Alterations of the Microvascular Network in Sclerotic Hippocampi From Patients With Epilepsy

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INTRODUCTION

Temporal lobe epilepsy (TLE) is a common form of intractable epilepsy; hippocampal sclerosis is the most frequent pathological finding in resected mesial temporal tissue (1). Hippocampal sclerosis is characterized by gliosis and neuronal loss that are most prominent in the cornu Ammonis fields CA1, followed by CA4/CA3, CA2, and the granular cell layer of the dentate gyrus (DG) (1, 2). These alterations are commonly accompanied by a dispersion of the dentate granular cell layer with ectopic neurons observed in the molecular layer (3). Certain alterations of the vasculature have also been reported in the sclerotic hippocampus; these are coupled to angiogenesis and alterations in the permeability of the blood-brain barrier (4–12). Indeed, through specific immunostaining for vascular endothelial growth factor and tyrosine kinase receptors, a significant increase in the density of blood vessels in the sclerotic hippocampus of patients with TLE has been reported (9). Alkaline phosphatase (AP) is a ubiquitous enzyme present in brain endothelial cells that has been used to visualize the capillary network of the cerebral cortex and subcortical structures in monkeys and in humans (13–20). In the course of our studies on the microvasculature of sclerotic hippocampal tissue obtained from TLE patients by surgical resection, we observed an impressive reduction in AP staining that suggested a depletion of the microvasculature.

To resolve this contradiction, we analyzed the density of blood vessels in surgical resection specimens from TLE patients and controls. To our knowledge, the most reliable histological method currently available to achieve this goal is to use toluidine blue-stained plastic semithin sections to estimate the volume fraction occupied by blood vessels in the sclerotic hippocampi. We also examined the pattern of collagen Type IV (collagen IV) immunoreactivity; collagen IV is a major component of the blood-brain barrier basal lamina (21). We also analyzed the fine structure of the microvascular network of the hippocampus from TLE patients by correlative light and electron microscopy.

We present evidence that there is a consistent and significant loss of microvessels within the CA1 field in the sclerotic hippocampus despite the apparent increase in the labeling of blood vessels seen with immunocytochemical markers. We propose that this loss of blood vessels should be considered as another pathological characteristic of hippocampal sclerosis.

MATERIALS AND METHODS

Tissue Processing

Human brain tissue was obtained either from autopsies (kindly supplied by Dr R. Alcaraz, Forensic Pathology Service, Basque Institute of Legal Medicine, Bilbao, Spain).
or postoperative tissue (Department of Neurosurgery, Hospital de la Princesa, Madrid, Spain). Patient consent was obtained in all cases in accordance with the Helsinki Declaration (22), and all protocols were approved by the institutional ethics committee of the Hospital de la Princesa. The tissue obtained at autopsy (2–3 hours postmortem) was from 5 normal males with no known neurological alterations (aged 23–69 years). Surgical tissue was obtained from 24 patients who had pharmacoresistant TLE; all of these patients had well-documented medical histories (Table 1).

Patients were presurgically evaluated according to the Hospital de la Princesa protocol, published elsewhere (24, 25). A tailored resection of neocortex and the amygdalo-hippocampal area according to the electrocorticography findings was performed following the surgical technique described (26).

Immediately after removal, surgical and autopsy tissue samples were fixed in cold 4% paraformaldehyde. Small blocks (~15 × 10 × 10 mm) were obtained across the entire rostrocaudal extent of the hippocampal formation and then immersed in a solution of 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 24 to 36 hours at 4°C. Serial coronal vibratome sections (50 μm) were subsequently obtained from the blocks.

### Histopathologic Analyses

Standard neuropathologic assessment of the surgically removed tissue demonstrated the presence of hippocampal sclerosis, as revealed in Nissl-stained or NeuN-immunostained sections in 19 cases. No histopathologic alterations were found in the hippocampal formation of 5 patients; this material has been used as control tissue in previous studies (27, 28). No abnormalities were observed in the autopsy tissue.

### AP Histochemistry

A specific histochemical technique was used to examine AP activity of the surgical and autopsy hippocampal specimens (29). Sections of the hippocampal formation (50 μm thick) were incubated in 100 mmol/L Tris-HCl solution (pH 9.5) with 100 mmol/L NaCl, 50 mmol/L MgCl₂, 0.53 mmol/L 4-nitro blue tetrazolium chloride (Sigma-Aldrich Chemie, Steinheim, Germany), and 0.38 mmol/L 5-bromo-4-cloro-3-indolyl-phosphate 4-toluidine salt (Sigma-Aldrich Chemie). The chromogenic reaction was allowed to proceed for 10 to 30 minutes at room temperature and then stopped by transferring the sections to 10 mmol/L Tris solution (pH 7.5) with 1 mmol/L EDTA and 10 mmol/L levamisole (Sigma-Aldrich Chemie). Sections were rinsed in Tris and mounted and subsequently dehydrated and cleared with xylene and coverslipped.

### Immunocytochemistry

Sections of the surgical and autopsy hippocampal formation tissue were batch processed using standard immunocytochemical techniques. Free-floating sections were pretreated in 1% H₂O₂ for 30 minutes to remove endogenous peroxidase activity and then for 1 hour in PBS with 0.25% Triton-X (Merck, Darmstadt, Germany) and 3% normal horse serum. The sections were then incubated overnight at 4°C with a mouse antibody against human collagen IV (1:1000; Sigma-Aldrich, St Louis, MO), a protein located in the basal lamina of blood vessels (21). The sections were processed by the avidin-biotin method using a secondary horse anti-mouse biotinylated antibody (1:200; Vector Laboratories, Burlingame, Calif) and the Vectastain ABC immunoperoxidase kit, with 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) as the chromogen. The sections were mounted, dehydrated, cleared with xylene, and coverslipped. Adjacent Nissl-stained sections were used to reveal the borders between the different hippocampal fields. No significant qualitative differences were observed in the immunocytochemical staining of sections from autopsy material and sections from the control biopsies.

The images were captured with a digital camera (Olympus DP70) attached to an Olympus light microscope; Adobe Photoshop CS3 Extended 10.0.1 software was used to generate the figure plates (Adobe Systems, San Jose, Calif).

### Quantitative Analysis of the Stained Blood Vessels

The vascular densities of the blood vessels positive for AP or immunoreactive of collagen IV were quantified as previously described (18). Briefly, we estimated the area occupied by blood vessels per unit area of the tissue. For this purpose, images were analyzed using the appropriated

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**Table 1. Summary of Clinical Data From Patients With Epilepsy and the Outcome of Surgery**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, Years; Sex; Side</th>
<th>Age at Onset; Duration, Years</th>
<th>Seizure Type</th>
<th>Seizure Frequency</th>
<th>Engel Scale (Time After Surgery in Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*H65</td>
<td>21, m, L</td>
<td>14, 7</td>
<td>PC</td>
<td>2–3/week</td>
<td>III (48)</td>
</tr>
<tr>
<td>H162</td>
<td>44, m, L</td>
<td>4, 40</td>
<td>PC</td>
<td>4–5/week</td>
<td>I (30)</td>
</tr>
<tr>
<td>H164</td>
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<td>1.5, 32.5</td>
<td>Gen</td>
<td>1/week</td>
<td>I (24)</td>
</tr>
<tr>
<td>H170</td>
<td>15, f, R</td>
<td>0, 15</td>
<td>PC, gen</td>
<td>Daily</td>
<td>I (30)</td>
</tr>
<tr>
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<td>1, 40</td>
<td>Gen</td>
<td>2–3/week</td>
<td>I (38)</td>
</tr>
<tr>
<td>H192</td>
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<td>11, 15</td>
<td>PC</td>
<td>Daily</td>
<td>I (34)</td>
</tr>
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<td>*H200</td>
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<td>4, 23</td>
<td>PC</td>
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<tr>
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<td>PC</td>
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</tr>
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<td>PC</td>
<td>3–4/week</td>
<td>II (24)</td>
</tr>
<tr>
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<td>PC, gen</td>
<td>Daily</td>
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<td>H233</td>
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<td>PC, gen</td>
<td>1/week</td>
<td>II (12)</td>
</tr>
<tr>
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<td>PC</td>
<td>1/week</td>
<td>I (12)</td>
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<tr>
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<td>20, 21</td>
<td>PC, gen</td>
<td>1–2/week</td>
<td>I (12)</td>
</tr>
<tr>
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<td>PC</td>
<td>1/week</td>
<td>I (12)</td>
</tr>
<tr>
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<td>PC</td>
<td>1/week</td>
<td>I (12)</td>
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<tr>
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<td>PC, gen</td>
<td>Irregular</td>
<td>I (12)</td>
</tr>
<tr>
<td>H241</td>
<td>43, f, L</td>
<td>4, 39</td>
<td>PC, gen</td>
<td>Irregular</td>
<td>I (12)</td>
</tr>
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<td>*H242</td>
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<td>18, 20</td>
<td>PC, gen</td>
<td>Daily</td>
<td>I (10)</td>
</tr>
<tr>
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<td>45, m, R</td>
<td>17, 28</td>
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<td>2/week</td>
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<td>*H249</td>
<td>23, f, R</td>
<td>18, 5</td>
<td>PC</td>
<td>Daily</td>
<td>I (24)</td>
</tr>
</tbody>
</table>

f, female; gen, secondarily generalized; L, left; m, male; PC, partial complex seizures; PS, partial simple seizures; R, right.

*Non-sclerotic. Engel Scale for surgical outcome: Class I, seizure-free; Class II, rare seizures; Class III, worthwhile improvement (23).
software (Scion Image, Beta 4.0.2, Scion Corporation, Frederick, MD). Measurements were made in the DG, the CA fields, and the subiculum of the coronal sections obtained; all hippocampal subfields were analyzed in 3 to 4 sections from the same patient. The vascular density was analyzed at a final magnification of 2,300× using an automatic command created to identify and measure all the field areas with gray levels within a range of the gray values indicative of vessel staining. This range was manually adjusted for each analysis because the staining intensities in different sections produced different gray ranges. Thus, a semiautomated procedure separated the stained areas from the background. The selection was visually checked, and corrections were made to avoid the selection of artifacts and to improve the process.

Finally, the individual values of the selected areas within each region and the value of the area of interest were extracted. The total vessel area was expressed as a percentage...
of the reference area (area of interest). Statistical comparisons between the groups were performed using analysis of variance or Kruskal-Wallis nonparametric test, depending on whether the data sets followed a Gaussian distribution and passed the test for homogeneity of variances (30). All statistical studies were performed with the aid of the Graph Pad Prism statistical package (Graph Pad Prism 4.0, Graph Pad Software, Inc, San Diego, Calif).

**Estimation of the Volume Fraction of Blood Vessels**

The volume fraction occupied by blood vessels ($V_v$) was estimated in semithin sections from the CA1 field of the nonsclerotic and sclerotic hippocampi. For this purpose, 50-μm-thick sections adjacent to those used for histopathologic assessment were embedded in Araldite to obtain 2-μm-thick semithin sections. The semithin sections were stained with 1% toluidine blue in 1% borax and examined under the light microscope to locate the area of interest. The volume of the blood vessels was measured by point counting in serial semithin sections of the CA1 region and applying the Cavalieri principle as in previous studies (31–35). Blood vessels were counted when the lumen and endothelial cells were clearly identifiable. Counting was performed using the CastGrid integrated stereological package (Olympus, 2000, Denmark) attached to an Olympus BX51 light microscope at 40× magnification. Comparisons were made by using the Student t-test with the aid of SPSS 13.0 software (SPSS, Inc, Chicago, IL).

**Electron Microscopy**

For electron microscopy, we followed the same protocol for collagen IV immunocytochemistry previously described, except that Triton-X was excluded from the buffers. Once the immunostaining was completed, the sections were treated with 1% osmium tetroxide and embedded flat in Araldite resin. Plastic-embedded sections were studied by correlative light and electron microscopy as described in detail elsewhere (36). Briefly, sections were photographed under the light microscope, and they were then cut into serial semithin (2 μm) sections using a Reichert ultramicrotome. The semithin sections were examined under the light microscope and rephotographed. Selected semithin sections were resectioned into serial ultrathin sections with a silver-gray interference color that corresponded to a thickness of approximately 60 to 70 nm (37). The ultrathin sections were collected on formvar-coated single-slot grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX electron microscope (JEOL, Peabody, MA). Photographs were taken with a digitalizing image system (Mega View III Side-mounted TEM Camera, Soft Imaging System GmbH, Munster, Germany) using the analiSIS imaging acquisition software (analiSIS 3.2, Soft Imaging System).

**RESULTS**

**AP Histochemistry**

Alkaline phosphatase staining labeled numerous blood vessels in all fields of the hippocampal formation. In the CA fields, AP staining revealed numerous capillaries in the stratum radiatum, whereas relatively few labeled capillaries were evident in the alveus, the stratum oriens, and the stratum pyramidale (Fig. 1). In the subiculum, AP labeling was mainly detected in the stratum pyramidale; in DG, the molecular and polymorphic strata had the strongest AP staining, whereas the granular layer was almost devoid of capillaries. The density of AP-stained blood vessels was estimated in the strata pyramidale and radiatum of the CA fields, the stratum pyramidale of the subiculum, and the strata molecular, granular, and polymorphic of the DG.

We compared the density of AP-positive blood vessels in the DG, the CA fields, and the subiculum from autopsy tissues with those from nonsclerotic and sclerotic hippocampal tissues (Figs. 1 and 2). No significant differences were identified between the autopsy and nonsclerotic tissue in any of the hippocampal fields (Table 2). By contrast, the density of these blood vessels was significantly lower in the CA1 of the sclerotic hippocampi in which the area occupied by AP-positive blood vessels was reduced by 70% and 60% compared with that of the nonsclerotic hippocampus and autopsy tissue, respectively. No significant differences were found in the DG, CA2, CA3/CA4, or subiculum (Figs. 1, 2; Table 2).

**Collagen IV Immunostaining**

Analysis of the microvascular network using collagen IV immunocytochemistry revealed numerous vascular

<table>
<thead>
<tr>
<th>TABLE 2. Hippocampal Vessel Densities</th>
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<tr>
<td>Hippocampal Fields</td>
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<tr>
<td>---------------------</td>
</tr>
<tr>
<td>DG</td>
</tr>
<tr>
<td>CA3/4</td>
</tr>
<tr>
<td>CA2</td>
</tr>
<tr>
<td>CA1</td>
</tr>
<tr>
<td>Subiculum</td>
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Vessel densities are expressed as the percentage of the area occupied by alkaline phosphatase-positive vessels per unit of area of the cortex (mean ± SEM) and statistical comparisons with the indicated p values.

CA, cornu Ammonis; DG, dentate gyrus; ns, not significant.
structures that appeared to have a similar distribution pattern in both the sclerotic and nonsclerotic hippocampi at low magnification. Nevertheless, in the sclerotic CA1, there was a higher density of labeled elements (Figs. 3, 4A, B). Indeed, estimation of the density of collagen IV-immunoreactive blood vessels in the strata pyramidale and radiatum of the CA1 field from autopsy tissues and nonsclerotic and sclerotic hippocampal tissues showed no significant differences between the autopsy tissue and nonsclerotic tissue. The density of these collagen IV-immunoreactive blood vessels was, however, significantly higher in the CA1 field from autopsy tissues and nonsclerotic and sclerotic hippocampal tissues showed no significant differences between the autopsy tissue and nonsclerotic tissue.

The density of these collagen IV-immunoreactive blood vessels was, however, significantly higher in the CA1 of the sclerotic hippocampi (mean ± SEM, 24.3 ± 2.67) compared with the nonsclerotic hippocampi (9.4 ± 0.25, p = 0.02), which represented an increase of 160% in the collagen IV-immunoreactive density. This finding was in agreement with the increase in blood vessel density reported in patients with TLE when immunostaining for the vascular endothelial growth factor and tyrosine kinase receptors was analyzed (9). Moreover, a detailed examination of the collagen IV-immunoreactive blood vessels revealed that in the stratum pyramidale of the CA1 field from the sclerotic hippocampi, there were notable alterations in the appearance of the collagen IV-immunoreactive elements (Figs. 4C–G). In the sclerotic CA1 region, immunostained blood vessels had a rough surface with numerous small spinelike protrusions compared with the typically smooth surface found in the nonsclerotic CA1 (Figs. 4C, E). These protrusions gave a

FIGURE 3. Photomicrographs of collagen IV-immunostained hippocampal sections. Low-power photomicrographs from control (A) and sclerotic hippocampal (B) sections show the distribution of collagen IV-immunostained blood vessels. Arrows indicate the cornu Ammonis 1 (CA1) field, also shown at higher magnification in Figures 4A, B. Note that the sclerotic hippocampal formation is smaller than the control hippocampus. bv in (B) indicates a blood vessel, also shown at higher magnification in Figure 5A. DG, dentate gyrus; Sub, subiculum. Scale bar = (A, B) 500 μm.

FIGURE 4. Photomicrographs of the cornu Ammonis 1 (CA1) field from a control (A) and sclerotic (B) hippocampus illustrating the increase in collagen IV-immunoreactive (ir) blood vessels in the sclerotic tissue. Higher magnification of the CA1 area from a control (C) and sclerotic (D) hippocampus (indicated with arrows in Figs. 3A, B) illustrating the morphological modifications in the sclerotic hippocampus. (E) Higher magnification of the blood vessel indicated by an arrow in (C) illustrates the smooth surface of the collagen IV-ir blood vessels in the control tissue. (F) Higher magnification of the blood vessel marked with a single arrow in (D) shows the presence of small spinelike protrusions on the surface of the collagen IV-ir blood vessel (arrowheads). (G) Higher magnification of the area indicated with a double arrow in (D) to show the abnormal tubular or vascular-like collagen IV-ir structures with a vacuolar or reticulated appearance. Scale bars = (A, B) 75 μm; (C, D) 30 μm; (E–G) 10 μm.
spiny appearance to the surface of the blood vessels located in CA1 (Figs. 4D, F). Furthermore, instead of the homogeneous staining of the blood vessel surface, numerous abnormal tubular or vascular-like structures appeared with a vacuolar or reticulated aspect (Fig. 4G). These morphological alterations were also occasionally observed in the transitional area between the CA1 and subiculum, as well as in other CA regions. In collagen IV–immunostained sections that were counterstained with toluidine blue, it was evident that the abnormal reticulated vascular-like structures were very abundant in regions of severe neuronal loss and gliosis, such as the medial portion of the CA1. These structures were sparse in other hippocampal fields despite the relatively moderate neuronal loss and gliosis observed in these regions (Fig. 5).

**Volume Fraction Occupied by Blood Vessels**

To estimate the actual volume fraction (V_v) occupied by blood vessels irrespective of their immunoreactivity for collagen IV or AP activity, 50-μm-thick sections (n = 2–3 per case) adjacent to those used for histopathologic assessment were embedded in Araldite to obtain 2-μm-thick semithin sections. These semithin sections were stained with 1% toluidine blue to visualize the blood vessels in the CA1 subfield from nonsclerotic and sclerotic hippocampi. In each case, an average of 33 semithin sections were studied (range, 26–40), covering a surface of 100 to 300 mm², to estimate the V_v occupied by blood vessels using the Cavalieri method (Fig. 6). In the normal CA1 field, numerous blood vessels were observed (Figs. 6A, C); they occupied an average V_v of 2.51% ± 0.05%. By contrast, CA1 blood vessels were infrequently found in the sclerotic CA1 field (Figs. 6B, D), where the V_v was only 0.96% ± 0.13%, representing a significant decrease of approximately 60% (p < 0.01). Thus, the remarkable decrease in V_v-stained blood vessels in the sclerotic CA1 field was consistent with the finding that the volume fraction occupied by blood vessels in this region was also strongly reduced.

**Electron Microscopy**

Collagen IV–immunostained sections were also studied in the CA1 region using correlative light and electron microscopy, where the most important alterations of blood vessels seem to occur. For this purpose, 16 semithin (2 μm) plastic sections from the nonsclerotic (n = 3 cases) and sclerotic (n = 5) CA1 region were photographed, and serial ultrathin sections were then obtained from this tissue. A total of 67 normal blood vessels and 54 blood vessels that had an abnormal appearance at the light microscope level were examined using this correlative light-electron microscopy method. At the electron microscope level, blood vessels in the nonsclerotic CA1 had a normal appearance, with a smooth surface (Fig. 7), whereas those in the sclerotic CA1 displayed similar alterations to those observed by light microscopy (Figs. 8 and 9). Thus, the surface of many blood vessels displayed abnormal protrusions of the basal lamina that were responsible for the spiny appearance of the blood vessels in this region (Figs. 8C, D). Interestingly, the

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**FIGURE 5.** Photomicrographs showing collagen IV immunostaining in the transitional cornu Ammonis 1 (CA1)/subiculum (Sub) region from a sclerotic hippocampus. (A, B) Low-power photomicrographs of a collagen IV–immunostained section before (A) and after counterstaining with toluidine blue (B), bv indicates the same blood vessel as in Figure 3B. Arrows indicate surviving neurons in the transitional CA1/Sub region (Fig. 2A). (C) Photomicrograph of the large boxed area in (B) shows the labeling of blood vessels and the presence of Nissl-stained neurons and glial cells. Arrows indicate pyramidal neurons in the transitional CA1/Sub region. (D) Higher magnification of the area boxed in (C) illustrating the presence of normal collagen IV–ir blood vessels adjacent to normal-looking neurons (arrows). (E) Higher magnification of the small boxed area in (B) illustrates morphologically altered blood vessels (arrowheads) concurrent with gliosis and neuronal loss in the medial CA1. Scale bars = (A, B) 300 μm; (C) 60 μm; (D, E) 30 μm.

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vascular-like collagen IV–immunoreactive processes with a vacuolar appearance were indeed atrophic branches of blood vessels, with the peculiarity that their reduced lumen was mainly filled with the processes of reactive astrocytes (Fig. 9). The nature of these structures suggests that collapse of the blood vessels had occurred in this region.

**DISCUSSION**

The main findings of the present study are that there is a consistent decrease in blood vessels (up to 70%) in the CA1 field of the sclerotic human epileptic hippocampus, as well as alterations in the remaining microvessels. Together, these results indicate a strong relationship between hippocampal sclerosis and alterations in the microvascular network in the CA1.

Alkaline phosphatase staining in the CA1 field of the sclerotic hippocampus from epileptic patients was severely diminished, whereas no significant differences were found in the DG, CA2, CA3/CA4, and subiculum. Furthermore, uniform AP staining was observed in all hippocampal fields from nonsclerotic epileptic patients, suggesting that the changes in AP activity are specifically related to hippocampal sclerosis and not to epileptic activity per se. Although we do not know the biologic relevance of this finding, it has been shown that AP activity varies in the rat brain when seizures are induced (38). Moreover, genetic defects in AP lead to epileptic seizures because of alterations in the normal AP activity (39–43). In addition, AP may be involved in the regulation of \( \gamma \)-aminobutyric acid (GABA) synthesis and neurotransmission in the cerebral cortex (29, 44). Thus, because the reduction of AP staining would suggest a modification in its activity, this might in turn imply the modulation of the GABAergic system. Because alterations of the GABAergic system have been proposed to explain the development of epilepsy (45–47), changes in AP activity may contribute to the impairment of the GABAergic system in the sclerotic hippocampus.

The decrease in the density of AP-labeled blood vessels contrasts with the higher density of vascular-like processes immunostained for collagen IV in the sclerotic hippocampus compared with the nonsclerotic hippocampus. This increase would suggest that angiogenesis has occurred. This would be consistent with the recently reported increase in blood vessel density in patients with TLE, observed through immunostaining for the vascular endothelial growth factor and tyrosine kinase receptors (9). Electron microscopy, however, revealed that collagen IV–immunoreactive elements in the sclerotic CA1 were mostly atrophic capillaries with a reduced or virtually absent lumen, many filled with processes of reactive astrocytes. Thus, normal capillaries were rarely observed in the CA1, and this observation was confirmed by the decrease of around 60% in the \( V_c \) occupied by blood vessels. This decrease coincided with the decrease in AP staining and demonstrates a real loss of microvessels. Together, these changes in CA1 vascularization indicate profound alterations of the microvasculature in this particular hippocampal field.

In a previous study using the same hippocampal tissue, significant reductions in the total neuronal density were observed in all subfields of the sclerotic hippocampi compared with the nonsclerotic hippocampi (28). Because differences in the density of AP-positive blood vessels were only significant in CA1, our results seem to indicate that there is no correlation between alterations of the AP activity and neuronal loss and gliosis outside of the CA1 field. Furthermore, in both the CA1 and the transitional CA1/subiculum region, collagen IV immunostaining revealed morphological alteration of blood vessels. Remarkably, these altered blood vessels were mainly found in those areas where neuronal loss was so severe that virtually no neurons were present (see later). It is well known that neurons, glia, and blood vessels are closely coupled both anatomically and functionally (48–50). Indeed, neurons, glia, and blood vessels have been considered together as a functional entity, the neurovascular unit (50).

Perturbations in the integrity of neurovascular function are thought to initiate several cascades associated with injury (51). Inflammatory processes are activated if normal blood-brain barrier permeability is disrupted (52), damaging the adjacent brain tissue, producing ion imbalances, and inducing...
cell death (51). Because neuronal loss occurs in all CA fields and in the DG of the sclerotic hippocampus, there is no clear relationship between neuronal death and vascular alterations. On the other hand, the greatest neuronal loss is observed in the CA1, particularly in the medial portion of the CA1, in which up to 80% of neurons may be lost in the sclerotic hippocampus (28). Thus, it is possible that specific impairment of the neurovascular unit in the CA1 causes the loss of neurons in this hippocampal field and that the adjacent blood vessels might then degenerate, becoming atrophic and nonfunctional elements, as reflected by the staining for AP and collagen IV in the CA1 and in the neighboring CA1/subiculum region. We cannot, however, determine whether the alterations of the vascular system precede neuronal damage in the sclerotic hippocampus, as reported in patients with leukoaraiosis (20).

The latter condition is a degenerative brain disease of the white matter in which significant blood vessel loss is evident before any visible damage to the cerebral cortex appears. Hence, more extensive local loss of blood vessels in the CA1 may make this region more susceptible to neuronal damage than other hippocampal fields.

Finally, interictal-like discharges are mostly initiated in the subiculum and in the area of transition to the CA1 (53). Indeed, it has been hypothesized that the histologically normal subiculum may serve as a hyperexcitable fringe area that would amplify the relatively few excitatory outputs from the sclerotic Ammon horn (47, 54–56). The subiculum represents an anatomical transition zone between Ammon horn and the entorhinal cortex, and it is the major output structure of the hippocampus (57, 58). At the boundary

**FIGURE 8.** Correlative light and electron microscopy photomicrographs of collagen IV–immunoreactive (ir) blood vessels in the cornu Ammonis (CA1) field of a sclerotic hippocampus. (A) Photomicrograph of a plastic-embedded section showing collagen IV–ir blood vessels. (B) Photomicrograph of a 2-μm-thick semithin plastic section obtained from the same section shown in (A). V1 and V2 indicate the same blood vessels labeled in (A). (C) Low-power electron micrograph taken after resection of the semithin section shown in (B) illustrating blood vessel V1. The arrow and arrowhead indicate small spinelike protrusions at the surface of the collagen IV–ir blood vessel and an atrophic branch, respectively. (D, E) Higher magnification of (C) illustrates the morphological alterations of the blood vessel V1. Arrow in (D) indicates the same protrusion as in (C). (E) Higher magnification of the atrophic branch in the blood vessel, which appears as an abnormal excrescence of the endothelial membrane. Scale bars = (A) 150 μm; (B) 75 μm; (C) 10 μm; (D) 2.5 μm; (E) 1 μm.
between the CA1 and subiculum from human temporal lobe epileptic tissue, some surviving neurons lack GABAergic perisomatic innervation, whereas others are hyperinnervated by abnormally dense axon terminals from interneurons (27). Moreover, changes in the density and proportion of parvalbumin-immunoreactive interneurons have been observed in the subiculum (28). In addition, there are alterations in the relative expression of NKCC and KCC cotransporters in this region. Such changes have been proposed as a neurochemical substrate for the depolarizing responses induced by GABAergic signaling through GABA<sub>A</sub> receptors in neurons within the subiculum and in the transitional CA1/subiculum region (53, 59). Taken together, our data regarding the morphological changes in the vascularization of the transitional CA1/subiculum regions further emphasize the importance of this region in the epileptogenicity of the sclerotic hippocampus.

In summary, profound vascular alterations are consistently observed in the sclerotic CA1 and, therefore, they can be considered as a major histopathologic feature of hippocampal sclerosis. Further electrophysiological studies, in conjunction with correlative microanatomical and neurochemical characterization of the epileptic tissue, will be necessary to define the functional significance of specific changes in the microvascular network of TLE patients. These results suggest that the vascular system may represent an appropriate target for therapeutic interventions in patients with TLE associated with hippocampal sclerosis.

**FIGURE 9.** Electron microscopy of a collagen IV-ir blood vessel from the cornu Ammonis 1 (CA1) field of a sclerotic hippocampus demonstrates its morphological alterations. (A, B) Low magnification of collagen IV-ir atrophic blood vessels. Note that the lumen has almost completely disappeared or it is filled by glial processes. (C, D) Higher magnifications of the boxed areas in (A) and (B), respectively. The asterisks indicate the typical processes of reactive astrocytes filling the blood vessels; arrows point to the abnormal appearance of the endothelial membrane in (C) and (D). Scale bars = (A) 1.4 μm; (B) 2 μm; (C) 0.5 μm; (D) 1.4 μm.
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