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Different types of connective tissue alterations associated with cervical artery dissections

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Abstract This study describes the technical handling and the diagnostic evaluation of skin biopsies in order to standardize the assessment of the delicate morphologic abnormalities that are found in patients with spontaneous cervical artery dissections (sCAD). Skin biopsies from 126 patients with sCAD and from 29 healthy relatives were analyzed. The morphology of the connective tissue was normal in 54 patients with sCAD (43%) and aberrant in 72 patients with sCAD (57%). These latter patients were classified into three groups: in 43 patients, we repeatedly observed composite collagen fibrils and elastic fibers with fragmentation and minicalcifications. In 13 further patients, the dermis was significantly thinner than in healthy subjects. The collagen fibers contained fibrils with highly variable diameters. In a third group of 16 sCAD patients, the abnormalities were restricted to the elastic fibers (with fragmentation and minicalcifications) without significant alterations in the morphology of the collagen fibrils. The finding of different morphologic classes of aberrations among patients suggests that the connective tissue defects are genetically heterogeneous. The segregation of the connective tissue phenotype in three families suggested an autosomal dominant pattern of inheritance.

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Introduction

Spontaneous cervical artery dissection (sCAD) is an important cause of stroke among young and middle-aged patients [22]. In a minority of the patients, the development of the dissection is triggered or caused by mechanical injury [17], but in most cases the pathogenesis of the dissection remains unclear.

Several risk factors have been associated with CAD, e.g., hereditary connective tissue disorders, in particular the vascular type of Ehlers-Danlos syndrome (EDS IV) [18, 23], morphologic alterations of the dermal connective tissue without clinical signs of a connective tissue disorder [3, 4], recent infection [5, 11], mild hyperhomocysteinemia [19, 20], or alpha-1-antitrypsin deficiency [21, 24]. The impact of these different risk factors is discussed controversially, and pathogenetic models for the development of an arterial dissection are not self-evident for most of the predisposing factors. Hence, the pathogenesis of CAD remains unknown in most cases [2].

The morphology of the arterial wall has only rarely been studied directly in patients, as most patients with sCAD do not require surgical intervention and recover well. However, the aberrant connective tissue morphology in skin biopsies from patients is thought to indicate a generalized structural defect of the connective tissue, leading to a weakness of the vessel wall.

In an attempt to evaluate the significance of connective tissue alterations for sCAD, we performed the present study. First, we introduced a standardized procedure for the handling and analysis of skin biopsies. Such a standardization is important, because the morphologic aberrations are mild, and their assessment delicate and difficult. Next, we proposed a classification of morphologic alterations based on the findings in 126 sCAD patients. Finally, we studied the possible inheritance of these morphologic alterations in four families.

Patients and methods

A consecutive series of patients with sCAD was included in this study. This series included 25 patients who were initially studied by Brandt and co-workers and 65 patients from a follow-up study from the same center [3, 4]. Another 36 new patients and 29 healthy relatives were added to the previous series. The diagnosis of sCAD was based on clinical signs and symptoms together with neurovascular findings. All patients had symptomatic sCAD with either local symptoms (i.e., neck or facial pain, Horner syndrome, cranial nerve deficits) or cerebral ischemia (transient ischemic attack/stroke) or a combination of both. Duplex sonography studies showing a high resistance flow pattern in the absence of atherosclerosis and visualizing an intimal flap suggested the diagnosis of CAD, which was confirmed in all patients by MRI of the neck (mural hematoma), spiral CT angiography (mural hematoma, string sign, or tapered extracranial occlusion), or digital subtraction angiography (string sign, long-segment pseudo-occlusion, or tapered extracranial occlusion). We included 1 patient in our study with severe stenosis of the internal carotid artery, probably due to dissection, without having confirmed his dissection by MRT (index patient from family C in Fig. 4). All patients were carefully examined for signs of a known hereditary connective tissue disorder such as a marfanoid habitus, hyperextensible joints, skin abnormalities, hypertrophic scars, or abnormalities on funduscopy. Healthy relatives from 4 patients were clinically examined for signs of a connective tissue disorder, and a skin biopsy was taken for investigation of the connective tissue.

Excision and processing of skin biopsy

The performance of skin biopsies was approved by the local ethical committees (University of Heidelberg, University of Basel) and required informed consent from each patient. Biopsies were taken from the outer aspect of the upper arm close (about 10 cm) to the elbow. The skin was thoroughly and repeatedly sterilized with 70% ethanol. An almond-shaped piece of skin (10×5 mm) was excised with vertical deep knife incisions extending into the subcutaneous fat tissue. The biopsy sample was subsequently cut into two pieces. One part was processed for transmission electron microscopy, the other part was immediately processed for tissue culturing of fibroblasts. Squashing of the excised piece of skin, either during excision or during the handling with the forceps, was carefully avoided as additional mechanical stress might be a possible source of morphologic artifacts.

Preparations for electron microscopy

Biopsy specimens were initially fixed in 3% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) at room temperature, cut into pieces of ca. 1 mm^3, and fixed in the same solution for several hours. After being washed in buffer solution, specimens were post-fixed in 1% OsO_4 in 0.1 M Na-cacodylate buffer (pH 7.4), rinsed in water, passed through a graded ethanol dehydration series, transferred to propylene oxide, embedded in epoxy resin (glycidether 100 or Epon 812), and cut after polymerization was completed into semithin and ultrathin sections. Semithin sections were stained with methylene blue and evaluated by light microscopy. We carefully documented which part of the skin (upper, middle, or deep dermis) was to be cut into ultrathin sections. Ultrathin sections were treated with uranyl acetate, contrasted with lead citrate, and examined with a Philips EM 400 transmission electron microscope.

Evaluation of the electron microscopic and histologic observations

At least three blocks of embedded tissue were cut into semithin sections, and parts from the upper, middle, and deep dermal regions were cut into ultrathin sections. About 100 collagen bundles and elastic fibers were inspected at magnifications of about x20,000 within each block. For a detailed analysis of elements of the connective tissue, we focussed on the reticular dermal region, where connective tissue structures are usually very regular and not significantly affected by environmental factors such as actinic elastosis. The structure of the collagen fibers (in cross-section) and of the elastic fibers should also be studied at high magnifications (>x10,000) to detect typical aberrations like composite collagen fibrils or minicalcifications in the elastic material. The morphology of the extracellular matrix components of patients must be compared with similar material from healthy, normal control persons. However, collagen fibrils and elastic fibers show some morphologic variation even in healthy subjects, which makes the electron microscopic diagnosis of a connective tissue disorder difficult. It is based on careful comparisons between patients and control subjects, on exact standardization (age, biopsy technique, fixation, dermal region analyzed, anamnestic data on skin exposure, and medication), and on multiple observations of numerous collagen bundles and elastic fibers of the same patient. Even in patients with strong aberrations, part of the collagen fibrils and elastic fibers look normal. Experience with skin samples from patients with known inherited connective tissue disorders, notably several subtypes of Ehlers-Danlos syndrome (EDS), is helpful in the classification of the skin samples from patients with sCAD, since most abnormalities in sCAD patients range in severity and aspect somewhere between normal and those in patients with EDS.

The morphology of the connective tissue was always inspected and evaluated by two experienced researchers and classified as normal or abnormal. Abnormalities might suggest the presence of a known connective tissue disease or might be weaker in intensity. If clinical and laboratory data suggested the presence of a known connective tissue disorder, complementary molecular genetic tests were done in some cases to confirm the diagnosis.

Results

Skin biopsies from 126 patients with sCAD and from 29 healthy relatives were prepared for histology and electron microscopy. Six specimens could not be evaluated, due to artificial modifications of the morphology caused by improper handling (two biopsies were not deep enough, one consisted only of fat, one was squashed too strongly, and two were damaged during transport because the tube with fixation solution was leaking). The quality of 149 out of 155 biopsies (96%) was good enough for a reliable histologic and electron microscopic analysis.

We found morphologic aberrations in 72 biopsies from patients with sCAD. On the basis of the morphology, we classified the aberrations into three groups (Figs. 1, 2, 3, Table 1): as 'EDS III-like' in 43 patients, showing regularly composite fibrils within mid-dermal collagen bundles and elastic fibers with fragmentation and minicalcifications (Fig. 1); as 'EDS IV-like' in 13 patients, revealing a significantly thin dermis and small caliber collagen fibrils in small, often loosely packed collagen bundles (Fig. 2); in a third group of 16 patients, only the elastic fibers showed abnormalities, i.e., pronounced fragmentation and focal electron-dense deposits (minicalcifications) (Fig. 3). Within each group the aberrations varied in strength (in quantity).

The segregation of the connective tissue alterations was analyzed in four pedigrees. In three families, we observed EDS III-like connective tissue aberrations (Fig. 4A–C). We analyzed 21 persons from these families. The 3 index pa-

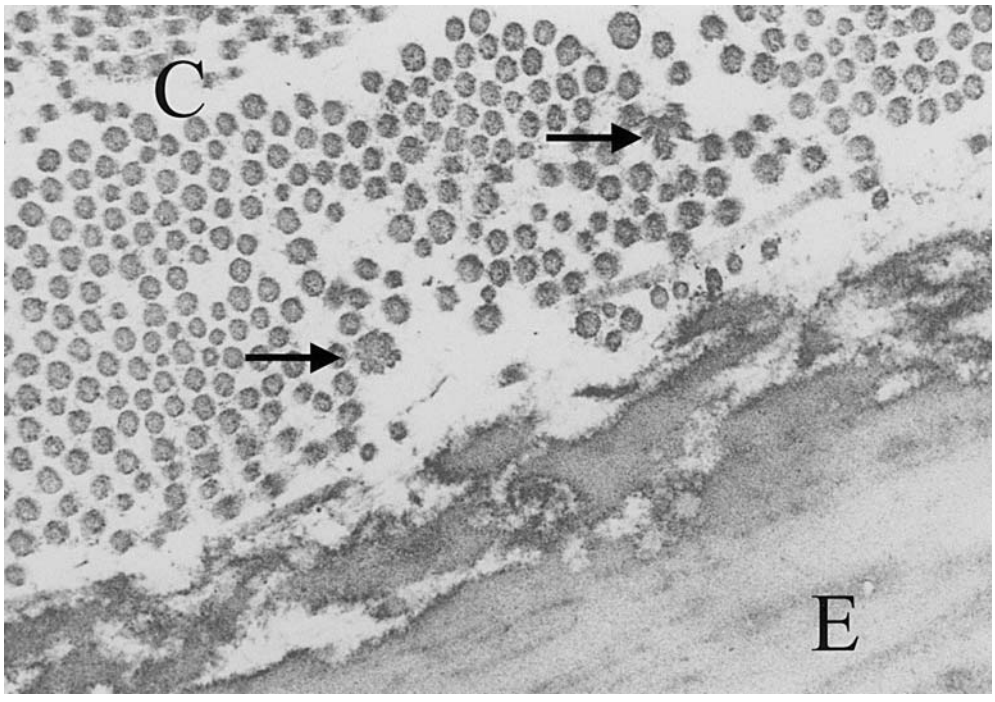

Fig. 1 The 'EDS III-like' pattern of connective tissue alterations in a skin biopsy from a patient with sCAD (x40,000). Collagen fibrils (*C*) are shown in cross-section. An elastic fiber (*E*) is shown in the *lower part* of the micrograph. The morphology of some collagen fibrils is irregular. *Arrows* point to 'composite fibrils'

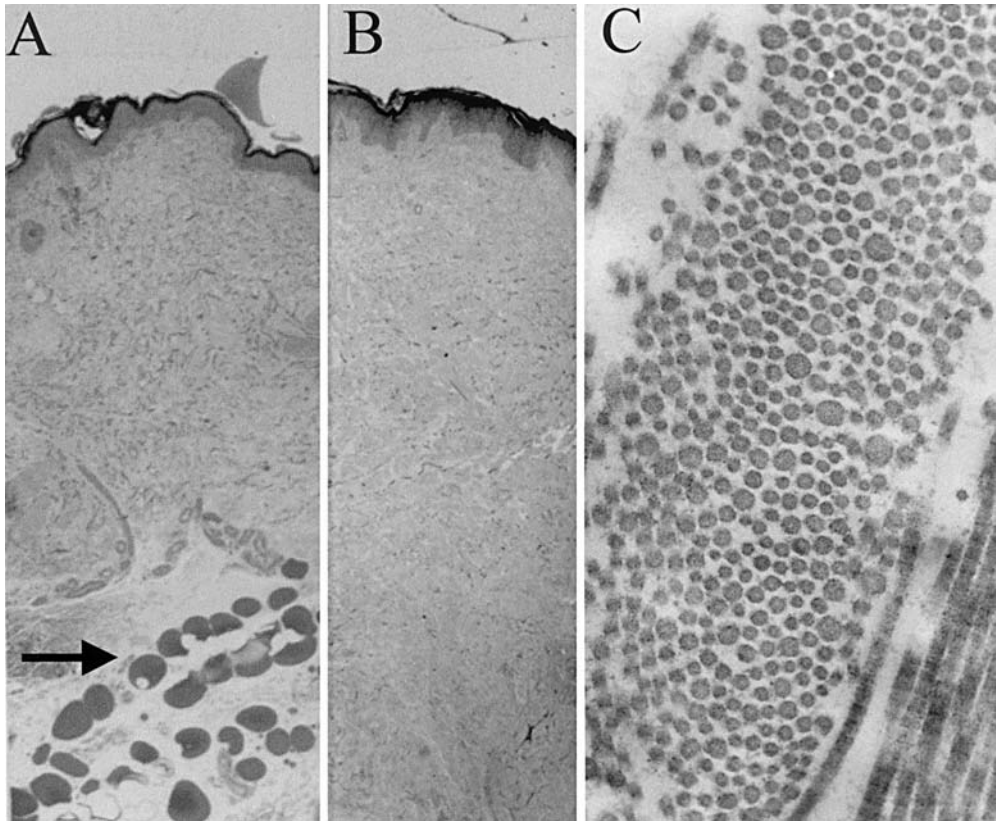

Fig. 2A–C 'EDS IV-like' connective tissue morphology in skin biopsies. Semithin sections of epidermis and dermis (x35) from a patient with spontaneous cervical artery dissections (sCAD) (**A**) and from a healthy control subject (**B**) under light microscopy after methylene blue staining. The dermis of the patient is thin (*arrow* indicates subcutaneous fat tissue). **C** Electron micrograph (x40,000) from the dermis of a patient with sCAD. The ample variation in diameter of the collagen fibrils, which are loosely organized into fibers, is the most salient deviation from normal in this type of connective tissue morphology

tients and 9 healthy relatives displayed similar aberrations, 8 further healthy relatives did not show any connective tissue alterations, and in 1 relative the aberrations were much weaker than in the index patient. The segregation in the family of the patient with EDS IV-like aberrations (Fig. 4D) seems to be different. Only two patients show clear and reproducible morphogic alterations. We also observed very weak connective tissue aberrations in both sons of a sister without an aberrant connective tissue phenotype. A further investigation of the father of these sons showed that the father did not carry an aberrant connective tissue phenotype.

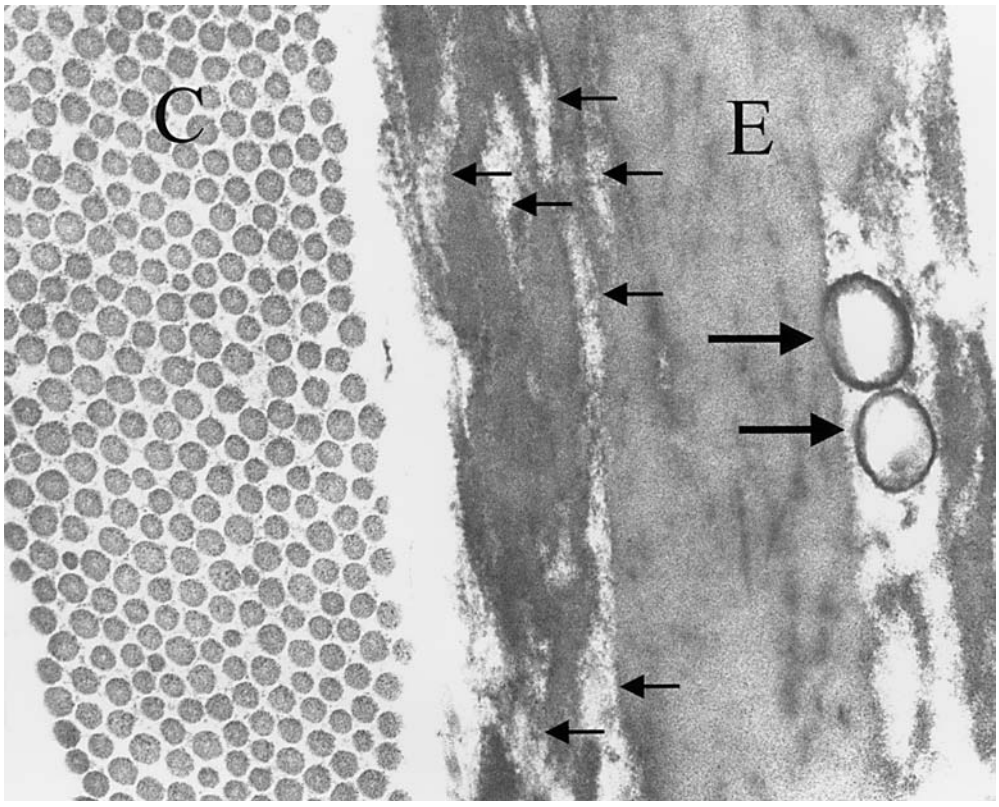

Fig. 3 Regular morphology of collagen fibers (*C*) with irregular elastic fibers (*E*) with calcareous inclusions (*large arrows*) and fragmentation (*small arrows*) (x40,000)

Table 1 Electron microscopic analysis of 126 skin biopsies of patients with spontaneous cervical artery dissections (sCAD). The electron microscopic findings were classified as 'EDS III-like', 'EDS IV-like' or 'only elastic fibers'. In the families of 10 patients with sCAD, a first-degree relative also had a history of sCAD (*EDS* Ehlers-Danlos Syndrome). The group of patients with sCAD of multiple vessels consists of patients with recurrent dissections as well as patients with multiple simultaneous dissections

Type of dissections	EDSIII-like	EDS IV-like	Only elastic fibers	Normal
sCAD of single vessel	33	7	11	36
sCAD of multiple vessels	10	6	5	18

Discussion

We described the procedure for the analysis of the connective tissue morphology in skin biopsies in detail. A connective tissue analysis was possible in almost all cases, unless the biopsies were not deep enough (did not reach into the subcutaneous fat tissue), were too small, or their fixation was incomplete.

For the electron microscopic and histologic examination of diagnostic skin biopsies, the findings in patients must be compared to findings in healthy control subjects. The distinction between normal and abnormal morphology might be difficult in some cases, as there is a continuous transition from perfectly normal to definitely abnormal. A connective tissue diagnosis can therefore rarely be made on the sole basis of isolated electron micrographs of a single patient. The assessment of the morphology depends as a rule on a variety of clinical, histologic and electron microscopic observations as well as on extended experience with other patients and with control subjects [6, 9, 26]. Such healthy control subjects should be matched according to age, sex, ethnic origin, sun exposure, health condition, and medication.

In this study, we classified the ultrastructural alterations in skin biopsies from patients into three different groups, which were compared to the findings in patients with known connective tissue disorders [1, 12, 13, 14]. In an earlier analysis of the ELN gene in patients with sCAD, we had already alluded to a possible differentiation between patterns of aberrant morphology and included some patients with aberrations only in the elastic fibers [14]. A subsequent study of connective tissue in patients with intracranial aneurysms revealed the existence of two different types of morphologic alterations [8]. We studied here the morphologic heterogeneity in a large series of sCAD patients and confirmed the existence of different classes of aberrations with quantitative differences within each class. Moreover, we discerned a third group of patients with aberrations restricted to the elastic fiber system resembling the findings of heterozygous carriers of recessive pseudoxanthoma elasticum (PXE-carrier-like) [1].

The analogy with the findings in patients with EDS III and EDS IV [12] and in heterozygous PXE [1] carriers suggests that genetic heterogeneity exists amongst patients with sCAD. In previous work, Brandt et al. [3, 4] only showed electromicrographs of EDS III-like connective

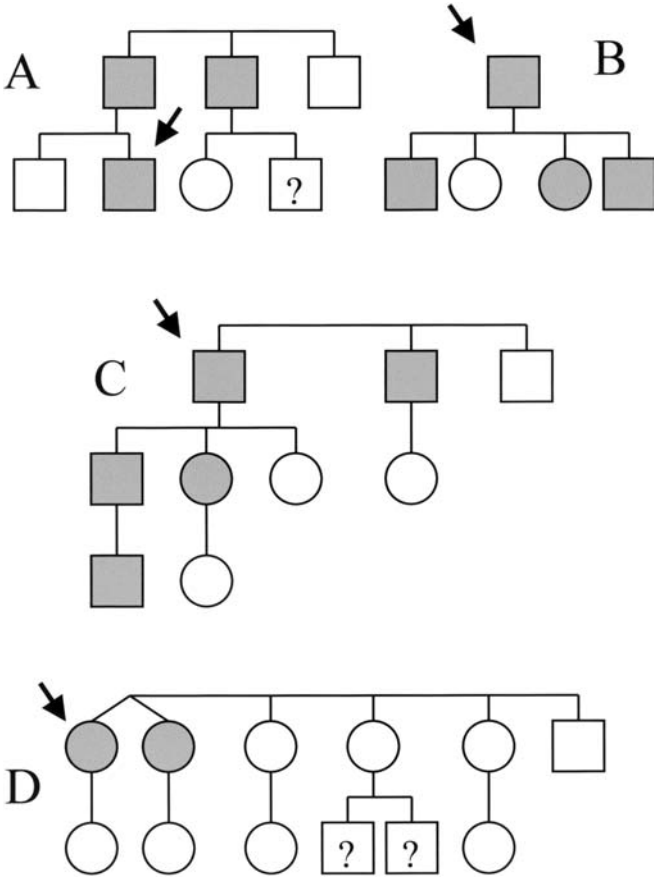

Fig. 4 Families with inherited connective tissue alterations. Index patients with sCAD are indicated with an *arrow*. The index patient of family *C* suffered from an ACI stenosis. An ACI dissection was not confirmed. *Shaded symbols*: carriers of aberrant connective tissue phenotype; *open symbols*: carriers of normal connective tissue in skin biopsies. The connective tissue aberrations in families *A*, *B* and *C* are of the EDS III-like pattern. In family *D*, the index patient and her sister have EDS IV-like connective tissue phenotype. The index patient suffered from sCAD as well as from an intracranial aneurysm. A further brother of the index patient (not shown) died from sCAD. His connective tissue phenotype is unknown. Three healthy relatives showed very slight abnormalities in a skin biopsy – these minor abnormalities are marked as '?' to indicate that the phenotyping of these subjects is unclear

tissue aberrations, the most common type found amongst patients with sCAD.

The segregation analysis in the three families suggested that the EDS III-like alterations are inherited. The 1:1 ratio of carriers and non-carriers as well as the segregation patterns within the families suggest an autosomal dominant inheritance. Hence, these morphologic connective tissue alterations were considered a reliable subclinical phenotype and might be associated with an unknown mutation. The analysis of a single family of a patient with EDS IV-like aberrations was not conclusive. The mode of inheritance is unclear, as are the penetrance of the phenotype and its stability. The segregation pattern is compatible with either an autosomal dominant or a recessive mode of inheritance. However, it is also possible that the connective tissue alterations in this family are not inherited at all. Because only a single pedigree of this type was analyzed, further material must be investigated in order to settle these questions.

The genetic basis of the connective tissue alterations is still unclear. Several genes that are involved in the biosynthesis of the extracellular matrix were analyzed in these patients, but no mutations were found in COL3A1, COL5A1, COL5A2, ELN, or ABCC-6. Hence, sCAD patients do not suffer from mild forms of classic or vascular EDS (types I/II and IV) [6, 7, 9, 15, 16, 25, 26] or from other known hereditary connective tissue syndromes. A small family with 'EDS III-like' aberrations was used for genetic linkage analysis and enabled the exclusion mapping of a large number of candidate genes involved in the biosynthesis and modeling of the extracellular matrix [10]. The refined phenotyping of the connective tissue aberrations and the enlarged pedigrees will be very useful for further genetic analyses.

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