Absence of collagen deficiency in familial cerebral aneurysms

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It has been suggested that a deficiency in the expression of type III collagen may play a role in the pathogenesis of cerebral aneurysms. To test this hypothesis in cases of familial cerebral aneurysms, fibroblast cell cultures were established and the expression of collagen types I and III was studied in a patient with three cerebral aneurysms whose mother and sister also had cerebral aneurysms. Cultured skin fibroblasts were labeled with tritiated proline. The collagens and procollagens were precipitated and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis after reduction to analyze procollagen chains. Control cell lines were analyzed simultaneously. Quantitation of the ratios of type III to type I procollagen synthesis was achieved by integration of the intensities of the pro-α1(III), pro-α1(I), and pro-α2(I) bands on fluorograms of electrophoretic gels of medium proteins.

There was no difference in type I and III procollagen levels observed between the cells from the aneurysm patient and those from the control cell lines. These data do not support the hypothesis that familial cerebral aneurysms are caused by a deficiency of type III collagen.

KEY WORDS • cerebral aneurysm • collagen deficiency • Ehlers-Danlos syndrome • familial disorder

The etiology of cerebral aneurysms has long been the subject of debate. Two general theories have been proposed to explain their occurrence. The degeneration theory holds that cerebral aneurysms result from an acquired degeneration of cerebral arteries under the influence of atherosclerosis and hypertension. The congenital theory proposes that cerebral aneurysms result from an inborn structural deficiency of the cerebral vessel harboring them. Other factors invoked in the formation of cerebral aneurysms include incomplete involution of fetal arteries at branching points of the adult circulation, and the rheological effects of cerebral blood flow acting as a jet at areas of sharp angulation of the arterial tree.

A possible congenital etiology for cerebral aneurysms has been suggested by the occurrence of these lesions in families and in identical twins and by their association with certain conditions with a well-established mode of inheritance such as polycystic kidney disease. More recently, the observation that some cerebral aneurysms may be associated with a minor deficiency of type III collagen and the demonstration of such a deficiency in patients with Ehlers-Danlos syndrome type IV (a genetically determined condition) has suggested to some that a minor deficiency in type III collagen may be important in the development of cerebral aneurysms. This deficiency could result from a defect in the type III collagen gene or in its expression. Such a genetically determined deficiency would be expected to occur in clearcut cases of familial cerebral aneurysms. The demonstration of such a deficiency would strongly support the concept of a genetic etiology of cerebral aneurysms. The demonstration of type III collagen deficiency in asymptomatic members of families with cerebral aneurysms could then provide a marker for the presence of aneurysms, which could be treated before rupture.

In the present study, the hypothesis that familial cerebral aneurysms are associated with a mild deficiency of type III collagen has been tested by evaluating type I and III collagens in skin fibroblasts obtained from a patient with this condition.

Clinical Material and Methods

Clinical Material

The relevant data of the family pedigree are given in Fig. 1. Individual I-1 died at 68 years of age from the autopsy-proven rupture of a giant aneurysm arising from the left middle cerebral artery (MCA). A small
right MCA aneurysm was also present. Her daughter, II-6, suffered a subarachnoid hemorrhage from a left MCA aneurysm as demonstrated on computerized tomography scans and confirmed at surgery. She also harbored aneurysms arising from the left internal carotid artery (ICA) bifurcation and right ICA, the latter aneurysm being at the level of origin of the posterior communicating artery; both aneurysms were also confirmed at surgery. This patient, the proband, consented to skin being harvested from the temporal region at the time of craniotomy for subsequent fibroblast culture and collagen analysis. Four of her five siblings underwent elective four-vessel cerebral angiography and one (II-3) was found to harbor a right ICA aneurysm at the origin of the anterior choroidal artery. Cerebral angiography was normal in three other siblings. Members of generation III are prepubescent and have not been submitted to angiography.

**Methods of Collagen Analysis**

**Cell Culture and Procollagen Labeling.** The fibroblasts were cultured in minimum essential medium supplemented with 12% fetal calf serum. The medium was changed every 3 days until the cells reached confluence. On the day before labeling, the cells were trypsinized, counted, and plated in two P35 petri dishes for each cell line at 2.5 x 10^5 cells/dish. Two control cell lines were included in each experiment. After 24 hours the cells were washed once with Dulbecco's modified minimum essential medium without serum, and preincubated for 1 hour in the same medium (0.8 ml) in the presence of ascorbate (50 μg/ml). After preincubation, 100 μl of medium containing 50 μCi 5-[3H]-proline was added to the medium and the cells were labeled for 17 hours.

**Procollagen and Collagen Preparation.** All of the preparations were performed on ice or at 4°C. The media were collected in 1.5-ml Eppendorf tubes and supplemented with a protease inhibitor cocktail containing (final concentrations): ethylenediaminetetra-acetic acid 25 mM, p-aminobenzamidine 1 mM, phenylmethylsulfonyl fluoride 1 mM, and N-ethyl-maleimide 10 mM. After clarification by centrifugation at 15,000 G for 1 minute, the supernatant was aliquoted into two equal parts. To each part, 50 μg of type I collagen cold carrier was added. The collagens and procollagens were precipitated by the addition of an equal volume of ammonium sulfate (352 mg/ml) to reach 30% saturation. The suspensions were left overnight with gentle mixing.

The precipitates were centrifuged at 15,000 G for 15 minutes and the pellet was washed twice with 1 ml ammonium sulfate (30% saturation). The pellets of one aliquot of each culture were suspended in 90 μl of medium containing 50 μCi 5-[3H]-proline and the cells were labeled for 17 hours.

**SDS-PAGE.** For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the non-pepsinized precipitates were directly suspended in electrophoresis sample buffer, denatured for 1 minute at 100°C, and dialyzed overnight against sample buffer. After reduction with 1% β-mercaptoethanol and further denaturation at 100°C for 3 minutes, the samples (15,000 cpm) were loaded onto the gel. To the pepsinized samples, 11 μl of electrophoresis buffer concentrated 10 times was added and the samples were denatured for 3 minutes at 100°C. The proteins were then subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250.
Collagen in familial cerebral aneurysms

tured for 3 minutes at 100°C, dialyzed overnight against
sample buffer, and separated using the delayed reduc-
tion method of Sykes, et al.8

Electrophoresis was performed as described by Loe-
mmler,2 with the addition of 2 M urea to the stacking
and running gels, using 5% acrylamide running gels in
a mini Protean II system.* The gels were stained for
10 to 15 minutes with Coomassie blue (0.05%) in
HAc:methanol:water 10:40:50 and destained for 60
minutes in methanol:HAc:water 15:7.5:77.5. The gels
were next impregnated with Amplify+ (following the
specifications of the manufacturer) and dried on a filter
paper. The gels were then exposed to preflashed (0.2 to
0.3 optical density) Kodak XAR-5 films for 4 to 15
days at -80°C. After the films were developed, their
electrophoretic profiles were quantitated.‡

Results

Results of analysis of the medium proteins from the
proband, two control samples, and a patient with Eh-
ers-Danlos syndrome type IV are shown in Fig. 2.
Visual inspection of these autoradiograms shows a clear
deficit of pro-a 1 (III) in the latter case, while no differ-
ce is noticeable between the control samples and the
samples from the proband (the familial multiple aneu-
rysm patient). This is further confirmed by the quanti-
tative data shown in Table 1. Results of the pepsinized
medium collagen analysis, although nonquantitative,
were also similar to those of the control samples.

Discussion

The rupture of a cerebral aneurysm is a devastating
event that usually strikes people during their most
productive years, and commonly results in permanent
disability or death. The etiology of cerebral aneurysms
remains the subject of controversy even though most
would agree that degenerative factors related to ather-
oscclerosis and hypertension are important in their de-
velopment. The possibility of genetic determination of
cerebral aneurysms is suggested by their occurrence in
identical twins, in members of the same family, and in
conditions with a clearly demonstrated genetic basis,
such as polycystic kidney disease.3 These latter condi-
tions, however, often produce severe hypertension, and
the development of aneurysms in patients afflicted with
elevated arterial pressure may reflect this phenomenon
rather than an inherent defect in the structure of the
cerebral blood vessel wall.7 The long-disputed theory
that cerebral aneurysms may be genetically determined
regained some vigor with the demonstration that certain
sporadic cerebral aneurysms may be associated with a
mild deficiency of type III collagen.4,5 As type III col-
lagen is mainly found in the media of the arteries, as
well as in the skin, the demonstration of such a defi-
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mined collagen imbalance in the etiology of cerebral
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sume that it would be present in patients with a family
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support this hypothesis. Similarly, in an ongoing study
of patients with multiple cerebral aneurysms (unpub-
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lagen deficiency in four female patients harboring 10
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type I ratios: 0.12, 0.12, 0.15, and 0.16:1).

The data reported here were obtained as part of a
screening program for collagen synthesis abnormalities
of over 60 human fibroblast cell lines. Most of these
cell lines originated from patients with generalized con-
nective tissue disorders such as osteogenesis imperfecta
and Ehlers-Danlos syndrome. For these cultures we
analyzed (by autoradiography of SDS-urea poly-
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the pepsinized medium collagens using the delayed
reduction technique, and the cell-layer proteins after
reduction.8 The most consistent results were obtained
by analysis of the medium proteins. Analysis of pepsin-
ized medium collagens was found useful in the detec-
tion of certain collagen structural abnormalities, but
gave inconsistent quantitative results, confirming the
observation made by Neil-Dwyer, et al.,9 in a similar
study. These workers found type III collagen deficiency
or possible type III collagen deficiency in 11 of 17
patients, most of whom were female. This discrepancy
with regard to the results of our study is unlikely to be
related to a difference in patient populations: Neil-
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ruptured. This was also the case for two members of
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context, it is important to note that no difference in the
presentation, epidemiology, evolution, or appearance

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* Amplify manufactured by Amersham Corp., Arlington
** Protein II system manufactured Bio Rad Labora-
† Instrument, Inc., Rockville, Maryland.
‡ Ultrasound XL microdensitometer manufactured by IKB
§ Visual inspection of these autoradiograms shows a clear
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of the aneurysms in patients with and those without collagen deficiency was noted by Neil-Dwyer, et al.

Our data do not support the hypothesis that familial cerebral aneurysms are caused by a genetically determined type III collagen deficiency. It also puts in doubt the necessity of invoking such a deficiency, mild or marked, to account for the formation or rupture of sporadic cerebral aneurysm. This observation is further supported by the paucity of reports of cerebral aneurysms in patients with Ehlers-Danlos syndrome type IV, who often have a marked deficiency of collagen type III (Fig. 2). Although the present findings indicate that the fibroblasts of patients with familial aneurysms express normal levels of type III collagen, the functional integrity of this molecule has not been studied. The possibility that such patients carry an inherited defect which may influence the physiological function of the molecule cannot be excluded. Such alterations could include differences in the primary structure and/or post-translational modifications of the type III collagen molecule, and could result from changes in the gene or in the intracellular or extracellular processing of the molecule. Furthermore, because the expression of type III collagen was assessed in skin fibroblasts rather than in the cells that populate the cerebral arteries, we cannot rule out an altered type III collagen specific to cerebral vessels. Detailed sequencing analysis of the type III collagen gene and protein, screening for alterations in protein modifications, ultrastructural examination of collagens, and the study of tissue-specific expression of type III collagen in patients with familial aneurysms may reveal abnormalities which are beyond the scope of the analysis presented here.

The possibility remains that the presence of cerebral aneurysms in the three individuals of the family studied here represents the fortuitous occurrence of sporadic aneurysms in related individuals, but this is highly unlikely. It is more unlikely since two individuals (I-1 and II-6) harbored multiple cerebral aneurysms, an unusual occurrence.

This analysis was unable to demonstrate type III collagen deficiency in a patient with multiple familial cerebral aneurysms who would be expected to express such a deficiency if it were of etiological importance. It is stressed that skin biopsy for collagen analysis is not an adequate screening procedure by which to exclude the presence of a cerebral aneurysm in family members at risk.

Acknowledgments

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References