Type III Collagen Deficiency in Saccular Intracranial Aneurysms
Defect in Gene Regulation?

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Background and Purpose—We sought to determine whether there are mutations in the COL3A1 gene in patients with saccular intracranial aneurysms with a type III collagen deficiency and whether there is an association between a marker in the COL3A1 gene and saccular intracranial aneurysms. One of the heritable factors possibly involved in the pathogenesis of saccular intracranial aneurysms is a reduced production of type III collagen, demonstrated earlier by protein studies.

Methods—We analyzed the type III collagen gene in a group of 41 consecutive patients with an intracranial aneurysm, of whom 6 patients had shown a reduced production of type III collagen in cultured diploid fibroblasts from a skin biopsy.

Results—No mutations could be demonstrated in the COL3A1 gene, especially not in the globular N- and C-terminal regions. A null allele was excluded in 25 patients, including 1 patient with a decreased type III collagen production. No differences were found between 41 patients and 41 controls in allele frequencies of a DNA tandem repeat polymorphism located in the COL3A1 gene.

Conclusions—It is concluded that the COL3A1 gene is not directly involved in the pathogenesis of most of intracranial aneurysms. The reduced type III collagen production in cultured fibroblasts found in some patients with an intracranial aneurysm is not explained by the present study and needs further exploration. (Stroke. 1999;30:1628-1631.)

Key Words: cerebral aneurysm ■ collagen ■ pathology

Subarachnoid hemorrhages have a high mortality rate.1,2 The majority of subarachnoid hemorrhages are caused by rupture of an intracranial saccular aneurysm.3 The pathogenesis of intracranial aneurysms has not been elucidated but is thought to be a multifactorial process.4,5 Several factors, such as smoking and hypertension, have been associated with the formation of intracranial aneurysms.6 The formation of intracranial aneurysms has been associated with pseudoxanthoma elasticum, autosomal dominant polycystic kidney disease, and Marfan syndrome, among others.7–9 However, the relation with Marfan syndrome may be fortuitous, as was recently demonstrated in a follow-up study.10

No deficiency of type III collagen production was observed in 5 patients with familial intracranial aneurysms.11,12 However, several studies have suggested that type III collagen deficiency is a risk factor for intracranial aneurysms.13–17 In none of these studies was there a molecular analysis of the type III collagen gene, except for the study performed by Kuivaniemi and coworkers,18 who analyzed part of the type III procollagen gene encoding the triple helix. In 40 patients, no mutations were found in this part of the gene.

In a previous protein study we observed a decreased production of type III collagen in cultured skin fibroblasts from 6 of 41 consecutive patients with intracranial aneurysms and in none of a group of 41 age- and sex-matched healthy controls.19 Here we present molecular analysis of the complete COL3A1 gene, including the regions encoding the globular N- and C-terminal parts of the protein. Mutations in the C-propeptide may affect or even prevent triple helix formation as well as protein stability, and mutations in the N-propeptide may affect the function of type III collagen by preventing removal of the N-propeptide. The propeptide regions were not analyzed in the previous study by Kuivaniemi et al.18

Patients with a normal level of type III collagen were also studied. It is possible that patients with mild forms of Ehlers-Danlos syndrome–vascular type have normal production of a structurally altered type III collagen as a result of a mutation of the COL3A1 gene.20

In Ehlers-Danlos syndrome–vascular type, inactive gene copies (null alleles) of COL3A1 result in a mild form of the disorder.21 To determine whether both COL3A1 alleles were

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active, we investigated whether polymorphisms in the 3'-untranslated part of type III collagen were present. Null alleles, which do not produce stable mRNA, lead to a low level of gene product.

Brega et al. investigated allele frequencies for type III collagen gene using an Ava II polymorphism (restriction fragment length polymorphism) in 19 patients with an intracranial aneurysm and in 15 controls. A diallelic polymorphism with fragments of 5.7 kb (allele A) and 4.3 kb (allele B) was found. Allele B was demonstrated in 11 patients and in only 2 controls. This polymorphism is located in an intron of the gene and probably has no biological effect. This association could indicate linkage disequilibrium with a mutation in the COL3A1 gene, which may occur when a mutation has arisen in a gene close to a DNA marker with a certain allele. If the mutation spreads through the population, it remains associated with this allele. If this would be the case, a subgroup of patients in whom type III collagen is involved in the formation of intracranial aneurysms should be identified by a specific allele of the linked polymorphism. These disequilibrium data suggest that an abnormal type III collagen is involved in the formation of some intracranial aneurysms. We studied a more polymorphic and informative DNA tandem repeat polymorphism in the COL3A1 gene.

Subjects and Methods

Patient Population

Forty-one consecutive patients with an intracranial saccular aneurysm admitted to the Department of Neurosurgery of the Academic Medical Center in Amsterdam and 41 healthy controls (age and sex matched) were included in this study. After informed consent was obtained, a skin biopsy was taken for fibroblast culture, and type III collagen production was determined. For comparison of allele frequencies, we calculated the difference, with 95% CI limits, using Fisher’s exact test.

DNA Analysis

The COL3A1 gene was analyzed on cDNA produced from cultured skin fibroblast RNA. In all patients the region of the gene encoding type III collagen was amplified fragments from the part of the cDNA encoding the N-propeptide and the sense strand of the PCR products on the Dynabeads and on the DNA sequence analysis of the complete N-propeptide and C-propeptide of type III collagen demonstrated no DNA sequence variations in any of the 41 patients.

Polymerase Chain Reaction–SSCP/Heteroduplex Analysis

Polymerase chain reactions (PCR) were performed on the immobilized cDNA to amplify the type III collagen cDNA in 20 overlapping fragments for SSCP/heteroduplex mutation analysis and DNA sequencing. For SSCP/heteroduplex analysis, fragments of ~350 bp were used. These fragments were analyzed on polyacrylamide minigels in an automated electrophoresis system (Phastsystem, Pharmacia, Upsala, Sweden). If a sample yielded additional bands in the single-stranded (SSCP) or double-stranded (heteroduplex) area of the gel, the region of the gene containing this fragment was subjected to sequence analysis. PCR primer sequences and reaction conditions are available upon request from one of the authors (G.P.).

DNA Sequencing

For DNA sequence analysis, PCR products were made with cDNA and/or genomic DNA primer sets, of which the primer was 5'-biotin labeled. Single-stranded DNA was prepared with streptavidin-coated Dynabeads (Dynal, Oslo, Norway) according to the manufacturer’s instructions. Dideoxy sequencing reactions with a Pharmacia T7 sequencing kit (Pharmacia, Upsala, Sweden) were performed on the sense strand of the PCR products on the Dynabeads and on the antisense strand that was washed off the Dynabeads and ethanol precipitated. Specific sequencing primers were used for each PCR fragment. S-labeled dCTP was used to detect the products by autoradiography after electrophoresis on 6% denaturing polyacrylamide gels.

C to T Polymorphism in Exon 33 of the COL3A1 Gene

To detect whether polymorphisms were present in exon 33, a restriction analysis according to Tromp et al. was used.

DNA Marker in Intron 25 of the COL3A1 Gene

Mays et al. described a 15-base DNA tandem repeat marker in intron 25 of COL3A1, and we studied this marker using CYS-labeled dCTP in the PCR reaction to label the PCR products. The estimated length of the alleles was assessed on an ALFExpress automated DNA sequencer (Pharmacia, Upsala, Sweden) and corresponded to an apparent repeat length of 16 bp.

Statistical Analysis

For statistical analysis, we used the Fisher’s exact test.

Results

Thirty-eight patients had a ruptured intracranial aneurysm, and 3 had an unruptured intracranial aneurysm. Cerebral angiography was performed in all patients except 1. This patient was operated on immediately because of a rapid clinical deterioration, and the presence of an aneurysm was confirmed during surgery. A positive family history for subarachnoid hemorrhages was present in 4 patients (10%).

After performing SSCP/heteroduplex analysis of PCR-amplified fragments from the part of the cDNA encoding the triple helix of type III collagen of all patients, we detected a fragment with an altered electrophoretic mobility in only 1 patient. The cultured fibroblasts of this patient previously showed a decreased synthesis of type III collagen. To characterize the nucleotide sequence of the altered fragment of this patient, DNA sequencing was performed. The data showed a T→C change at position 2793 (numbering according to Ala-Kokko et al.). This was confirmed in genomic DNA. However, the T→C change is a silent mutation and does not lead to an amino acid substitution of type III procollagen. Polymorphisms in exon 33 were detected in 16 of the 40 patients by a restriction analysis according to the method of Tromp et al.
Allele Frequency Using 16 Base Tandem Repeat Marker in Intron 25 COL3A1 Gene in 41 Controls and 41 Consecutive Patients With Intracranial Aneurysms

<table>
<thead>
<tr>
<th>Allele</th>
<th>Controls</th>
<th>Patients</th>
<th>Difference, %</th>
<th>95% CI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>226</td>
<td>0</td>
<td>1</td>
<td>+1.2</td>
<td>−1.2 to 3.6</td>
</tr>
<tr>
<td>242</td>
<td>34</td>
<td>37</td>
<td>+3.7</td>
<td>−18.8 to 11.5</td>
</tr>
<tr>
<td>258</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>−13.1 to 13.1</td>
</tr>
<tr>
<td>274</td>
<td>24</td>
<td>21</td>
<td>−3.7</td>
<td>−17.3 to 10.0</td>
</tr>
<tr>
<td>290</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>−5.8 to 5.8</td>
</tr>
<tr>
<td>306</td>
<td>1</td>
<td>0</td>
<td>−1.2</td>
<td>−3.6 to 1.2</td>
</tr>
</tbody>
</table>

To determine whether both copies of the gene produced stable RNA, we looked for polymorphisms in the 3'-untranslated part of type III collagen. SSCP/heteroduplex analysis of PCR-amplified fragments of the untranslated part of type III collagen showed heterozygosity in 25 of the 41 patients; 1 of them was shown earlier to have a decreased type III collagen production.19

In all 25 patients the heterozygous polymorphism could also be demonstrated in the cDNA, indicating that both alleles of COL3A1 were expressed and produced stable mRNA.

To investigate abnormal allele frequency in the type III collagen gene, we used a highly variable, tandem repeat marker in intron 25 of the COL3A1 gene with 6 alleles. We found no difference in allele frequency in our patients compared with the control group (Table ), nor was a difference in allele frequency found in patients with decreased type III collagen production compared with patients with normal production.

**Discussion**

Several studies have shown a decreased level of type III collagen in patients with intracranial aneurysms.13–17 Our earlier study of type III collagen protein analysis in diploid fibroblast cultures obtained from 41 patients with intracranial aneurysms also showed a significantly decreased level in 6 patients,19 supporting the hypothesis that decreased production of type III collagen plays a role in the formation of intracranial aneurysms in some patients.

SSCP/heteroduplex analysis, screening the complete type III collagen coding sequence, showed only 1 silent mutation (2793T→C), which does not lead to a change in the amino acid sequence. This result is in agreement with data presented by Kuivaniemi et al,18 who observed no mutations when sequencing the triple-helix encoding part of the type III procollagen gene in 40 patients with intracranial aneurysms. However, in this study the N-propeptide part or the C-propeptide part of the type III collagen gene was not analyzed. The globular part of the C-propeptide is essential for the formation of the triple helix in fibrillar collagens.29,30 A mutation in the C-terminal part may theoretically lead to a failure of association of the procollagen monomers with an intracellular breakdown of the mutated pro-α1 chain, or it may lead to an abnormal association with all 3 α1 chains being destroyed.31 Therefore, the C-propeptide of the type III collagen gene was sequenced, showing no changes. Our data strengthen the conclusion of the study of Kuivaniemi et al18 that the type III collagen gene is not likely to be involved in the formation of intracranial aneurysms. In the collagen mutation database, 3% of the mutations are large deletions.32 Single or multi-exon deletions can be demonstrated in cDNA, as we found in our collagen mutation studies (J.S.P. van den Berg, MD, et al, unpublished data, 1999). Large PCR products from cDNA in these patients did not show evidence of deletions or exon skipping. However, we may have missed any large deletions that encompass the entire gene or lead to unstable messenger RNA.

Null alleles leading to reduced type III collagen secretion have been described.33 These patients showed a normal or mild clinical phenotype.21 In 25 patients, including 1 patient with decreased production of type III collagen, the presence of a null allele could be excluded, making it unlikely to be a major contributor to the phenotype in this study group.

In none of the 6 patients with an intracranial aneurysm and a decreased level of type III collagen was a mutation in the type III collagen found with SSCP/heteroduplex. SSCP analysis, with PCR fragments of 350 nucleotides, detects >80% of the mutations.33,34 Combining SSCP with heteroduplex analysis will lead to a higher detection rate. However, polymorphisms in exon 33 were detected with a restriction analysis according to Tromp et al26 and not with SSCP/heteroduplex analysis.

The Ehlers-Danlos syndrome–vascular type phenotype varies from classic (“acrogeric”) presentation to almost no visible abnormalities (“atypical”).35 In patients with classic Ehlers-Danlos syndrome–vascular type, mutations in the type III collagen gene are frequently detected, but they are often not found in the atypical form of the disease.36 Our patients with decreased production of type III collagen may be considered to have atypical Ehlers-Danlos syndrome–vascular type. The decreased production of type III collagen in patients with this syndrome may be due to defects during posttranslational modification or altered collagen metabolism, eg, elevated gelatinase activity.37

The reported association of intracranial aneurysms with type III collagen polymorphism22 was not confirmed in the present study, since none of the 6 alleles of a highly variable marker showed a difference in frequency in the patients compared with the control group. This shows that, at least in the Dutch population, there is no indication of linkage disequilibrium in the region of COL3A1. It is therefore unlikely that a single mutation in this gene plays a role in susceptibility to intracranial aneurysms.

Although reduced production of type III collagen is a contributory factor in the formation of intracranial aneurysms in some patients, determination of the exact causative molecular mechanisms of this aberration awaits further studies.

**Acknowledgments**

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