Human Evidence That the Cystatin C Gene Is Implicated in Focal Progression of Coronary Artery Disease

Per Eriksson, Hiroyuki Deguchi, Ann Samnegård, Pia Lundman, Susanna Boquist, Per Tornvall, Carl-Göran Ericsson, Lott Bergstrand, Lars-Olof Hansson, Shu Ye, Anders Hamsten

Objective—Overexpression of elastolytic cysteine and aspartic proteases, known as cathepsins, is implicated in atherogenesis. The potential significance of imbalance in expression between cathepsins and their inhibitor cystatin C in cardiovascular disease has been highlighted by the demonstration of cystatin C deficiency in human atherosclerosis and abdominal aortic aneurysms.

Methods and Results—We identified and characterized physiologically relevant polymorphisms in the promoter region of the cystatin C gene that influence cystatin C production and used these polymorphisms as a tool to examine the significance of cystatin C in coronary atherosclerosis in vivo in humans. Seven polymorphisms, all in strong-linkage disequilibrium, were identified in the cystatin C gene, of which 2 promoter polymorphisms (−82G/C and −78T/G) were functional in vitro in electromobility shift and transient transfection assays. Genotyping of 1105 individuals (237 survivors of a first myocardial infarction before age 60 and 2 independent groups comprising a total of 868 healthy individuals) revealed that the plasma cystatin C concentration was significantly lower in carriers of the mutant haplotype. Furthermore, the mutant haplotype was associated with a higher average number of stenoses per coronary artery segment in unselected postinfarction patients (N=237) undergoing routine coronary angiography.

Conclusions—These results provide human evidence for an important role of cystatin C in coronary artery disease. (Arterioscler Thromb Vasc Biol. 2004;24:551-557.)

Key Words: cystatin C • genetics • coronary artery disease • promoter • remodeling

Degradation of the extracellular matrix through the action of specialized proteolytic enzymes is a prerequisite of vascular remodeling. Normal physiological remodeling allows the adult blood vessel to adapt and repair, whereas perturbations of vascular remodeling are an important component of the pathogenesis of atherosclerosis, plaque rupture, restenosis, and aneurysm formation. In the past several years, the roles in these processes of serine proteases, matrix metalloproteinases, and their respective inhibitors have started to emerge.1 Overexpression of some elastolytic cysteine proteases also has been implicated in atherogenesis,2 and the potential significance of imbalance between cathepsins and their inhibitor cystatin C remains unknown, and the available clinical information is restricted to the finding of an association between the serum cystatin C concentration and size3,9 and annual expansion rate9 of small abdominal aortic aneurysms (AAA).

Against this background, we identified and characterized physiologically relevant polymorphisms in the promoter region of the cystatin C gene that influence cystatin C production and used these polymorphisms as a tool to examine the...
significance of cystatin C in coronary atherosclerosis in vivo

Methods

Gene Sequencing
Four PCR fragments spanning the proximal promoter, the signal peptide and exons 1, 2, and 3, respectively, were generated (Figure 1) for the nucleotide sequencing of the cystatin C gene. The PCR fragments were used as templates for further amplifications as part of the Taq DyeDeoxy Terminator Cycle sequencing system (Perkin Elmer, Applied Biosystems Division). Nested primers were used for the analysis of overlapping sections of 200 to 300 base pairs (bp) in both directions.

Genotyping
Genotyping for the −82 G/C and +4 A/C polymorphisms was performed using a PCR fragment amplified by the forward primer, 5′-GATGGATGGGGAGGACAG, and the reverse primer, 5′-CGGATGGCCAGGAG, and cleaved with restriction enzyme SstII (Figure 2). The −5 G/A polymorphism was investigated using the same PCR fragment as mentioned and the restriction enzyme DdeI. The −78 T/G polymorphism was screened for using a Taq DyeDeoxy Terminator Cycle sequencing system (Perkin Elmer, Applied Biosystems Division) (see previous). Genotyping of the +148 G/A polymorphism was performed using a PCR fragment amplified by the forward primer, 5′-TCTATCTAGCTCAAGCCTCTCG, and the reverse primer, 5′-TCTGCTCGCTTGTGTAGCCTCTCG, and subsequently digested with the enzyme SacII (Figure 2).

Electromobility Shift Assay
Nuclear extract preparations were prepared according to Alksnis et al. All buffers were supplemented freshly with 0.7 μg/mL leupeptin, 16.7 μg/mL aprotinin, 0.5 mM PMSF, and 0.33 μM 2-mercaptoethanol. The protein concentration in the extract was estimated by the method of Kalb and Bernlohr. The sequences of the probes were AAAAGGGAGTGCAAGGCAGGCCG/GGGGTGGGGGGGGCGGC and GCCCTCGCCGCTAGA/AGTATCGCA/CGCAGGTCCTCCTC for the polymorphisms located at positions −82 to −78 and the polymorphisms located at positions −5 to +4, respectively (polymorphisms are shown in bold). All 4 possible combinations of polymorphisms in each region were used. Incubation for electromobility shift assay was conducted essentially as described. Final concentrations in binding reactions were as follows: 10% glycerol, 10 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl2, 0.5 mM EDTA, and 1 mM DTT. DNA-protein complexes were separated from unbound DNA probe on native 7% polyacrylamide gels (acrylamide:bisacrylamide w/w: 80:1) in low-ionic-strength buffer containing 22.5 mM Tris, 22.5 mM borate, and 0.5 mM EDTA (pH 8) by electrophoresis at 200 V for 2 hours.

Transient Transfection Experiments
Two DNA constructs covering the region from −259 to +37 of the cystatin C promoter and thereby harboring 4 of the polymorphic sites were designed by PCR (forward primer 5′-GATGGATGGGGAGGACAG and reverse primer 5′-CGGATGGCCAGGAG, and ligated into a pGEM-T-Easy cloning vector (Promega). The two combinations, the wild type −82G/−78T/−5G/+4A and the mutant −82C/−78G/−5A/+4C haplotypes, could be picked up from genomic DNA. An EcoRI fragment harboring the insert was subsequently ligated into a LITMUS 28 vector (New England Biolabs). The constructs were then cut and ligated into a Kho I-restricted and Hind III-restricted pGL2–Basic Vector (Promega). The correct sequence of the inserts was tested by DNA sequencing. Transient transfection was performed as described. The human monocyte/macrophage cell line U937 was maintained in 7.5% FCS-RPMI 1640 medium. Cell density was kept between 0.2 to 1 million cells/mL. For differentiation experiments, 10–20 mL cells were seeded in 10-cm2 dishes at 2 × 105 cells/mL. Cell density was kept between 0.2 to 1 million cells/mL. For differentiation experiments, 10–20 mL cells were seeded in 10-cm2 dishes at 2 × 105 cells/mL. Luciferase levels were expressed as arbitrary units after standardization against Renilla luciferase levels (pRL-TK vector, Promega; co-transfected to normalize for variation in transfection efficiency). Transfection with pGL2–Basic Vectors without insert served as negative controls. Transfections were performed in triplicate in 3 independent experiments.

Biochemical Analysis
Plasma for measuring cystatin C by a particle-enhanced nephelometric immunoassay (Dade Behring) was prepared from venous blood drawn into tubes containing Na2EDTA (final concentration 4 mmol/L). Established biochemical risk indicators were determined as described.

Clinical Cohorts
Two clinical cohorts were investigated in the study. A random sample of 639 healthy 50-year-old white men living in the northern parts of Stockholm was examined to establish allele frequencies for all cystatin C polymorphisms and the relation between cystatin C genotype and plasma cystatin C concentration in healthy individuals. The second cohort was part of a study that comprised a total of 393 unselected survivors of a first myocardial infarction before age 60 years who were admitted to the coronary care units of 3 hospitals in the northern part of Stockholm. Exclusion criteria were type-1 diabetes mellitus, renal insufficiency (serum creatinine >200 μmol/L), any known chronic inflammatory disease, drug addiction, psychiatric disease, or inability to comply with the protocol. For each postinfarction patient, a sex-matched and age-matched control person was recruited from the general population of the same catchment area. Three months after the index cardiac event, patients and controls underwent examination, and all patients included at 2 of the
hospitals (n=293) were offered routine coronary angiography. A total of 252 postinfarction patients (86%) agreed to be included in the coronary angiography substudy, of whom 237 were successfully genotyped and used in the present study. Coronary angiography was performed during the initial admission if needed for clinical reasons; if not, then it was performed 3 months later. Angiograms divided into 15 coronary segments were analyzed by the Medis QCA system. In each segment reference diameter, minimal lumen diameter, percent diameter stenosis, mean segment diameter, segment length, plaque area, segment area, and number of significant (>50%) stenoses were measured.

The Ethics Committee of the Karolinska Hospital approved the studies of the cohorts. All participants gave their informed consent.

Statistical Methods
Statistical analyses were performed using the StatView software (SAS). Allele frequencies were estimated by gene counting. A χ²-test was used to compare the observed numbers of each cystatin C genotype with those expected for a population in Hardy-Weinberg equilibrium. Normalized linkage disequilibrium coefficients (D’) were calculated according to Ott. Haplotype frequencies were estimated using the HaploTyper program, which uses a Bayesian algorithm. Differences in continuous variables between groups were tested by analysis of variance (ANOVA), with the Scheffe F test used as a post-hoc test. Correlations were analyzed by a linear regression method.

Results
Detection of Common Polymorphisms Within the Promoter and Exon 1 of the Cystatin C Gene
All three exons of the cystatin C gene together with a 214-bp fragment of the proximal promoter region, a promoter fragment previously demonstrated to be essential for basal promoter activity, were sequenced in both directions using DNA samples from 24 healthy subjects. Seven different polymorphisms were detected, of which the majority have been described before: −82 G/C (a G-to-C substitution at position −82), −78 T/G, −5 G/A, +4 A/C, +87 C/T, +148 G/A, and +213 G/A (Figure 1). No polymorphisms were detected in exons 2 and 3. Of polymorphisms located in the signal peptide and exon 1, only the G-to-A substitution at position +148 results in an amino acid change (Thr to Ala).

Allele Frequencies and Degrees of Linkage Disequilibrium
Genotyping for the −82 G/C, −5 G/A, +4 A/C, and +148 G/A polymorphisms was performed in 639 healthy, population-based men aged 50 years. In addition, 157 randomly selected individuals from the same cohort were sequenced for the −78 T/G polymorphism because no gel-based or other method could be designed for this polymorphism. The sequencing confirmed the results obtained by restriction enzyme digestion for the polymorphisms located at positions −82, −5, and +4. The +87 C/T and +213 G/A polymorphisms were not further analyzed because these polymorphisms do not change the amino acid sequence and therefore are probably not of functional importance. All polymorphisms were found in Hardy-Weinberg equilibrium. Allele frequencies and pair-wise linkage disequilibrium coefficients are shown in Table 1. Complete or almost complete allelic associations were observed between all polymorphisms studied. Haplotype analyses revealed 3 major haplotypes: −82G/−78T/−5G/+4A/+148G (wild type, frequency of 0.7571), −82C/−78G/−5G/+4C/+148A (mutant in all positions except −5, 0.1914), and −82C/−78G/−5A/+4C/+148A (mutant in all positions, 0.0457).

Associations Between Cystatin C Polymorphisms and Plasma Concentrations of Cystatin C
The relationships between cystatin C polymorphisms and the concentration of circulating cystatin C were first analyzed in 672 healthy 50-year-old men. As shown in Table 2, the less frequent −82 G-allele, −78 G-allele, −5 A-allele, +4 C-allele, and +148 A-allele were associated with a significantly lower plasma concentration of cystatin C, which is in accordance with the strong-linkage disequilibrium between the different polymorphisms. Individuals homozygous for the wild type haplotype had significantly higher plasma concentrations of cystatin C than heterozygous individuals and non-carriers. Secondly, circulating cystatin C concentrations were measured in 237 postinfarction patients undergoing routine coronary angiography and 229 age-matched and sex-matched controls (Table 2). The same genotype–pheno-type associations were observed, ie, presence of the rare cystatin C alleles was associated with lower plasma cystatin C concentrations. Identical allele-specific associations were observed in patients and controls. Furthermore, patients had lower plasma cystatin C concentrations compared with the controls (0.79±0.14 μg/mL [N=237] versus 0.84±0.11 μg/mL [N=229]; P<0.0001). Of note, there was no statistically significant difference in allele frequencies between patients and controls (−82G/C: 0.78/0.22 versus 0.76/0.24; NS).

Polymorphisms Influence Binding of Nuclear Proteins and Transcriptional Activity of the Cystatin C Promoter
Electromobility shift assay demonstrated that the −82 G/C and −78 T/G polymorphisms influence the binding of nuclear proteins. As demonstrated by Figure 3A lanes 1 to 4 and lanes 13 to 16, nuclear proteins derived from U937 cells bind with stronger affinity to the mutant haplotype (−82C/−78G) than to the wild-type haplotype (−82G/−78T). Mutating only one site resulted in an intermediate affinity (Figure 3A, lanes 5 to 12). In contrast, the −5 G/A and the +4A/C polymorphisms did not influence the binding of nuclear proteins (Figure 3A, lanes 17 to 24). Transient transfection studies were performed to evaluate the effect of the different cystatin C variants on promoter activity in vitro. As shown in

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele Frequencies</th>
<th>D’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>−82 G/C</td>
<td>0.78/0.22</td>
<td>−</td>
</tr>
<tr>
<td>−78 T/G</td>
<td>0.77/0.23*</td>
<td>−</td>
</tr>
<tr>
<td>−5 G/A</td>
<td>0.95/0.05</td>
<td>−</td>
</tr>
<tr>
<td>+4 A/C</td>
<td>0.78/0.22</td>
<td>−</td>
</tr>
<tr>
<td>+148 A/G</td>
<td>0.75/0.25</td>
<td>−</td>
</tr>
</tbody>
</table>

*Allele frequency was determined by sequencing of 157 individuals. All normalized linkage disequilibrium coefficients (D’) are significant, P<0.0001.
The association between plaque area did not differ according to cystatin C genotype. Diffuse CAD (average mean segment diameter or average severity of stenoses (average minimal lumen diameter) or not shown). In contrast, angiographic parameters reflecting dilation was found for single polymorphisms (Table 3 and data not shown). The same association between cystatin C genotype and severity of coronary artery disease (CAD) in healthy individuals and patients with recent myocardial infarction. The corollary of these findings is that these polymorphisms, a G-to-C substitution at position 78, were found to influence the binding of nuclear factors and affect the basal rate of gene transcription in an allele-specific manner in vitro and to be included in the analysis. However, there was no significant correlation between plasma cystatin C concentration and average number of stenoses per coronary artery segment ($r = -0.112$, ns).

**Association of Cystatin C Genotype With Severity of Coronary Artery Disease**

There was a significant association between cystatin C genotype and severity of coronary artery disease (CAD) in the postinfarction patients. As shown in Table 3, the mutant haplotype was associated with an increased average number of stenoses per coronary artery segment. The same association for single polymorphisms (Table 3 and data not shown). In contrast, angiographic parameters reflecting severity of stenoses (average minimal lumen diameter) or diffuse CAD (average mean segment diameter or average plaque area) did not differ according to cystatin C genotype. The association between $-82G/-78T/-5G/+4A$ haplotype and number of stenoses per segment remained statistically significant when established risk indicators (body mass index, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, insulin, and proinsulin) were included in the analysis. However, there was no significant correlation between plasma cystatin C concentration and average number of stenoses per coronary artery segment ($r = -0.112$, ns).

**Discussion**

In this study, the cystatin C gene was examined to detect functional polymorphisms that can be used to further elucidate the significance and functions of cystatin C in pathological vascular remodeling in humans. Two common promoter polymorphisms, a G-to-C substitution at position $-82$ and a T-to-G substitution at position $-78$, were found to influence the binding of nuclear factors and affect the basal rate of gene transcription in an allele-specific manner in vitro and to be associated with the plasma concentration of cystatin C in healthy individuals and patients with recent myocardial infarction. The corollary of these findings is that these polymorphisms are useful tools to define the pathophysiological roles of cystatin C in vivo in humans. Subsequent genotype–phenotype association studies in patients with premature...
myocardial infarction indicated that the mutant haplotype is associated with a greater number of coronary artery stenoses. Patients with premature CAD (MI before age 60 years) were studied because it can be assumed that various causal factors and pathogenic mechanisms would be more readily identified when they have exerted their effect at a fairly young age. Also, the confounding influence of age per se and of age-related degenerative disorders on CAD should be reduced in young patients.

This is the first study of whether common genetic variation in cystatin C gene expression is associated with development of vascular disease. Recent work has demonstrated an association between homozygosity for the $-82C/-78G$ haplotype and late-onset Alzheimer disease. Furthermore, an association between cystatin C genotype and age-related macular degeneration has been demonstrated. The gene for cystatin C also has been extensively scanned for sequence variation causing hereditary cystatin C amyloid angiopathy, a rare autosomal dominant disorder in which cystatin C is deposited as amyloid fibrils in the cerebral arteries, leading to massive brain hemorrhage. The disease-causing mutation was identified as a T-to-A substitution in exon 2, resulting in a leucine-to-glutamine change at position 68.

Before the present work, to our knowledge, no data existed on how polymorphisms influence the expression of the cystatin C gene and/or the activity of cystatin C. Our results suggest that the association between cystatin C genotype and plasma cystatin C concentration is caused by an altered promoter activity of the cystatin C gene. The mutant $-82C/-78G/-5A/+4C$ haplotype had significantly lower promoter activity in vitro, consistent with the association found in vivo between presence of the mutant haplotype and lower plasma cystatin C concentrations. Electromobility shift assay demonstrated that polymorphisms affected the binding of nuclear proteins to the $-82/-78$ region, whereas there was no effect

TABLE 3. Cystatin C Genotype and Severity of Angiographically Determined Coronary Artery Disease in Postinfarction Patients

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Homozygous</th>
<th>Heterozygous</th>
<th>Non-carriers</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>132</td>
<td>81</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Mean number of stenoses per segment</td>
<td>0.15±0.12</td>
<td>0.18±0.17</td>
<td>0.20±0.23</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Mean % stenosis</td>
<td>31.6±8.7</td>
<td>32.3±10.7</td>
<td>34.2±7.4</td>
<td>NS</td>
</tr>
<tr>
<td>Minimum lumen diameter (mm)</td>
<td>2.33±0.44</td>
<td>2.34±0.50</td>
<td>2.26±0.26</td>
<td>NS</td>
</tr>
<tr>
<td>Mean segment diameter (mm)</td>
<td>3.03±0.46</td>
<td>3.05±0.49</td>
<td>2.94±0.29</td>
<td>NS</td>
</tr>
<tr>
<td>Cystatin C ($\mu$g/mL)</td>
<td>0.82±0.14</td>
<td>0.75±0.12</td>
<td>0.67±0.11</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age (y)</td>
<td>53±5</td>
<td>53±5</td>
<td>53±6</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.0±3.95</td>
<td>26.59±3.08</td>
<td>29.41±3.97</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.29±1.04</td>
<td>3.38±0.96</td>
<td>3.39±1.15</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.07±0.29</td>
<td>1.12±0.32</td>
<td>1.05±0.29</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/L)</td>
<td>2.00±1.39</td>
<td>1.78±0.99</td>
<td>1.89±0.56</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma insulin (pmol/L)</td>
<td>48.6±27.1</td>
<td>51.1±30.2</td>
<td>50.3±18.8</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma proinsulin (pmol/L)</td>
<td>6.29±5.36</td>
<td>6.36±7.20</td>
<td>4.61±2.21</td>
<td>NS</td>
</tr>
</tbody>
</table>

Means±SD are shown.

BMI indicates body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

*Analyses performed on individuals with significant stenosis (N=186).

P values are ANOVA comparisons of the three genotype groups.
on binding to the −5/-4 region. Thus, the stronger affinity of nuclear proteins to the −82C/−78G haplotype suggests that the lower transcriptional activity may be caused by binding of a transcriptional repressor to the mutant haplotype. Database analysis could not, however, reveal the identity of the potential repressor.

Because the promoter polymorphisms are in complete allelic association with the +148 G/A substitution causing a switch from Thr to Ala at position −2 within the signal peptide, we cannot exclude that this polymorphism also contributes to the lower plasma concentration of cystatin C. However, the mutation at position −2 is unlikely to have functional implications because positions −1 and −3 relative to the cleavage site are important for release to occur correctly whereas the amino acid at position −2 is of minor importance (the −3, −1 rule).26

Previous studies suggesting a role for cysteine and aspartic proteolytic activity in vascular disease are mainly based on detection of overexpression or underexpression of these proteases in affected vascular tissue. However, it has recently been shown that deficiency of cathepsin S reduces atherosclerosis in low-density lipoprotein receptor-deficient mice.27 Our results demonstrating an association between a genetically determined decrease in cystatin C expression with increased severity of CAD is in line with the notion that cathepsins play a causal role in atherosclerosis. The results are also in accordance with the reports of increased expression of cathepsin S and cathepsin K,2 decreased cystatin C expression1 in human atheroma, and with the similar finding of overexpression of cysteine and aspartic proteases in developing atheroma in mice.28 The fact that the mutant cystatin C haplotype was related to the average number of stenoses per coronary artery segment, but not to mean minimal lumen diameter or mean segment diameter or plaque area, suggests that imbalance between cathepsin and cystatin C expression may promote plaque rupture. This interpretation is based on the assumption that the number of stenoses per coronary segment is likely to reflect more closely the frequency at which plaque rupture occurs than the other QCA measurements, because the majority of coronary stenoses represent previous episodes of plaque rupture.29,30

In the present study, we could detect a lower plasma concentration of cystatin C in postinfarction patients than in controls. This is in agreement with a report of significantly lower circulating cystatin C levels in asymptomatic subjects with dilated aorta,3 but it contrasts with an earlier epidemiological study in which plasma levels of cystatin C were unassociated with the risk of peripheral arterial disease.31

The finding that the plasma concentration of cystatin C was not significantly correlated with the number of stenoses per coronary segment suggests that allele-specific regulation of cystatin C expression in the arterial wall acts in concert with other remodeling mechanisms predisposing to plaque rupture. Because the number of patients assessed by quantitative coronary angiography was fairly limited, replication of the cystatin C allele-specific haplotype-specific association with number of stenosis per coronary segment is needed in a larger sample to rule out the possibility of a false-positive finding. Also, the relation between plasma cystatin C concentration and severity of CAD needs to be re-examined in a larger cohort.

In all, these restrictions notwithstanding the results generated in this study provide evidence that cystatin C may play an important role in pathological remodeling of coronary arteries and plaque rupture in humans.

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