Some patients with intracranial aneurysms have a reduced type III/type I collagen ratio

A case-control study

J.S.P. van den Berg, MD; M. Limburg, MD, PhD; G. Pals, PhD; F. Arwert, PhD; A. Westerveld, PhD; R.C.M. Hennekam, MD, PhD; and K.W. Albrecht, MD

Article abstract—A reduced production of type III collagen has been reported in previous studies to be associated with intracranial aneurysms. The purpose of this prospective case-control study was to assess the possible role of a reduced type III/type I collagen ratio as a risk factor for having an intracranial aneurysm. The study group consisted of 41 consecutively admitted patients with intracranial aneurysms. Intracranial aneurysms were demonstrated by intra-arterial digital subtraction cerebral angiography or during operation. The control group consisted of 41 healthy volunteers matched for age and sex. Fibroblasts were cultured from skin biopsies from patients and control subjects, and the type III/type I collagen ratios were determined. The type III/type I collagen ratios in the controls ranged from 5.5 to 19.8%, with a median ratio of 10%, and none had a ratio below 5.5%. The type III/type I collagen ratios in patients ranged from 1.1 to 25.1%, with a median ratio of 10.5%, and eight patients (19.5%) had a low (<5.5%) ratio \( p = 0.005, \) Fisher's exact test. Our findings support the hypothesis that a reduced production of type III collagen may contribute to the formation of intracranial aneurysms in some patients.

Subarachnoid hemorrhages represent approximately 7% of all strokes\(^1\) and account for 45% of the strokes between the ages of 35 and 44 years.\(^2\) Over the last four decades, the incidence of subarachnoid hemorrhages has not changed, and the mortality rate has remained high.\(^3,4\) Many survivors remain in a disabled state.\(^5\) Most subarachnoid hemorrhages are caused by rupture of an intracranial aneurysm.\(^6\) Early detection and preventive surgery may lead to an increased lifespan.\(^5\) Mortality and morbidity for surgery of unruptured intracranial aneurysms are relatively low.\(^6\) Understanding of the underlying mechanisms leading to the formation of intracranial aneurysms is important in view of the development of preventive strategies.

One hypothesis considers the pathogenesis of intracranial aneurysms to be a multifactorial process in which both intrinsic factors, such as vessel wall abnormalities, and extrinsic factors, such as hypertension, play a role.\(^7,8\) A reduced production of type III collagen is a possible candidate for such an intrinsic factor. Several observations suggest a relationship between a defect of type III collagen and the pathogenesis of intracranial aneurysms. Type III collagen is responsible for the tensile strength of the arteries, especially when the strain on the vessel wall becomes high.\(^10-12\) Mutations in the type III collagen gene, encoding the type III procollagen, produce the Ehlers–Danlos type IV phenotype.\(^13\) Ehlers–Danlos type IV patients show a diversity of clinical symptoms, including skin laxity and ruptured intracranial aneurysms.\(^14,15\)

Several protein-based studies have revealed a relationship between a reduced production of type III collagen and intracranial aneurysms.\(^16-21\) However, Kuivaniemi et al.\(^22\) using DNA sequence analysis of part of the gene encoding the triple-helical domain of type III collagen in 40 patients with intracranial aneurysms, did not observe mutations and concluded that structural mutations in the gene encoding for type III collagen are not a common cause of intracranial aneurysms.

To investigate a possible significance of a reduced production of type III collagen on the protein level, we determined the type III/type I collagen ratios from skin fibroblasts of patients with intracranial aneurysms and compared these with type III/type I collagen ratios in control subjects. Our study ques-
tions were as follows: is the presence of intracranial aneurysms associated with a reduced production of type III collagen, and, if so, is there a difference in the clinical features of these patients with a normal and reduced production of type III collagen?

Methods. In a prospective study, we included all consecutive patients with an intracranial aneurysm admitted to the Department of Neurosurgery of the Academic Medical Center in Amsterdam. The diagnosis had to be confirmed by a three-vessel intra-arterial digital subtraction angiography or at craniotomy. Hypertension, polycystic kidney disease, or other renal abnormalities; symptoms fitting Marfan syndrome; Ehlers–Danlos syndrome type IV; pseudoxanthoma elasticum; and family history of the same factors were recorded from the medical history. Patients were examined by two experienced clinical geneticists (R.C.M.H. and the late J.W. Oorthuys) for signs of Ehlers–Danlos syndrome type IV, Marfan syndrome, and pseudoxanthoma elasticum. The cerebral angiograms were independently studied by two investigators who scored the localization and size of the aneurysm. The largest measured diameter in any projection was taken as the size of the aneurysm. In case of any discrepancy, the angiograms were discussed, and a consensus between the two observers was obtained. The control group consisted of 41 healthy volunteers (personnel from the Academic Medical Center) matched for age and gender and without a history of intracranial aneurysms. The study was approved by the Hospital Medical Ethical Committee.

Biochemical and molecular studies. After informed consent, a skin biopsy was taken for fibroblast culture. Fibroblasts were cultured to confluence in 25 cm² flasks in Ham’s F10 (Life Technologies, Bethesda, MD) with 10% fetal calf serum. Metabolic labeling was performed for 20 hours in 2 mL BME prolin free media (Life Technologies) containing 5% dialyzed fetal calf serum, 37 μg/mL L-ascorbic acid, 2 μCi/mL L-[U¹⁴C]-Prolin (Amersham International, Little Chalton, U.K.). After ethanol precipitation of 333-μL aliquots of media, the pellets were dissolved in 150 μL 0.5 M acetic acid. One aliquot of each sample was treated with pepsin for the analysis of collagen; untreated aliquots were used for procollagen. The cells were trypsinized, centrifuged, washed, and frozen as dry pellets. The pellets were homogenized in 150 μL 0.5 M acetic acid. After centrifugation, the supernatants were used for electrophoresis. After liquid scintillation counting, 20,000 cpm aliquots of the samples (between 5 and 10 μL) were lyophilized, dissolved in 20 μL of sample loading buffer (Promega Corp., Madison, WI), and subjected to SDS-PAGE in a Protein II electrophoresis system (Bio-Rad, Hercules, CA). After 90 minutes at 200 V (10°C), the samples were reduced in the gel by addition of 30 μL of dithiothreitol solution (7.7 mg/mL in 40% saccharose) in each slot (reduction of samples before electrophoresis leads to overlap of type I and III collagens). After another 90 minutes at 400 V, the gels were dried and analyzed with a Phosphor-Imager (Molecular Dynamics, Sunny Valley, CA). The signals from the radioactively labeled collagen chains were quantitated in arbitrary but linear units using a Phosphor-Imager.23 The ratio of type III/type I collagen, defined as the α(III)/α(I) + α2(I) + α1(III) ratio, was then computed. In all subjects, the final ratios are the average results of three independent separate labeling experiments.

All collagen determinations were done within four passages of the biopsy.

All type III/type I collagen ratios encountered in the control group were considered to represent normal values. As a result, all values found in the patient group that were below the lowest value of the control group were considered to represent abnormally low type III/type I ratios. In patients with abnormally low ratios of type III/type I collagen, intracellular type III procollagen was assessed by electrophoretic analysis of the content of lysed cells. Structurally abnormal type III procollagen has an abnormal electrophoretic mobility.24

In the statistical analyses, Fisher’s exact test and Wilcoxon rank sum test were used.

Results. Most patients (38/41) presented with a ruptured intracranial aneurysm. In all patients except one, a cerebral angiography was performed. This patient was operated on immediately because of a rapid clinical deterioration, and the presence of an aneurysm was confirmed. In the three patients without presenting subarachnoid hemorrhage, the indications for a cerebral angiography were intracerebral hemorrhage, atypical facial pain with a positive family history for intracranial hemorrhages, and headache with transient neurologic signs of visual disturbance and dysarthria. In this patient, brain CT showed a lesion compatible with an intracranial aneurysm. The median age of the patients was 49 years (ranging from 26 to 79 years). Other characteristics of the patients are shown in table 1. The three patients with a positive family history for polycystic kidney disease also had a positive family history for hypertension. Cerebral angiography in one patient also showed a small arteriovenous malformation. The median age of the control subjects was 43 years (ranging from 26 to 56 years), and there were 16 men and 25 women.

The results of the protein analysis are presented in figure 1. The type III/type I collagen ratios in the control subjects ranged from 5.5 to 19.8%, with a median of 10%. The resulting cutoff value below which type III/type I collagen ratios will be considered abnormally low was 5.5%. The type III/type I collagen ratios in the patients ranged from 1.1 to 25.1%, with a median of 10%, and eight patients (19.5%) had a very low (<5.5%) ratio (figure 2). This difference is statistically significant (p = 0.005, Fisher’s exact test). The ratios of the type III/type I collagen in patients and control subjects were compared on a group level and there was no evidence that the samples of the ratios of the patients and control subjects were derived from different distributions (two-tailed p = 0.51, Wilcoxon rank sum test).

The patient with the small arteriovenous malformation had a normal type III/type I collagen ratio. In fibroblasts from the eight patients with type III/type I collagen ratios of less than 5.5%, intracellular type III procollagen was found to be low, and there were no indications of an excess of structurally abnormal type III procollagen.

We examined whether patients with a very low type III/type I collagen ratio (<5.5%) differed in clinical characteristics from the patients with a normal type III/type I collagen ratio (table 2). Apart from a trend favoring patients with a very low type III/type I collagen ratio to have a positive family history for polycystic kidney disease, no statistically significant differences were found.
Table 1 Characteristics of 41 consecutive patients with intracranial aneurysms

<table>
<thead>
<tr>
<th></th>
<th>Number of patients (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men:women</td>
<td>16:25</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>19 (46)</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
</tr>
<tr>
<td>Subarachnoid hemorrhage</td>
<td>4 (10)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>20 (49)</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Multiple aneurysms</td>
<td>6 (15)</td>
</tr>
<tr>
<td>Localization of aneurysms</td>
<td></td>
</tr>
<tr>
<td>ACA left/right</td>
<td>0/4</td>
</tr>
<tr>
<td>ACoA</td>
<td>14</td>
</tr>
<tr>
<td>ICA left/right</td>
<td>4/5</td>
</tr>
<tr>
<td>MCA left/right</td>
<td>6/6</td>
</tr>
<tr>
<td>Basilar artery</td>
<td>5</td>
</tr>
<tr>
<td>PICA left/right</td>
<td>2/1</td>
</tr>
<tr>
<td>Clinical outcome</td>
<td></td>
</tr>
<tr>
<td>Discharge to rehabilitation center</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Discharge to nursing home</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Home</td>
<td>31 (75)</td>
</tr>
<tr>
<td>Deceased</td>
<td>6 (15)</td>
</tr>
</tbody>
</table>

ACA = anterior cerebral artery; ACoA = anterior communicating artery; ICA = internal carotid artery; MCA = middle cerebral artery; PICA = posterior inferior cerebellar artery.

Discussion. We examined a possible relationship between intracranial aneurysms and a reduced production of type III collagen through protein analysis. We found no difference in the median type III/type I collagen ratios between patients and control subjects. However, we found significantly more persons with low type III/type I collagen ratios in the patient group than in the control group. This is in keeping with the a priori formulated hypothesis. We examined the clinical characteristics of patients with normal and low type III/type I collagen ratios and found no differences.

Using protein analysis, Leblanc et al. found normal type III collagen production in five patients with familial intracranial aneurysms of different families. However, there are several reports finding a reduced production of type III collagen in fibroblasts or low amounts of collagen type III in arterial wall samples of patients with intracranial aneurysms. Oestergaard and Oxlund used postmortem samples of the middle cerebral and brachial arteries of 14 patients who died of a ruptured intracranial aneurysm and similar samples of a control group of 14 patients who died of causes unrelated to aneurysm rupture. Electrophoresis and spectrophotometry revealed a reduced amount of type III collagen in samples of the middle cerebral arteries in six patients of the ruptured aneurysm group and in none of the control group. However, the studied population was biased because only deceased patients were included. There was a difference between the type III/type I collagen ratios determined from the middle cerebral artery and the brachial artery taken from the same patient. The authors did not supply explanation for this disparity. Majamaa et al. determined the synthesis of type III collagen in skin fibroblast cultures from 11 patients with an intracranial aneurysm (plus 9 control subjects) and found no reduced pro-
duction of type III collagen, although they reported a production of unstable type III collagen in 2 patients. In this study, six patients had a first- or second-degree relative with an intracranial aneurysm, including the two patients with unstable type III collagen production. In another study by Majamaa et al. in 12 patients with aneurysmal subarachnoid hemorrhages (plus 8 control subjects), protein analysis showed combined procollagen production in two cell lines to be reduced. Despite widely varying methodology and several possible flaws in design and conduct, these studies indicated a role of reduced production of type III collagen in the formation of intracranial aneurysms.

Two patients with a low type III/type I collagen ratio (≤5.5%) from our study also had a positive family history of polycystic kidney disease, and one had polycystic kidney disease. Adult polycystic kidney disease (ADPKD) is a disease with a firmly established association with intracranial aneurysms. At least a third locus for ADPKD is still unmapped. The gene for type III collagen is located on chromosome 2. A genetic relation between ADPKD and EDS type IV is possible.

Five of eight patients with a low type III collagen had one or more risk factors for intracranial aneurysms. This is in keeping with the theory that intracranial aneurysms have a multifactorial cause. This theory is based on the following clinical and epidemiologic data. Independent acquired risk factors for intracranial aneurysms are smoking, hypertension, and age. If twins have intracranial aneurysms, they often occur in analogous or mirrored locations. Familial aggregation of intracranial aneurysms has been described on several occasions. First-degree relatives of patients with an intracranial aneurysm in particular have a two- to threefold increased chance of harboring an aneurysm. Familial aneurysms have characteristics different from those that occur spontaneously. They occur at a lower age, are located less often in the anterior cerebral artery ramifications, and may rupture at a smaller size. Among family members, aneurysms arise more often from the same arterial distribution and rupture more often within the same decade of life. However, indications exist for genetic heterogeneity. In a recent review on the pattern of inheritance in all published families, no single genetic mode could be identified. Other links to the genetic nature of intracranial aneurysms are the abovementioned association with ADPKD and with heritable connective tissue disorders. Intracranial aneurysms occur in the setting of pseudoxanthoma elasticum, osteogenesis imperfecta, Ehlers–Danlos syndrome type IV, and neurofibromatosis type 1. The suggestion of Adamson et al. on atherosclerosis being a single cause seems less likely.

Our determinations of collagen production in cell cultures will give a rough indication of the various processes happening in the arterial walls. However, our data suggest that a defect in the production of type III collagen plays a role in the pathogenesis of intracranial aneurysms. Although the reduced type III/type I collagen ratios are likely related to type III collagen deficiency, we have not demonstrated this.
has to be elucidated. We found no indications for intracellular accumulation of structurally abnormal type III procollagen in cultured skin fibroblasts with a type III/type I collagen ratio of 5.5% or less.

Kuivaniemi et al. performed DNA sequencing analysis on type III collagen cDNA in 40 patients with a high prevalence of familial aneurysms (29/55, or 53%). They found no mutations. Protein analysis was performed in one individual with a variant at amino acid 435. The protein production was normal. Unfortunately, the collagen production in the other patients was not estimated. Only the part of the gene coding for the triple-helical domain of collagen III was studied. This leaves open the possibility of mutations in the remainder of the gene (e.g., the region coding for the C-propeptide) and the possibility of defects during the posttranslational modification of type III collagen.

Our results support the theory that reduced production of type III collagen is involved in the pathogenesis of intracranial aneurysms in some patients.

Acknowledgements
M.L. is a Clinical Investigator of the “Nederlandse Hartstichting.” This project is dedicated to J.W. Oorthuys (deceased). We thank A. Gorrissen for helping to collect patient data.

References

Figure 2. Polyacrylamide gel electrophoresis of a patient with a low type III collagen (A) and a control subject with a normal type III collagen (B). Arrow 1 = type III collagen α1(III); arrow 2 = type V collagen α1(IV); arrow 3 = type I collagen α1(I); arrow 4 = type I collagen α2(I).


