with 95% CI 0.88–1.12); for all studies combined, the risk was increased by 20% for the DD genotype (OR 1.21 with 95% CI 1.11–1.32). The authors discuss some limitations of the meta-analysis; information on the use of ACE inhibitors was either absent or limited in the studies included in the meta-analysis; this may be an important limitation because it is widely recognized that ACE inhibition influences plasma ACE levels, blood

pressure, and risk for ischemic cardiovascular disease. Another limitation of the meta-analyzed studies (except the U.S. Physicians Health Study) is that only nonfatal cases were included. Therefore, if the DD genotype is associated with increased risk for CAD and fatal MI, the meta-analysis results may show an underestimation of the influence of the DD genotype. A previous study by Evans et al⁵¹ addressed this issue and found increased D allele frequency in cases of fatal MI in the Belfast arm of the ECTIM study; however, the study of Evans et al did not meet the inclusion criteria for this meta-analysis.

Many other studies showing a positive disease association for ACE polymorphism were also excluded from this recent meta-analysis. Unlike previous meta-analyses, the study of Agerholm-Larsen et al⁹² had very restrictive exclusion criteria; studies were excluded if participants were nonwhite, if plasma ACE activity and blood pressure were not measured in the controls, if disease associations were not reported for all three ACE genotypes, if information on SD or SEM was not available, or if studies were conducted in diabetics and hypertensive patients.^{92,93} The last two exclusion criteria in particular may flatten the association of the D allele with the end points analyzed, ie, risk of MI, ischemic heart disease, and ischemic cerebrovascular disease. On the other hand, combining heterogeneous study populations from around the world creates a background of genetic heterogeneity. Even if studies on nonwhite populations were excluded, the meta-analysis populations would not be all Caucasian. For example, the U.S. Physicians Health Study, included in the meta-analysis, enrolled a predominantly (but not exclusively) white male population.

Negative findings in disease association studies and meta-analyses underline the important concept that multiple interacting factors, genetic and environmental, contribute to the development of CAD and MI and may overcome the modest risk increase conferred by a single genetic polymorphism in the overall population.⁹³ In contrast, the risk may be significantly increased in selected subgroups of patients. In this respect, the risk for CAD and MI conferred by interaction between different genetic polymorphisms and mutations becomes relevant.

Genotypic Interactions

Multiple polymorphisms in the angiotensinogen gene have been reported.¹¹ A common polymorphism is a T-to-C substitution in exon 2, position 704, resulting in a methionine-to-threonine substitution at position 235; the AGT M235T polymorphism may be a marker for blood pressure variation. The 235T allele is associated with high plasma angiotensinogen concentrations and hypertension,^{11,94–96} whereas other studies are nonconfirmatory.^{97,98} Homozygosity for the AGT 235T was found to be an independent risk factor for CAD, conferring an approximately twofold increased risk in a case-controlled study of a Caucasian population from New Zealand.⁹⁹ This study included 422 patients with documented CAD, 50% of which had MI, and 406 age- and sex-matched controls. An increased risk for CAD was seen in 235T

homozygotes (OR 1.7), as was an increased risk for MI (OR 1.8). Adjustment for several risk factors increased the estimate for CAD risk to 2.6 and that for MI to 3.4.⁹⁹ The AGT 235T allele was the only disease-associated polymorphism reported to correlate with the extent of coronary lesions in the study of Jeunemaitre et al.⁸⁹ The positive associations of the AGT 235T allele with plasma AGT levels, hypertension, and CAD were recently confirmed by Winkelmann et al.¹⁰⁰ in a case-controlled study of 301 Caucasian male subjects; homozygosity for the AGT 235T allele was associated with a 1.5-fold risk for CAD and MI. The significant association with coronary risk persisted after adjustment for other risk factors, including age, smoking, glucose, and apolipoprotein B.¹⁰⁰ In contrast, the study reported by Ichihara et al.¹⁰¹ on 327 Japanese patients with CAD and 352 matched controls did not find an association between AGT genotype and CAD.

A polymorphism in the angiotensin II type 1 receptor (AGTR₁) gene involving an A-to-C substitution at position 1166, located at the 5' end of the 3' untranslated region of the AGTR₁ gene, was found at increased frequency in patients with hypertension,¹⁰² and it was also shown to be associated with increased aortic rigidity in hypertensives.¹⁰³ A significant interaction between AGTR₁ and ACE polymorphisms was found by Tiret et al.¹⁰⁴ in a cohort of 613 patients with MI from the ECTIM study, compared to 723 age-matched controls. The OR for MI was 1.52 for subjects with ACE DD genotype and AC heterozygosity for AGTR₁, and 3.95 for homozygotes for both ACE DD and AGTR₁ CC genotypes. In a subgroup of patients defined as low risk by traditional risk factors, the synergy between these two polymorphisms was even stronger: for patients with the DD and AC genotypes the OR for MI was 7.03, and for patients with the DD and CC genotypes, the OR for MI was 13.3.¹⁰⁴ This type of synergistic effect was confirmed by other studies in CAD patients⁴⁸ and in determining aortic stiffness in hypertensives¹⁰³; other studies did not confirm this synergy in MI,¹⁰⁵ restenosis after coronary angioplasty,¹⁰⁶ left ventricular hypertrophy,¹⁰⁷ hypertrophic cardiomyopathy,¹⁰⁸ CAD and MI,¹⁰⁹ or arterial wall thickness.^{110,111}

Conclusions

Disease associations of ACE genotype represent an area of intense clinical research since 1992, when Cambien et al.⁴² first reported that the DD genotype is a risk factor for myocardial infarction. A very large number of subsequent studies found positive disease associations, whereas others did not confirm these findings. Meta-analyses did not resolve the controversy, and multiple study limitations should be taken into consideration in searching for an explanation of contradictory findings. The weight of the large number of studies that report positive disease associations and, perhaps more importantly, the enormous number of studies that demonstrate the beneficial effects of ACE inhibitors in cardiovascular diseases cannot be ignored. It is possible that a single genetic polymorphism such as ACE may confer a modestly increased risk that

may be added to, amplified, or even overcome by other acquired environmental and/or additional genetic factors. Therefore, the risk associated with a given polymorphism may be clinically important in subsets of patients, for example, in patients with a certain genetic profile, including multiple deleterious polymorphisms or mutations, or in patients with additional acquired risk factors. The small number of polymorphisms in the RAS genes that appear to be clinically significant may change dramatically in the coming years with completion of the Human Genome Project, setting the stage for complex genetic profiling and risk stratification in patients with cardiovascular disease.

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