Novel Cerebral Lesions in the Eker Rat Model of Tuberous Sclerosis: Cortical Tuber and Anaplastic Ganglioglioma

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Abstract. The Eker rat is a model for human tuberous sclerosis (TSC) caused by a mutation in the Tsc2 gene. We describe here histological and immunohistochemical findings of the brain lesions in Eker rats, with emphasis on 2 novel lesions found in this study: a cortical tuber and an anaplastic ganglioglioma. The rat cortical tuber resembled those of humans, and further confirmed the value of this animal model as a tool for investigating the molecular pathology of tuberous sclerosis. On the other hand, the rat anaplastic ganglioglioma had features of a malignant neoplasm that are absent from human subependymal giant cell astrocytomas.

Key words: Animal model; Cortical tuber; Eker rat; Ganglioglioma; TSC2; Tuberin; Tuberous sclerosis.

INTRODUCTION

Tuberous sclerosis (TSC) is characterized pathologically by the development of benign tumors (hamartomas) in the skin (angiofibroma), kidney (angiomyolipoma), heart (rhabdomyoma), and various other organs (1). Malignant tumors occur only rarely, but a small number of patients develop renal cell carcinoma (2). In the brain, lesions typical of TSC include cortical tubers, clusters of heterotopic neurons in the white matter, and subependymal nodules or giant cell astrocytomas (SGCAs). The hallmark common to these lesions is the presence of abnormal giant cells. Histological and immunohistochemical studies have demonstrated that the giant cells randomly show either neuronal or glial features, or both, indicating their abnormal differentiation (1, 3).

TSC is an autosomal dominantly inherited disorder caused by mutations of either of 2 genes: TSCI on chromosome 9q34 (4) or TSC2 on 16p13.3 (5). The respective genes were cloned in 1997 and 1993, and their products were named hamartin (6) and tuberin (7). Recent studies have shown that the 2 proteins colocalize and interact with each other (8, 9). In order to elucidate their pathogenetic role in the neurologic manifestations of TSC, their expression in the control tissues and TSC lesions has been investigated extensively (10±17). The results, however, have been variable and often difficult to interpret. The discrepancies may have resulted from the genetic heterogeneity of human TSC (TSCI vs TSC2, deletions vs point mutations, etc.), as well as from the different methods employed in the studies.

These problems may be circumvented by the use of a naturally occurring animal model of TSC, the Eker rat. This autosomal dominant disorder is caused by a germ-line mutation in the rat Tsc2 gene (18). Carriers of the mutation (Tsc2<sup>+/−</sup>) develop multiple renal cell carcinomas bilaterally in the kidneys (19, 20). Little attention has been paid to the brain pathology of Eker rats, but a pioneering work recently found 2 types of intracerebral lesions, subcortical and subependymal hamartomas, in 19 (44%) and 9 (21%), respectively, of the 43 brains examined (21). The similarity of these lesions to some of the human TSC lesions noted above indicates the usefulness of this animal model in the molecular neuropathologic studies of TSC.

In the present study, we detected in the Eker rat cerebrum 2 novel lesions, a cortical tuber and anaplastic ganglioglioma, in addition to the 2 types of hamartomas described above. The presence of a cortical tuber is important, as tubers are epileptogenic and presumably associated with autism and other neurological symptoms of TSC (22). To further elucidate the similarities and differences between the rat and human lesions, we immunohistochemically characterized the lesions using antibodies against various neuronal and glial markers and against tuberin.

MATERIALS AND METHODS

Animals

Founder rats carrying the Eker mutation were kindly provided by Dr. Alfred G. Knudson (Fox Chase Cancer Center, Philadelphia, PA). Eker rats were bred on a Long Evans background at the Animal Facility of the Cancer Institute since 1991. All animals were housed and treated in accordance with institutional
Eker rats were diagnosed as carriers (Tsc2<sup>2<sup>ex</sup></sup>) by detecting the mutation in the Tsc2 gene (18). Neither seizures nor abnormal behaviors were evident on routine observation of these rats. Nineteen carriers and 6 noncarriers (Tsc2<sup>+/+</sup>), from 17 to 24 months of age, were killed and underwent complete necropsy. Kidney tumors were found in all of the carriers, but in none of the noncarriers. For Western blotting experiments, the brains of 5 carriers and 2 noncarriers were frozen and stored at −80°C. The remaining brains for immunohistochemistry were cut into 4 consecutive coronal sections and fixed in 10% neutral formalin. They were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E) and Kluèver-Barrera stainings.

**Antibodies**

Two antibodies, anti-Tub-NT and anti-Tub-CT, were raised against human tuberin and characterized previously (12). Preliminary experiments confirmed their cross-reactivity with rat tuberin. For immunohistochemistry, we used a panel of antibodies that recognized neuronal and glial antigens, consisting of mouse monoclonal antibodies against nonphosphorylated neurofilament (NF P−) (clone SMI-32, Sternberger Monoclonals, Baltimore, MD), phosphorylated neurofilament (NF P+) (clone SMI-31, Sternberger), neuron-specific enolase (NSE) (Chemicon, Temecula, CA), calbindin-D28k (clone CL-300, Baltimore, MD), phosphorylated neurofilament (NF P<sub>2</sub>), and some for NF P<sub>1</sub>. They were all stained with vimentin. Subsequent procedures of biotin-streptavidin-peroxidase staining were the same as those described elsewhere (13).

**Immunohistochemical Properties of the Abnormal Large Cells in the Eker Carrier Cerebral Lesions**

<table>
<thead>
<tr>
<th>Lesion (#)</th>
<th>NF P−</th>
<th>NSE</th>
<th>CaBD</th>
<th>GFAP</th>
<th>Vimentin</th>
<th>Tuberin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical tuber (1)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Subcortical hamartoma (2)</td>
<td>−/+</td>
<td>−/+</td>
<td>−/−</td>
<td>−/−</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>Subependymal hamartoma (2)</td>
<td>−/+</td>
<td>−/+</td>
<td>−/−</td>
<td>−/−</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>Anaplastic ganglioglioma (1)</td>
<td>−/−</td>
<td>+/+</td>
<td>−/+</td>
<td>−/+</td>
<td>−/−</td>
<td>−/+</td>
</tr>
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Abbreviations: NF P−, nonphosphorylated neurofilament; NSE, neuron-specific enolase; CaBD, calbindin-D28k; GFAP, glial fibrillary acidic protein; +/−, positive in more than half of the cells; −/+−, positive in a small number of cells.

**RESULTS**

Gross examination of the dissected brains revealed enlargement of the lateral ventricles in 5 of the 19 Eker carriers, and in none of the 6 noncarrier rats. Microscopic observation of 14 carrier brains disclosed a cortical tuber in 1, subcortical hamartomas in 2, subependymal hamartomas in 2, and an anaplastic ganglioglioma in 1. All of these lesions were characterized by the presence of abnormal large cells. Their immunohistochemical properties are summarized in the Table. In 4 noncarrier brains, there were no histological abnormalities.

**Cortical Tuber and Subcortical Hamartoma**

A cortical tuber was found in the primary motor area of a 19-month-old carrier. Routine stainings showed that the tuber was 1.5 mm in size, and ill defined. Focal loss of normal lamination, decrease in neuronal density, and slight astrogliosis were noted (Fig. 1A). There were many cytomegalic neurons, about 35 μm in size, with Nissl substance in the center or periphery of their abundant cytoplasm. Their large nuclei had a single, prominent nucleolus (Fig. 1B). Immunohistochemically, there were many axons positive for NF P+ (Fig. 1D). Most of the cytomegalic neurons were positively stained with anti-NF P− (Fig. 1E, F), whereas none showed immunoreactivity for GFAP (Fig. 1G; Table).

A subcortical hamartoma was present in the white matter just beneath the tuber (Fig. 1A). This 0.7 mm sized hamartoma had a clear margin, showed hypomyelination and focal calcification, and consisted of abnormal large cells intermingled with elongated cell processes. The cells had abundant, pale cytoplasm that often showed vacuolation. Their nuclei varied in size, and were occasionally lobulated (Fig. 1C). Axonal swelling was noted in the periphery. Immunohistochemically, there were few axons stained with anti-NF P+ in the hamartoma (Fig. 1D). Many of the large cells were weakly positive for GFAP (Fig. 1H), and some for NF P− (Fig. 1E).
There was another subcortical hamartoma in the motor area of another Eker carrier, 0.3 mm in size, without an accompanying tuber. This nodule consisted of large, eosinophilic cells, which often had a prominent nucleolus and intercalating processes. These cells were weakly stained for GFAP, but not for neuronal markers (Table).

Tuberin immunoreactivity was absent from the tuber (Fig. 2A) and subcortical hamartomas (Fig. 2B). In the former, neither cytomegalic nor normal-sized neurons showed positivity. In the rest of the cerebral cortex, however, there were many immunoreactive neurons (Fig. 2C), the density of which was comparable to that of noncarriers (Fig. 2D). These findings were in agreement with the results of Western blotting, which showed no difference in tuberin content between carriers and noncarriers (Fig. 2E).

Subependymal Hamartoma and Anaplastic Ganglioglioma

Two Eker carriers had subependymal hamartomas, one in the caudoputamen, and another in the nucleus accumbens. The former was 0.3 mm in size, and consisted of large cells with abundant, pale cytoplasm. Their nuclei contained a nucleolus, and were occasionally multiple (Fig. 3A). About half of these cells were positively stained for GFAP (Fig. 3C), and none for NF P− (Fig. 3B). The latter nodule was 0.2 mm in size, and consisted of basophilic neuron-like cells (with Nissl substance) and eosinophilic balloon cells (Fig. 3D). Both types of cells showed random immunoreactivities for neuronal and astrocytic antigens, irrespective of their morphology (Table); some were positive for NF P− (Fig. 3E), and many for GFAP (Fig. 3F). In both nodules, these abnormal large cells were intermingled with abundant, elongated processes that were positive for GFAP. There were no axons immunoreactive for NF P+.

A 21-month-old carrier had a tumor, 4 to 8 mm in size and well circumscribed, arising near the splenium of the corpus callosum. The tumor cells were densely packed and pleomorphic. They had abundant cytoplasm with Nissl substance in the periphery, and a large, clear nucleus showing marked atypism and containing a prominent nucleolus. Some of the cells had multiple nuclei. Mitotic figures and nuclear fragmentation were frequently seen. These cells did not show a particular pattern of alignment, and were separated by eosinophilic interstitial tissue (Fig. 4A). Small deposits of hemosiderin and calcification were seen occasionally. Immunohistochemically, the tumor cells were positive for synaptophysin (Fig. 4B) andNSE, but not for calbindin (Table). A small number of them showed intense immunoreactivities for NF P− (Fig. 4C), GFAP (Fig. 4D), and vimentin (Fig. 4E). Labeling indices for Ki-67 and ssDNA were 9.2% and 1.9%, respectively. Tuberin staining was negative (Fig. 4F). Based on these histological and immunohistochemical findings, this tumor was named anaplastic ganglioglioma.

DISCUSSION

Recent studies have revealed that both TSC1 and TSC2 encode tumor suppressors. The TSC2 gene has 2 coiled-coil domains and a region with homology to GTPase-activating protein (GAP) (7, 9). Its product, tuberin, has GAP activities specific for Rap1, a member of the Ras superfamily that may function as a positive mitogenic signaling molecule (24), and for Rab5, a modulator of fluid-phase endocytosis (25). Tuberin plays a role in cell cycle control by inhibiting the transition from G0/G1 to S phase (26). A Drosophila homolog of TSC2, gigas, also regulates the cell cycle by blocking DNA replication or promoting mitosis (27). The TSC1 gene has no homology to TSC2 or other known vertebrate genes. Its product, hamartin, has a transmembrane domain and a coiled-coil domain (6). Although the molecular mechanism by which hamartin exerts tumor suppressor activity remains unknown, hamartin and tuberin bind to each other in vitro and in vivo, and the interaction is mediated by their coiled-coil domains (9).

The development of TSC-associated hamartomas, such as renal angiomyolipomas and cardiac rhabdomyomas, can be explained by Knudson’s two-hit theory (28). Patients with TSC carry a germline, loss-of-function mutation in either the TSC1 or TSC2 gene, the type of which is variable among families (29–33). Subsequently, a somatic mutation causing the loss of the normal allele gives rise to the renal and cardiac hamartomas, which often

Fig. 1. A cortical tuber and a subcortical hamartoma in the primary motor area of an Eker carrier. A: There were many cytomegalic neurons in the cortical tuber. Cortical lamination was disturbed, and cell density was high because of astrogliaosis. The subcortical hamartoma showed myelin pallor (Klüver-Barrera, 45X). B: Cytomegalic neurons contained Nissl substance. Some of them were balloon-like (Klüver-Barrera, 225X). C: The abnormal large cells in the subcortical hamartoma had eosinophilic cytoplasm, which often contained vacuoles (arrow). There was abundant calcification (H & E, 225X). D: Axons positive for NF P+ were present in the tuber (top) and white matter (bottom), but not in the hamartoma (middle) (NF P+ immunoperoxidase, 56X). E and F: In the tuber (E, top, and F), most of the cytomegalic neurons were intensely immunoreactive for NF P−. There was only weak labeling in the hamartoma (E, bottom), associated with abnormal large cells and axonal spheroids (NF P− immunoperoxidase, E, 90X; F, 180X). G and H: In the tuber (G), GFAP immunoreactivity was localized in scattered astrocytes. Cytomegalic neurons were negative. The abnormal large cells of the hamartoma (H) were weakly stained for GFAP (GFAP immunoperoxidase, 180X).
Fig. 2. Tuberin immunoreactivity was absent from the cortical tuber (A) and subcortical hamartomas (B) of an Eker carrier. However, positive labeling was noted in the normal-appearing cerebral cortex (C), the intensity of which was comparable to that of noncarriers (D) (A–D, tuberin immunoperoxidase, 180×). Western blotting of cerebral homogenates also showed no difference in tuberin content between 5 carriers and 2 noncarriers (E).

show loss of heterozygosity at 9q34 (34, 35) or 16p13.3 (14, 36) and the resultant disappearance of tuberin or hamartin (13, 14, 16).

In contrast, the mechanism underlying the development of TSC brain lesions, particularly cortical tubers, remains obscure. Recent genetic studies have detected LOH involving TSC1 (34, 35) or TSC2 (14, 37) in SGCAs, but not in cortical tubers (37). Immunohistochemically, abnormal giant cells of SGCAs show marked loss of tuberin immunoreactivity (13, 14), but those of cortical tubers often demonstrate significant staining for tuberin (even in patients with a proven TSC2 mutation) that is stronger than that of nearby, normal-sized neurons (10, 13, 17). Subtotal loss of tuberin, as detected by Western blotting, involves not only tubers but also histologically normal cortices (12). Since these features of cortical tubers apparently contradict the two-hit model for their histogenesis, precise analyses of the molecular phenotype of their pathognomonic giant cells are awaited. In this context, the presence of a cortical tuber in the Eker rat model, the germline mutation of which is already known (18), is of great importance.
Fig. 3. Subependymal hamartoma found in 2 Eker carriers, one in the caudoputamen (A–C), and another in the nucleus accumbens (D–F). A and D: The former hamartoma consisted predominantly of astrocytic cells, some of which had multiple nuclei (A), while the latter was a mixture of neuron-like (arrow) and balloon cells (arrowheads) (D) (H & E). B and E: Immunoreactivity for NF P– was absent from the former (B), but was weakly positive in the latter (E) (NF P– immunoperoxidase). C and F: Both hamartomas were positively stained for GFAP (GFAP immunoperoxidase). 225×.

The present study detected for the first time 2 lesions—a cortical tuber and anaplastic ganglioglioma—in addition to subcortical and subependymal hamartomas, which had been found previously (21). The morphological features of the rat cortical tuber, such as cortical dyslamination and cytomegaly, were similar to those of human tubers, although the former, unlike the latter, was not visible macroscopically and did not bulge above the neighboring cortex. The rat tuber was different from the subcortical and subependymal hamartomas in the histological and immunohistochemical properties of the abnormal giant cells composing them. In the cortical tuber, all the cells appeared to be cytomegalic neurons, and were positive exclusively for neuronal antigens. In the subcortical and subependymal hamartomas, many of the cells resembled astrocytes, as described previously (21), whereas some in this study were noted to express neuronal antigens, such as NF P– and NSE. This regional difference in the properties of giant cells mimics, to some degree, that observed previously in human TSC brains.
Fig 4. Anaplastic ganglioglioma in the brain of an Eker carrier. A: The cell density was high. Tumor cells showed pleomorphism and nuclear atypism. Some of them had multiple nuclei (arrows), and others showed mitotic figures (arrowheads) (H & E, 450×). B: Many of the tumor cells were positive for synaptophysin (Synaptophysin immunoperoxidase, 180×). C: A very small number of the tumor cells were intensely positive for NF P− (NF P− immunoperoxidase, 360×). D: GFAP immunoreactivity was positive in some tumor cells, and in perivascular astrocytes (upper right) (GFAP immunoperoxidase, 180×). E: Some of the tumor cells were positively labeled for vimentin (Vimentin immunoperoxidase, 180×). F: There was no tuberin immunoreactivity (Tuberin immunoperoxidase, 180×).
(38), although their exclusively neuronal appearance in the rat tuber is rare in human lesions.

This study also noted some differences between the brain lesions of Eker rats and those of human TSC patients. The most notable of them was the presence of an anaplastic ganglioglioma in the brain of an Eker carrier. Although some histological and immunohistochemical aspects of this tumor were reminiscent of human SGCA, its biological nature as a malignant neoplasm was manifested by marked nuclear atypism, active mitosis (a high labeling index for Ki-67), apoptotic cell death (positive labeling for ssDNA) and microhemorrhage, findings never seen in SGCA. In human TSC, there have been several anecdotal case reports of the occurrence of malignant gliomas, such as glioblastoma multiforme and ependymoma (1, 39, 40). However, these cases have been deemed incidental because of their extreme rarity. In general, the incidence of malignancies, such as renal cell carcinoma and splenic hemangiosarcoma (41), is much higher in the Eker rats than in human TSC patients. The occurrence of malignant brain tumors, such as the one found in this study, may also result from this increased susceptibility.

We also noted that tuberin expression in the cerebrum of Eker rats is altered in a somewhat different way from that of human TSC patients. Cytomegalic neurons of a rat cortical tuber, as well as the giant cells of hamartomas and tumor cells of gangliogliomas, showed no immunoreactivity for tuberin, in contrast to the aforementioned positivity of human lesions (10, 13, 17). On the other hand, there was diffuse loss of tuberin involving histologically normal cerebral regions of human TSC patients (12, 13), but not of Eker carriers. The molecular basis underlying these differences is unclear, but it is plausible that the physiological level of tuberin expression varies among species, and that certain human TSC2 mutations give rise to immunoreactive tuberin protein that is functionally deficient.

From the neuropathological viewpoint, the Eker rat model appears to be particularly useful in several lines of experiments. First, it would be interesting to study the molecular pathology of the abnormal giant cells (in particular, cytomegalic neurons in the cortical tuber), preferably by manipulation of single cells, as applied recently for human TSC lesions (42). A microdissection analysis is under way in our laboratory. Second, the Eker rat model is suitable for developmental studies because brains of carrier fetuses and pups are readily available. It should be interesting to observe early changes leading to the development of cortical tubers. The low incidence of this lesion may pose a problem, but the chance of discovery would be increased by sectioning the materials serially. Third, subjects homozygous for the Eker mutation are available for neuropathological examination. The Tsc2/CkI-6 state is lethal in midgestation, and the brains of homozygous embryos show exencephaly and focal, papillary overgrowth of the neuroepithelium (43). Future studies should aim to analyze the biochemical consequences of the graded loss of tuberin using Tsc2/CkI-6 and Tsc2/CkI-6+/− cells.

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