Intracranial aneurysms and connective tissue disorders
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Type III Collagen deficiency in Saccular Intracranial Aneurysms: defect in gene regulation?

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Introduction

Subarachnoid haemorrhages have a high mortality.1,2 The majority of subarachnoid haemorrhages is 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 amongst others with pseudoxanthoma elasticum, autosomal dominant polycystic kidney disease, and Marfan syndrome.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 there was a molecular analysis of the type III collagen gene, except for the study performed by Kuivaniemi and coworkers. They analysed part of the type III procollagen gene encoding the triple-helix. In 40 patients no mutations were found in this part of the gene.18

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 have not been analysed 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 mild forms of Ehlers Danlos syndrome vascular type have a normal production of a structurally altered type III collagen, due to 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 if both COL3A1 alleles were active we investigated if polymorphisms in the 3'-
untranslated part of type III collagen were present. Null alleles, that 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 (RFLP) in 19 patients with an intracranial aneurysm, and in 15 controls. A diallelic polymorphism with fragments of sizes 5.7 kilobase (allele A) and 4.3 kilobase (allele B) was found. Allele B was demonstrated in 11 patients and only in 2 controls.\textsuperscript{22} This polymorphism is located in an intron of the gene and has probably no biological effect. This association could indicate linkage disequilibrium with a mutation in the COL3A1 gene. This 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 which 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.\textsuperscript{23}

**Subjects and Methods**

**Patient population.** Forty-one consecutively 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.\textsuperscript{19} After informed consent a skin biopsy was taken for fibroblast culture, and type III collagen production was determined.\textsuperscript{19}

**Reverse transcription.** Total RNA was isolated from $1 \times 10^6$ cultured fibroblasts using RNAzol (Life Technologies, Bethesda, Maryland, USA) according to the manufacturer's instructions. First strand cDNA was prepared using oligo-dT coated magnetic beads (Dynal AS, Oslo, Norway) to capture the mRNA according to Raineri et al.\textsuperscript{24} Reverse transcription was performed on the captured mRNA after washing of the beads, using the oligo-dT on the beads as primer and Superscript II Reverse transcriptase (Life technologies, Bethesda, Maryland, USA).
DNA analysis. The COL3A1 gene was analysed on complementary DNA (cDNA), produced from cultured skin fibroblasts RNA. In all patients the region of the gene encoding the triple helix was screened with single strand conformation polymorphism (SSCP) and heteroduplex analysis, followed by DNA sequencing of aberrant fragments. The regions encoding the N- and C-terminal parts were sequenced in all patients. SSCP/heteroduplex analysis was also performed on genomic DNA and cDNA in the 3'-untranslated region of COL3A1 to detect polymorphic sites. If a patient was heterozygous for a polymorphism this was used to determine expression of both gene copies in the cDNA.

PCR-SSCP/heteroduplex analysis. PCR reactions 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 about 350 bp were used. These fragments were analysed on polyacrylamide minigels in an automated electrophoresis system (Phastsystem, Pharmacia, Upsala, Sweden).²⁵ 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 (GP).

DNA sequencing. For DNA sequence analysis PCR products were cDNA or/and genomic DNA made with primersets, of which the primer was 5'-biotin labelled. 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, Upsula, 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.²⁵S-labelled dCTP was used to detect the products by autoradiography after electrophoresis on 6% denaturing polyacrylamide gels.

C to T polymorphism exon 33 of the COL3A1 gene. To detect if polymorphisms were present in exon 33 a restriction analysis according to Tromp et al was used.²⁶

DNA marker in intron 25 COL3A1 gene. Mays et al described a 15-base DNA tandem repeat marker in intron 25 of COL3A1,²³ and we studied this
marker using CY5 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 comparison of allele frequencies we calculated the difference, with 95% confidence interval limits, using the Fisher’s exact test.\(^{27}\)

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**Results**

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

By carrying out 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 in only one patient a fragment with an altered electrophoretic mobility. The cultured fibroblasts of this patient previously showed a decreased synthesis of type III collagen.\(^{19}\) In order 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.\(^{28}\)). 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 using a restriction analysis according to Tromp et al.\(^{26}\)

To determine if 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 untranslated part of type III collagen showed heterozygosity in 25 of the 41 patients; one 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.

In the study for abnormal allele frequency in the type III collagen gene using a highly variable, tandem repeat marker in intron 25 of the COL3A1 gene, with 6 alleles, no difference in allele frequency was found in our patients compared to the control group (Table 1), nor was this found in the patients with a decreased type III collagen production compared to a normal production.

Table 1. 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% confidence limits (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>226</td>
<td>0</td>
<td>1</td>
<td>+1.2</td>
<td>-1.2 / 3.6</td>
</tr>
<tr>
<td>242</td>
<td>34</td>
<td>37</td>
<td>+3.7</td>
<td>-18.8 / 11.5</td>
</tr>
<tr>
<td>258</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>-13.1 / 13.1</td>
</tr>
<tr>
<td>274</td>
<td>24</td>
<td>21</td>
<td>-3.7</td>
<td>-17.3 / 10.0</td>
</tr>
<tr>
<td>290</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>-5.8 / 5.8</td>
</tr>
<tr>
<td>306</td>
<td>1</td>
<td>0</td>
<td>-1.2</td>
<td>-3.6 / 1.2</td>
</tr>
</tbody>
</table>

**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 a 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 one 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, 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 were not analysed. The globular part of the C-propeptide is essential for the formation of the triple helix in fibrillar collagens. 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 proc1 chain or it may lead to an abnormal association with all three α1 chains being destroyed. Therefore, the C-propeptide of the type III collagen gene was sequenced, showing no changes. Our data strengthen the conclusion of the Kuivaniemi study 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. Single or multi-exon deletions can be demonstrated in cDNA as we found in our collagen mutation studies (unpublished results). Large PCR products from cDNA in these patients did not show evidence for 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 a reduced type III collagen secretion have been described. These patients showed a normal or mild clinical phenotype. In 25 patients, including one patient with a 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 a mutation in the type III collagen was found using SSCP/heteroduplex. SSCP analysis, with PCR fragments of 350 nucleotides, detects more than 80% of the mutations. 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 al. and not with SSCP/heteroduplex analysis.

The Ehlers Danlos syndrome vascular type phenotype varies from classical ("acrogeric") presentation to almost no visible abnormalities ("atypical"). In patients with classical 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.\textsuperscript{36} Our patients with decreased production of type III collagen may be considered as having atypical Ehlers Danlos syndrome vascular type. The decreased production of type III collagen in the patients with “atypical” Ehlers Danlos Syndrome vascular type may be due to defects during post-translational modifications or an altered collagen metabolism e.g. elevated gelatinase activity.\textsuperscript{37}

The reported association of intracranial aneurysms with a type III collagen polymorphism\textsuperscript{22} was not confirmed in the present study, as none of the 6 alleles of a highly variable marker showed a difference in frequency in the patients compared to 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 a reduced production of type III collagen is a contributive factor to the formation of intracranial aneurysms in some patients, the exact causative molecular mechanisms of this aberration await further studies.

References


Type III collagen deficiency in a family with intracranial aneurysms

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