Ultrastructural Connective Tissue Aberrations in Patients With Intracranial Aneurysms

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Background and Purpose—An unknown connective tissue defect might predispose for the development and rupture of intracranial aneurysms in some patients. This study of connective tissue samples of a series of patients with intracranial aneurysms investigates the morphology of the extracellular matrix with methods that are currently used in the routine diagnosis of inherited connective tissue disorders.

Methods—Skin biopsies from 21 patients with intracranial aneurysms, many with multiple aneurysms, were studied by electron microscopy. None of the patients included in this study showed clinical signs of a known connective tissue disorder.

Results—In 7 patients (33%), we observed repetitive aberrations in the morphology of collagen fibrils and elastic fibers of the reticular dermis. The observed ultrastructural findings were somewhat similar to those typically observed in patients with Ehlers-Danlos syndrome (EDS) and in a subgroup of patients with spontaneous cervical artery dissections. The patterns of abnormalities fell into 2 classes: 4 patients displayed abnormalities that resembled those found in patients with EDS type III, and the electron microscopic findings in the skin biopsies from 3 patients resembled those of EDS type IV patients. The sequence of the COL3A1 gene from the patients with EDS type IV–like alterations of the connective tissue morphology was analyzed. No mutation was detected.

Conclusions—Connective tissue alterations were found in skin biopsies from a minority of patients with intracranial aneurysms. Electron microscopic investigation of skin biopsies from patients and their relatives might become valuable for clinical diagnostics, identification of persons at risk, and basic studies of the pathogenesis of this vascular disease.

Key Words: aneurysm ■ connective tissue disorders ■ hereditary disease ■ microscopy, electron microscopy

Subarachnoid hemorrhage (SAH) resulting from the rupture of an intracranial aneurysm (IA) is a severe disease with high mortality and morbidity. About 3% to 6% of the population >30 years of age harbor an unruptured aneurysm,1,2 and up to 30% of patients with IA have multiple aneurysms.3-5 The rupture rate of asymptomatic aneurysms is estimated to be between 0.05% (small aneurysms in patients with no prior SAH) and 1% (aneurysms >10 mm) per year.6 SAH accounts for 5% of stroke deaths and for more than one quarter of potential life-years lost because of stroke.1,2

It is largely unknown why only some people develop IA and most do not. Some acquired changes in the arterial wall are likely to play a role because hypertension, smoking, and alcohol abuse are risk factors for SAH.3 Genetic factors are also thought to increase the risk of IA development and rupture because IA is associated with a variety of inherited conditions. For instance, the relative risk for a ruptured aneurysm is increased in the classic and vascular types of Ehlers-Danlos syndrome (EDS types I/II and IV) and in autosomal dominant polycystic kidney disease (PKD1 and PKD2).7-11 Some patients with IA without a known genetic disorder have a family history of IA, which furthermore suggests a role of genetic factors in those patients who do not suffer from a known connective tissue disorder.12-15 Various research groups tried to identify candidate genes harboring disease-causing mutations or functional polymorphisms. However, no genetic factors have been found to be significantly associated with IA in nonsyndromic patients.16-20 It is reasonable to expect a stronger impact of inborn risk factors in younger patients and in patients with multiple IAs. We therefore selected for this pilot study younger patients with multiple IAs or with a family history and excluded patients with known lifestyle risk factors.

Skin can still be regarded as a “window” to heritable disorders of connective tissue.21 Biochemical and ultrastruc-
tural investigations of a skin biopsy are useful for the diagnosis of connective tissue disorders, particularly as long as specific molecular tests are not yet available. In this study, we investigated skin biopsies of patients with IAs by light and electron microscopy. We analyzed the morphology of the connective tissue with the same histological and electron microscopic methods used for the histological and ultrastructural diagnosis of EDS, pseudoxanthoma elasticum, and cutis laxa.22,23

Materials and Methods
Patients with IA were carefully examined for clinical signs of a known hereditary connective tissue disorder. We selected 21 patients (14 women, 7 men; mean age, 44 years) without clinical signs of connective tissue disorder for this study. Of these, 17 suffered from SAH caused by ruptured IA. The diagnosis of IA was confirmed by digital subtraction angiography in all patients.

For the collection of the skin biopsies and the subsequent handling and preparation of the material, we followed a standardized protocol developed for the diagnosis of connective tissue disorders. Biopsies were obtained by open, deep knife biopsy from the outer aspect of the upper arm close to the elbow. Specimens were prepared for light microscopy and electron microscopy as described elsewhere.22 As a control group, 10 patients (6 men, 4 women; mean age, 41 years; no signs of connective tissue disorder) were included in this study. For the analysis of the part of the COL3A1 gene that encodes the α(III)-chain, we isolated RNA from cultured fibroblasts and synthesized cDNA with random hexamers as described elsewhere.25 The α(III) encoding region was amplified in 6 overlapping fragments with the following primers: GTTCTCTGCGATGACATAATATGTGACGAA/ TGGAGAACCTGGCAGAGATGGCGTCCC/AGTAGGACCCCTTGTACGGCCAGGACTT/TTCCTGGTCCTCCTGGACTTCCGGGCA, TGACCATACATTGCTCGAGCACCGTCA, TGGTGAGCCGAGACCCCAAGACTT/TTCTCTCGCTCTCTCTGGACTCTCGGCA, TGGAGAACTCAGAGATGCCGTCCCA/AGTGAAGACCCCTT/GGCCCCATCTTCCCT, CCTGCTGTCCAGAAATGCCTTGGAGAAA/GGCCCTTGGGCCACCAGCGCATCCTTTC, GTTGCTCTCCAGGAGGCTTTGGGAGAGT, CTTGAGAGTTGGTGGTGCCGCTCCTCCACCACACCCCAG, and TGTGCAGACGGTGACACTCCGAGCAGTCA/ACAGAATACCTTGATAGCATCCA. The sequence of each fragment was analyzed in both directions and compared with the published consensus sequence of the COL3A1 gene (accession number X14420).26

Results
In 7 of 21 patients with IA, we found definite and repetitive alterations of the morphology of the connective tissue in the reticular dermis. The Table summarizes the clinical data of the patients and specifies the ultrastructural findings of the skin biopsies.

Electron micrographs of the reticular dermis of 2 patients are shown in the Figure. Several fibers in the collagen bundles of the skin of patient 1 were irregular with a so-called flowerlike appearance in cross section (arrows). Similar morphological aberrations are typically found in patients with EDS type III (hypermobility type).22 A different but again abnormal morphology was found in the connective tissue of patient 6. Irregular contours of collagen fibers were rarely found, but their diameter was slightly reduced and they were more variable than usual. The packing of the bundles was less dense and regular than in healthy control subjects. Moreover, the thickness of the reticular dermis was reduced. Such electron microscopic aberrations are characteristic of patients with EDS type IV (vascular type).22 The morphology of the elastic fibers was also abnormal in these patients (data not shown). In patient 1, elastic fibers were fragmented with electron dense calcified inclusions. In patient 6, compared with the collagen bundles, the elastic material was enriched in mass but did not show abnormal calcification or fragmentation (resembling EDS type IV). Despite the resemblance of the ultrastructural morphology, none of these patients with IA showed other typical symptoms of these EDS subtypes apart from the vascular symptoms. In 7 of our patients, we found such mild but reproducible aberrations. In 4 other patients,9,12,15,20 the electron microscopic findings were difficult to interpret. They were classified as normal, although the connective tissue morphology seemed to be somewhat irregular, but the aberrations were very mild.

The sequence of the whole α(I)III encoding region of the COL3A1 gene of the 3 patients with a EDS type IV–like electron microscopic morphology2,6,17 was analyzed after amplification of the COL3A1 cDNA and subsequent direct cycle sequencing. No mutations were found in the whole α(I)III encoding region of these patients.

Discussion
Electron microscopic connective tissue alterations were found in 7 of 21 patients with IA. The organ of interest, the wall of the cerebral arteries, was not investigated in this study because biopsy material from the arterial wall of patients is rarely available. Moreover, the histology of the arterial wall is dramatically modified in and around the aneurysm and rarely reflects the constitutional texture of the wall but visualizes the pathological reactions on aneurysm formation and rupture. We therefore investigated skin biopsies from the patients, which we consider the window to heritable disorders of the connective tissue. Comparable alterations in skin biopsies were not observed in a series of 10 control subjects with ischemic stroke or in a large series (>3000 individuals) of diagnostic skin biopsies that were analyzed at the electron microscopy laboratory of the Dermatology Department of the University of Heidelberg.

For this first investigation of skin biopsies from patients with IA, we excluded patients with known risk factors to focus on patients with a possible constitutional predisposition for IA but without signs of known connective tissue disorder. Our series of patients is apparently a nonrandom sample with a possible bias in the frequency and grade of connective tissue abnormalities because most patients selected for this study carried multiple aneurysms, whereas the proportion of them has been estimated to be about one third of the total population of patients.3–5 The results of this actual investigation should therefore not be generalized to the population of all patients with IA. This pilot study merely documents that electro microscopic connective tissue disorder display alterations in the morphology of the connective tissue in the reticular dermis. A systematic and prospective study is needed to estimate the prevalence of this connective tissue disorder among all subjects with IA.

The standardization of tissue sampling, specimen preparation, and evaluation of electron microscopic observations is important for proper diagnosis of connective tissue disorders by electron microscope. We compared the ultrastructural
morphology with a series of age-matched control subjects with stroke of other origin, evaluated a deep knife biopsy from the upper elbow, and analyzed only deeper parts of the reticular dermis to control for those factors that could increase the variability of the results, such as mechanical stress, aging, or ultraviolet ray exposure. Under these conditions, the presence of repetitive and constant alterations in the morphology of connective tissue is significant and must be interpreted as a pathological finding. Despite extensive experience with diagnostic skin biopsies, we had difficulty evaluating the ultrastructural findings of 4 patients. They were all classified as normal, although we could not exclude with certainty minor pathological findings. The finding of clearly pathological patterns of connective tissue morphology among 7 of 21 patients (33%) therefore might even be an underestimation.

The alterations in connective tissue in the reticular dermis of patients with IA have similarities with alterations found in patients with spontaneous cervical artery dissection.23,27 The ultrastructural morphology of the connective tissue suggests that some patients with IA and with spontaneous cervical artery dissection carry a defect in the extracellular matrix, leading to a structural defect in the arterial wall and predisposing for vascular diseases. The finding of electron microscopic alterations in the reticular dermis, however, is not specific for a particular disease. Comparable alterations are found in patients with EDS types II, III, and IV24 and in heterozygous carriers of pseudoxanthoma elasticum.23

### Clinical, Vascular, and Electron Microscopic Observations of Patients With IA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Name</th>
<th>Sex</th>
<th>Age, y</th>
<th>Clinical Diagnosis</th>
<th>Corresponding Vascular Abnormalities</th>
<th>Morphology of Connective Tissue in Skin Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ü.M.</td>
<td>M</td>
<td>23, 28</td>
<td>SAH IV</td>
<td>IA of ACA,* IA of left ICA</td>
<td>Like in patients with EDS III</td>
</tr>
<tr>
<td>2</td>
<td>B.M.</td>
<td>F</td>
<td>49</td>
<td>SAH III</td>
<td>IA of ACA,* IA of medCA*</td>
<td>Like in patients with EDS IV (no mutation in COL3A1)</td>
</tr>
<tr>
<td>3</td>
<td>F.P.</td>
<td>F</td>
<td>38</td>
<td>SAH III</td>
<td>IA of right ICA,* fusiform dilatation of BA</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>D.M.</td>
<td>F</td>
<td>38</td>
<td>SAH II</td>
<td>IA of right ICA*</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>H.P.</td>
<td>M</td>
<td>43</td>
<td>SAH IV</td>
<td>IA of antCA*</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>W.G.</td>
<td>F</td>
<td>57</td>
<td>SAH IV</td>
<td>IA of ACA,* IA of right ICA, IA of left ICA</td>
<td>Like in patients with EDS IV (no mutation in COL3A1)</td>
</tr>
<tr>
<td>7</td>
<td>R.-S.B.</td>
<td>F</td>
<td>35</td>
<td>SAH III</td>
<td>IA of distal right ICA*</td>
<td>Normal</td>
</tr>
<tr>
<td>8</td>
<td>S.A.</td>
<td>F</td>
<td>49</td>
<td>SAH II</td>
<td>IA of medCA right,* IA of PCA, fusiform dilatation of medCA left</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>K.P.</td>
<td>F</td>
<td>55</td>
<td>SAH I</td>
<td>IA of ACA, IA of medCA left, IA of medCA right, IA of BA, IA of antCA</td>
<td>Normal</td>
</tr>
<tr>
<td>10</td>
<td>H.B.</td>
<td>F</td>
<td>55</td>
<td>SAH IV</td>
<td>IA of medCA left, IA of ACA,* IA of ICA left, IA of medCA right, fusiform dilatation of ICA right</td>
<td>Normal</td>
</tr>
<tr>
<td>11</td>
<td>E.A.</td>
<td>F</td>
<td>65</td>
<td>Brainstem symptoms; brother, sister, and (probably) mother died of SAH</td>
<td>Fusiform dilatation of BA</td>
<td>Normal</td>
</tr>
<tr>
<td>12</td>
<td>T.H.</td>
<td>M</td>
<td>67</td>
<td>Recurrent TIA</td>
<td>IA of VA right</td>
<td>Normal</td>
</tr>
<tr>
<td>13</td>
<td>B.I.</td>
<td>F</td>
<td>48</td>
<td>SAHII</td>
<td>IA of medCA left*</td>
<td>Normal</td>
</tr>
<tr>
<td>14</td>
<td>M.S.</td>
<td>F</td>
<td>47</td>
<td>SAH</td>
<td>IA of antCA left,* IA of ICA left</td>
<td>Normal</td>
</tr>
<tr>
<td>15</td>
<td>W.-W.B.</td>
<td>F</td>
<td>36</td>
<td>SAH IV</td>
<td>IA of right ICA,* IA of anterior choroid artery</td>
<td>Normal</td>
</tr>
<tr>
<td>16</td>
<td>S.M.</td>
<td>M</td>
<td>21</td>
<td>SAH V</td>
<td>IA in postCA*</td>
<td>Normal</td>
</tr>
<tr>
<td>17</td>
<td>G.E.</td>
<td>M</td>
<td>48</td>
<td>SAH; brother had 2 SAHs and clipped IAs</td>
<td>No IA detected</td>
<td>Like in patients with EDS IV (no mutation in COL3A1)</td>
</tr>
<tr>
<td>18</td>
<td>D.A.</td>
<td>M</td>
<td>61</td>
<td>Recurrent brainstem symptoms; father died of SAH</td>
<td>Fusiform dilatation of BA*</td>
<td>Like in patients with EDS III</td>
</tr>
<tr>
<td>19</td>
<td>V.D.</td>
<td>F</td>
<td>22</td>
<td>Lyme disease suspected</td>
<td>IA in P1 segment links*</td>
<td>Mild but resembling the morphology in EDS III</td>
</tr>
<tr>
<td>20</td>
<td>W.I.</td>
<td>F</td>
<td>34</td>
<td>SAH V</td>
<td>IA of A pericallosa,* IA of medCA</td>
<td>Normal</td>
</tr>
<tr>
<td>21</td>
<td>F.E.</td>
<td>M</td>
<td>41</td>
<td>SAH II</td>
<td>IA of ICA right,* stenosis of ICA left</td>
<td>Like in patients with EDS III</td>
</tr>
</tbody>
</table>

ACA indicates anterior communicating artery; ICA, internal carotid artery; antCA, medCA, and postCA, anterior, medial, and posterior cerebral artery; BA, basilar artery; PCA, posterior communicating artery; TIA, transient ischemic attack; and VA, vertebral artery.

*Clinical symptoms were probably caused by this vascular abnormality.
The morphological alterations observed in the patients with definitely positive electron microscopy fell into 2 qualitatively different types: in 4 patients, we observed a pattern of abnormalities that is characteristic of patients with the hypermobility type of EDS (type III), and the aberrations in 3 other patients resemble those found in patients with vascular EDS (EDS type IV). Mutations in COL3A1 were not found in several studies of patients with IA.\(^\text{26,29}\) Typical COL3A1 mutations were also excluded in those 3 patients, despite their EDS type IV–like connective tissue. The patients with EDS type IV–like morphology therefore do not suffer from EDS type IV but appear to suffer from a different disorder with comparable electron microscopic findings in the skin but with a possibly different pathomechanism. The patients with EDS type III–like ultrastructural findings in the skin apparently do not suffer from hypermobility EDS because other typical symptoms were not present. However, as long as the molecular basis of EDS type III is unclear, there is no molecular test to exclude this subtype of EDS as an underlying disorder in patients with IA. In addition, a minor variant of EDS type III is possible.

The morphology of the collagen fibers of patients with EDS type IV differs essentially from the pattern seen in patients with the hypermobility type of EDS, and different genes are mutated in these patients. In analogy to the genetic heterogeneity of EDS subtypes, we speculate that our series of patients might also suffer from genetically heterogeneous diseases and that at least 2 different molecular defects will be found among them. In conclusion, a defect in the extracellular matrix, possibly predisposing for aneurysm formation, could be demonstrated in a subgroup of patients with IA.

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