Mutations in the Tropoelastin Gene (ELN) Were Not Found in Patients With Spontaneous Cervical Artery Dissections

Caspar Grond-Ginsbach, PhD; Constanze Thomas-Feles, MD; Inge Werner; Ralf Weber, MD; Frank Wigger, MD; Ingrid Hausser, PhD; Tobias Brandt, MD

Background and Purpose—The majority of patients with spontaneous cerebral artery dissection show ultrastructural alterations in dermal collagen and elastic fibers.

Methods—We studied the gene encoding tropoelastin (ELN) by reverse transcription–polymerase chain reaction and subsequent sequence analysis in 10 patients with abnormalities in their elastic fibers.

Results—No mutations were found in the whole coding region of the ELN gene. The simultaneous visualization and quantification of ELN splice variants by gene scanning enabled the analysis of the regulation of alternative splicing of ELN mRNA. No differences could be detected between fibroblast cultures of the patients and a control subject.

Conclusions—Neither mutations in the ELN gene nor dysregulation of its activity appears to be the cause of the connective tissue disorder that is found in most patients with spontaneous dissections. (Stroke. 2000;31:1935-1938.)

Key Words: dissections ■ RNA splicing, alternative ■ sequence analysis ■ tropoelastin

Spontaneous dissections of the cervical arteries (sCADs) are increasingly recognized as a cause of stroke among young and middle-aged patients.1 Ultrastructural abnormalities were found in skin biopsies of the majority of patients with sCADs, which suggests a predisposing generalized connective tissue disorder.2 Major findings in the dermal connective tissue included enlarged and irregular collagen fibrils and pronounced elastic fiber fragmentation. The morphology of the ultrastructural aberrations resembled the morphology found in Ehlers-Danlos syndrome type II and III for the collagen changes and that found in the marfanoid or pseudoxanthoma elasticum phenotypes for the elastic fiber abnormalities. Some known connective tissue disorders predispose for sCAD, but most sCAD patients do not display symptoms of a known hereditary syndrome.3 It is not known whether the multiple ultrastructural defects in the dermal connective tissue from sCAD patients depend primarily on a disorder of the collagen fiber system (with morphological alterations of the elastic fibers as secondary effects), whether aberrations of the elastic fibers are the fundamental defect, or whether some other factors in the assembly of the extracellular matrix are directly involved in the phenotype.

Elastic fibers are complex structures, composed of microfibrils and amorphous material. Fibrillin and various microfibril-associated proteins are found in the fibrillar part, whereas the amorphous part of the elastic fibrils consists mainly of elastin, a cross-linked form of posttranslationally modified tropoelastin.4 Several pathological conditions (supravalvular aortic stenosis, cutis laxa, and moyamoya disease) have been correlated with mutations in the gene encoding tropoelastin (ELN)5–8 or with dysregulation of ELN gene activity.9

In the present study, we investigate by direct sequence analysis the coding region of the ELN gene in sCAD patients with moderate to severe alterations in the morphology of the elastic fibers. Fibroblast cultures from 10 patients were analyzed. The pattern of alternative splicing of the ELN mRNA of the patients was also compared with ELN mRNA from a healthy control person.

Subjects and Methods
From an initial series of 25 patients with sCADs, we selected 10 patients with pronounced morphological alterations in the dermal elastic fibers for the present study. None of the patients showed signs of a known connective tissue disorder.

Skin biopsies were taken from the outer side of the upper arm by open deep-knife biopsy. Part of the material was processed for electron microscopy according to Anton-Lamprecht.10 From another part of the biopsy, fibroblasts were cultured in MEM supplied with 10% FCS according to standard procedures.

RNA was prepared from cultured skin fibroblasts with RNA-zol (AGS-Heidelberg), and cDNA was synthesized with mulV Reverse Transcriptase and random hexamers (Perkin-Elmer). A mixture of deaza-dGTP and dGTP (3:1) was used for the first-strand cDNA synthesis. The whole coding sequence of the ELN cDNA was amplified by use of synergy Taq with proofreading activity from Natutech in a 20 µL volume in the presence of 7% dimethyl sulfoxide by nested polymerase chain reaction (PCR) in overlapping fragments. To each amplification, 0.5 µL cDNA was added, which
corresponds to an initial 10 to 25 ng mRNA. After 22 cycles of amplification, the DNA was diluted 1:100. One microliter was amplified with a set of nested primer for another 22 cycles. After an initial denaturation for 1 minute at 94°C, the denaturation in each round of amplification was reduced to 5 seconds; annealing lasted 15 seconds at a temperature that was 5°C lower than the temperature of the primers. Primer extension was performed at 68°C for a duration of 1 minute for 1000 bp.

PCR primers were named according to their position on the sequence M36860 by Fazio et al., except for primer 18855 (3’ untranslated region), which was designed on the basis of the sequence U63721, and +99 (exon 26A), which was designed on the basis of the sequence U93037. Forward primers are as follows: 00006 (ATAAAACGAG-CCAG), and CAGGTGTCCCTAGTG), 01891 (CTCTCGGTGGAGTAGGCATC-GGGGTTGGAGCTGGGGGCTTTCC), 01295FAM (GGAGTCGGGGACT), 00998 (GGAGCTGCTGCAGGCTTAGT), 01240 GTGCGGAG), 00027 (GGGCTGGGGCATTTCT), 00453 (GAGTCGCT), 01936 Stroke August 2000

The Table summarizes relevant clinical data of the patients. Patients: Clinical History and Dermal Connective Tissue Diagnosis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at Time of Dissection, y</th>
<th>Clinical Diagnosis</th>
<th>EM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>35</td>
<td>L-VA</td>
<td>++ +</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>43</td>
<td>R-ICA</td>
<td>++ +</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>52, 54</td>
<td>L-ICA/R-ICA</td>
<td>++ +</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>38</td>
<td>R-ICA</td>
<td>++ +</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>42, 42</td>
<td>L-ICA/R-VA</td>
<td>+ +</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>43</td>
<td>L-VA</td>
<td>+ +</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>50</td>
<td>L-ACI</td>
<td>+ +</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>31, 32</td>
<td>L-VA/R-VA</td>
<td>+ +</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>47</td>
<td>L-ACI</td>
<td>0 +</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>59</td>
<td>L-ACI</td>
<td>++ +</td>
</tr>
</tbody>
</table>

EM indicates electron microscopic diagnosis of a deep-knife dermal biopsy; M, male; L-VA and R-VA, left and right vertebral artery, respectively; L-ICA and R-ICA, left and right internal carotid artery, respectively; Coll, collagen fibrils; Elast, elastic fibers; and 0, +, +++, and +++++, semiquantitative evaluation of the abnormal morphology (0 to +++ indicates normal to highly pathological range).

Alternative Splicing of ELN mRNA in Cultured Fibroblasts

We established fibroblast cultures from dermal biopsies of patients and a control person and analyzed the coding region of the ELN gene by reverse transcription PCR. After reverse transcription of total RNA from cultured fibroblasts, the whole coding region of the ELN gene was amplified in 4 overlapping fragments. Three of these amplified fragments were processed directly for cycle sequencing analyses. However, the 3’ end of the cDNA is alternatively spliced, and various fragments of different length are produced by PCR. In cultured dermal fibroblasts, we observed 2 major cDNA variants of different length (peaks B and D in Figure 2). After cloning and sequencing of the PCR fragments, these were interpreted as different splice variants. Exons 22, 24A, and 26A were never found in 20 analyzed clones from region 0027 up to region 2236 of the ELN cDNA, encompassing the whole coding region. Exons 23, 32, and 33 were absent in samples of the messengers because of alternative splicing. Major splice products can be visualized and quantified by gene scanning of regions 1295 through 2236 of the cDNA: the full-length mRNA (E, 9.7%), the mRNA without exon 32 (D, 42.0%), the mRNA without exons 32 and 33 (C, 3.3%), the mRNA without exons 32 and 33 (B, 34.8%), and the mRNA without exons 23, 32, and 33 (A, 4.2%). These 5 splice variants encompass ≈95% of the total amount of amplified ELN cDNA. However, various other splice variants are seen as small peaks in Figure 2. Moreover, with the aid of a PCR primer that anneals in exon 26A (an exon that is known to be rarely expressed), a product was generated in the cDNA samples of most patients (data not shown). Hence, an even higher number of splice variants of the ELN transcript is produced in at least some patients.
The overall pattern of splice variants in cultured fibroblasts of different patients and of the control subject is similar (Figure 2, A through D).

**Sequence Analysis of the Coding Region of the ELN mRNA**

PCR fragments were cycle-sequenced in both directions. The obtained results were compared with the published consensus sequence.¹¹ No mutation was detected in any of the patients. Not analyzed were the sequences of exon 22, exon 24A, and exon 26A, because we never or rarely found these sequences expressed in our cDNA.

**Discussion**

In earlier molecular genetic investigations of sCAD patients, mutations were found neither in the COL3A1 gene¹³,¹⁴ nor in COL5A1.¹⁵ Because pronounced alterations of the elastic fibers were also a frequent ultrastructural finding in patients with sCAD,² we hypothesized a mutation in the elastin-encoding gene ELN. The present investigation of the whole coding region of the ELN gene did not lead to the identification of mutations. Moreover, the pattern of alternatively spliced mRNAs and the relative proportion of the alternative transcripts seems normal for all samples tested. Therefore, neither mutations in the ELN gene nor dysregulation of its expression seems to be a relevant factor in the etiology of spontaneous cervical artery dissections.

For the present study, we selected sCAD patients without signs of a known connective tissue disorder and with strong morphological alterations of the dermal elastic fibers. In this subset of patients, we found 3 patients with recurrent dissections. Moreover, 1 of them had a first-degree relative (father) with a dissection too. The patients in the present study were selected according to such restrictive criteria that the falsification of our hypothesis of possible mutations in the ELN gene in patients with sCAD seems to be definite. It is remarkable that no female patients were found in this sample. It has been observed in a large series of patients with sCAD (T. Brandt, E. Orberk, R. Weber, unpublished data) that morphological connective tissue alterations are found more frequently in male sCAD patients than in female sCAD patients. Moreover, in the sample of female sCAD patients with morphological connective tissue aberrations, these aberrations are generally of a somewhat milder degree than those found in male patients.

The ELN gene was examined in the present study on the basis of RNA from cultured fibroblasts. This enabled both the rapid sequence analysis of large parts of the coding regions and the analysis of various splice products. However, the regulation of the transcriptional activity and of alternative splicing is known to be tissue specific. The expression of the ELN gene in cultured fibroblasts might differ substantially from the in vivo situation in the dermal connective tissue or in the tissues at risk (the vessel walls of the arteria carotis and arteria vertebralis).

Our investigation of the coding region of the ELN gene did not explain the observations made in dermal biopsies of patients with spontaneous cervical artery dissections. The cause for the abnormal morphology of the elastic fibers remains unclear. Either some other structural components of the elastic fiber or some of the many factors that regulate its biosynthesis might be defective, or the abnormal morphology of the elastic fiber is secondary.

Until now, the search for mutations in sCAD patients was not successful. However, an autosomal dominant inheritance of an ultrastructural connective tissue disorder was recently demonstrated in some families of patients with sCAD.¹⁶ The study of such pedigrees might enable the identification of further candidate genes that are involved in the pathogenesis of this disease.
Acknowledgments

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References

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