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RESEARCH ARTICLE

Co-Localization of TSC1 and TSC2 Gene Products in Tubers of Patients with Tuberous Sclerosis

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Two genes, mutations in which result in the phenotype of tuberous sclerosis (TSC), have recently been cloned. TSC2 on chromosome 16p13.3 encodes the protein tuberin, which appears to have growth regulating properties. TSC1 on chromosome 9q34 encodes hamartin which, as yet, has no specified cellular functions. Polyclonal antibodies were raised to synthetic peptides representing portions of tuberin and hamartin and used in immunoblots and immunohistochemical studies to localize the proteins in surgically resected neocortical tubers from four TSC patients. On Western blots of autopsy brain specimens, K-562 cell, and NT2 lysates, each antibody labelled a single band at the expected molecular weight. In immunohistochemical protocols on paraffin embedded tissue, antibodies to both tuberin and hamartin prominently labelled atypical and dysmorphic neuroglial cells that are a defining feature of TSC tubers. Some abnormal cells within cortical tuber sections were labelled with both tuberin and hamartin antisera. Our results suggest that tuberin and hamartin are both robustly expressed in similar populations of neuroglial cells of TSC tubers, even in the presence of TSC1 or TSC2 germline mutations. The roles of these gene products in normal and abnormal cortical development, tuber pathogenesis and the generation of seizures remain to be defined.

Introduction

Within the past several years, two genes (TSC1 and TSC2) associated with the multisystem disorder tuberous sclerosis (TSC) have been cloned and characterized (30). The first of these to be cloned, TSC2, is on chromosome 16p13.3 and encodes a protein, tuberin, which appears to have growth regulating properties (6, 37). Both the TSC2 transcript and tuberin are widely localized within epithelial cells, lymphocytes and endocrine glands throughout the body in both TSC patients and unaffected controls (22, 25, 38). Within the central nervous system (CNS) of both humans and rodents, TSC2 transcript and tuberin are abundant within neurons of the cerebral and cerebellar cortex, spinal cord and brainstem (especially motor neurons), choroid plexus epithelium and ependymal cells, and possibly astrocytes (8, 9, 19, 36). Tuberin appears to be overexpressed in dysmorphic neuron-like cells which are a defining feature of cortical tubers found in TSC patients (19).

The TSC1 gene on chromosome 9q34 has recently been cloned, and encodes a protein (hamartin) that as yet has no defined function (31). 'Loss of heterozygosity' studies suggest that it may encode a tumor suppressor molecule (2, 14). The purpose of this investigation was to (a) develop and characterize antibodies to hamartin and tuberin, (b) to use these immunoreagents to localize, using immunohistochemistry, sites of expression of hamartin in surgically resected cortical tubers from children with intractable epilepsy associated with TSC, and (c) to compare patterns of expression of hamartin and tuberin within these lesions. We have specifically chosen to focus initially on immunolocalization of these two important proteins in cortical tubers because tubers are a defining cerebral lesion of TSC, and, at least in a subset of TSC patients, are the apparent site at which epileptic seizures originate. Furthermore, tubers of TSC bear a striking resemblance, in terms of both architectural disorganization of the neocortex and cytologic components, to (non-familial) dysplastic cortical lesions associated with intractable

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Pt. #	Age	Location of tuber
1.	6 months	Frontal cortex/insula
2.	39 months	Frontal (right)
3.	1 year	Parieto-temporal -occipital
4.	14.3 years	Frontal

All patients were female; presented with intractable epilepsy and had other stigmata of tuberous sclerosis, age given is at the time of surgery.

Table 1. Clinical data on patients from whom tubers were resected.

seizure disorders in infants and children (7, 23, 33, 34). Thus insights into the pathogenesis of TSC tubers may be instructive in understanding the structural neurobiology of cortical dysplasia (CD).

Materials and Methods

Preparation and Characterization of Antibodies. Polyclonal antibodies were prepared to synthetic peptides representing amino acids #775-794 of the alpha-tuberin molecule (6) and amino acids (a.a.) #1107-1130 of hamartin (31) (Zymed Laboratories, South San Francisco, CA). For each peptide, rabbits were immunized with a conjugate of synthetic peptide and keyhole limpet hemocyanin (KLH) in a regimen consisting of one priming and three booster immunizations over 2-3 months. Resulting immunoreactive sera were affinity purified. Briefly, peptide-conjugated affinity gels were made using the Sulfolink Kit (Pierce, Rockford, IL). Prepared gels were batch-incubated with 25-30 mL of rabbit anti-peptide-KLH for 1 h at room temperature with gentle rocking. Column run-through was collected, and the gels washed with 50 mL of phosphate-buffered saline (PBS). Elution of antibodies was performed using 3 M KSCN, pH 7.0 (anti-tuberin) or 0.1 M glycine, pH 2.5 (anti-hamartin). Fractions that were determined by spectrophotometry (A280) to contain sufficient protein were pooled and dialyzed overnight against PBS containing 0.1% NaN_3. Dialyzed antibodies were concentrated to 1 mg/mL using centriplus-30 concentrators (Amicon, Beverly, MA), then stored at -20°C until use.

Immunoblotting. Frozen human frontal lobe autopsy tissue from two (non-TSC) patients was thawed in 2.5 volumes (by weight) of ice cold sucrose homogenization buffer (0.32 M sucrose, 5 mM HEPES, 3 mM EGTA [ethylene glycol-bis(beta-aminoethyl ether)-N, N, N', N'-tetraacetic acid], 0.5 mM MgSO4 and 1 mM PMSF [phenylmethylsulfonyl fluoride]) in Eppendorf tubes. It was then homogenized and heavy particulate components were sedimented at 5000 g x 8 minutes. The supernatant was removed and the tissue pellet rehomogenized in 2.5 volumes of the sucrose homogenization buffer. Homogenate was again centrifuged at 5000 g x 8 min. The supernatant was removed, combined with the first supernatant fraction and centrifuged (1 h at 100,000 g) in a Beckman model L5-75 ultracentrifuge with an SW 60 swing rotor. Cytosolic supernatant was collected and the membrane pellet resuspended in sucrose homogenization buffer. Tissue and fractions were stored at 4°C throughout the procedure. Protein quantitation was performed using the Bio-Rad assay (Bio-Rad, Hercules, CA). Protein fractions were separated on 5% stacking/12% resolving Na dodecyl sulphate (SDS)-denaturing gels and transferred to PVDF (polyvinylidene difluoride) membrane for immunoblotting. Nonspecific binding was blocked with 8% nonfat milk/0.2% Tween-20 in PBS. The membrane was incubated with anti-hamartin at a dilution of 1:400 (2.5 μg/mL) in blocking solution. Rabbit horseradish peroxidase (HRP)-conjugated secondary antibody was incubated with the membrane at a dilution of 1:1000 and detected by enhanced chemiluminescence (New England Biolabs, Beverly, MA, luminol reagent). In some cases, the anti-hamartin peptide antibody was preadsorbed with the peptide (to which antibody had initially been made) in order to specifically block hamartin immunolabelling; antibody was incubated with excess hamartin [1107-1130], 20 μg peptide per μg antibody in 500 mL PBS, for 1 h at 4°C with gentle agitation.

K-562 (ATTC #CCL-243) cells (1.4-2 x 10^8) were pelleted and washed with PBS, then incubated in 1 mL of ice cold lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 1 mM dithiothreitol [DTT], 1 mM PMSF) for 30 min with occasional agitation. The lysate was cleared by centrifugation at 10,000 g (10 min, 4°C). NT2 (Ntera2/D1) cell (Stratagene, La Jolla, CA) pellets were lysed in 1.5 x lysis buffer (Analytical Luminescence Laboratory, San Diego, CA), and protein quantitation was performed as mentioned. Homogenates were mixed 1:1 with 2 x SDS sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2 % bromophenol blue, 20% glycerol) and heated at 100°C for 5 min. Lysates were separated on SDS-denaturing gels and transferred to nitrocellulose or PVDF membranes for blotting. Membranes were blocked with tris-buffered saline (TBS), 3% normal goat

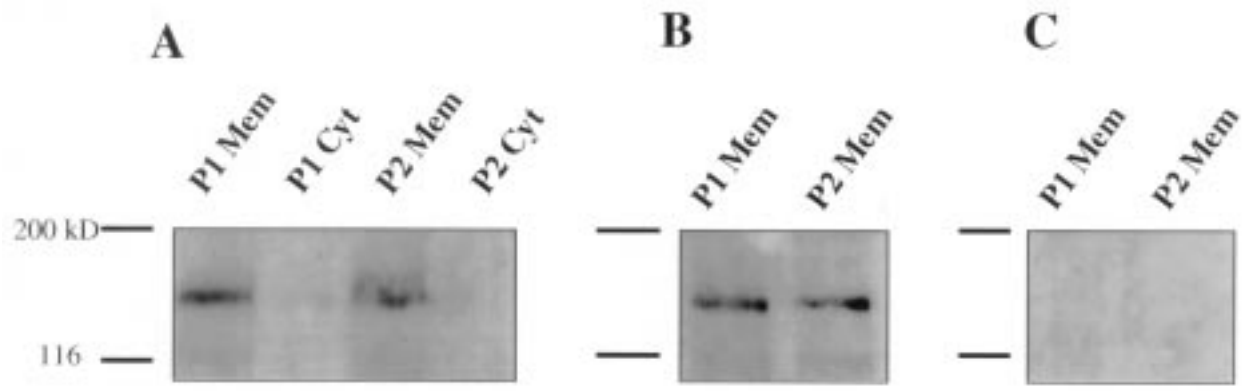

Figure 1. Western blot analysis of protein fractions using anti-hamartin antibody. Specimens of human (autopsy) brain (frontal lobe) from non-TSC patients (P1 = patient 1, P2 = patient 2) were fractionated into membrane ('mem') and cytosolic ('cyt') components. Equal amounts of total protein were loaded into all lanes on each gel prior to electrophoresis and immunoblotting (all lanes 50 μg in panel A, 100 μg in panels B,C). Hamartin was detected using antibody to a synthetic peptide representing amino acids 1107-1130. Hamartin is seen only in the membrane fractions at an approximate molecular weight of 130-150 kDa. Panel A shows selective labelling of membrane fractions. Panel B shows immunoblot of two membrane fractions incubated with anti-hamartin antibody at a primary dilution of 1:400. Panel C shows a portion of the same blot shown in 'B', incubated with anti-hamartin antibody at 1:400 dilution preadsorbed with the C-terminus peptide to which antibody had initially been made. Numbers to left of the blots show molecular weight (MW) size markers.

serum (NGS) and 3% bovine serum albumin (BSA) or 8% BSA, 1 mM PMSF, and 0.1% Tween-20. After blocking, the membrane was incubated with anti-tuberin antibody at 15 μg/mL in blocking solution. Immunoblotted proteins were detected by incubation with biotinylated goat anti-rabbit secondary antibody, avidin-biotin conjugate (Vector, Burlingame, CA) and diaminobenzidine substrate or HRP conjugated secondary antibody and ECL. In blocking experiments, antiserum was incubated with a two-fold excess of tuberin [775-794] in 1 mL TBS for 1 hr at room temperature with gentle rocking. To estimate the molecular weight (MW) of tuberin, prestained MW standards (Bio-Rad) were used.

Immunohistochemistry. Clinical data on patients from whom tubers were resected are presented in Table 1. All patients were investigated and focal neocortical resections were carried out using standard protocols as part of the UCLA Pediatric Epilepsy Surgery Program, details of which have previously been extensively published (7, 23, 28). All four patients fulfilled conventional diagnostic criteria for TSC (11, 12) but in no case is it
known whether the patient has a TSC1 or TSC2 mutation. All surgical tissue was handled as described in detail previously for cortical dysplasia surgical specimens (7, 23). For a preliminary assessment of tuberin and hamartin in the normal CNS, 10 fetal and 15 pediatric brains (non-TSC) from 20 week gestational age to 8 years old (20, 21, 24, 28, 29, 30, 31, 32, 36, and 41 week gestational ages, 8 day, 1, 2, 3, 4, 5, and 7 months old, 1, $1^1/_2$, 2, 3, 4, $4^1/_2$, 5 and 8 years old) were obtained from autopsy materials in UCLA files. Formalin fixed and paraffin embedded sections including cerebral cortex and white matter, basal ganglia, thalamus, germinal matrix, hippocampus, cerebellum, and brainstem were used in a preliminary study of the regional expression of tuberin and hamartin in the CNS.

Immunohistochemistry was carried out on paraffin sections cut at 6-8 μm thickness. Sections were dewaxed and rehydrated in graded alcohol concentrations. Endogenous peroxidase activity was blocked with 0.3% H_20_2 at room temperature (30 min in PBS). Antigen retrieval was performed in boiling 0.1 M citrate, pH 6.0/0.01% NaN_3 for 40 min. Sections were washed in PBS, treated with 0.2% Triton-X-100 in PBS (5 min) and washed again in PBS. After blocking in 5% BSA/3% NGS for 1 h at room temperature, sections were incubated overnight at 4°C with affinity-purified antibody diluted 1:700 to 1:750 (anti-tuberin) or 1:400-1:700 (anti-hamartin) in blocking solution. After vigorous washing, the sections were incubated with anti-rabbit biotinylated secondary antibody diluted 1:500 in PBS at room temperature for 30 min. Immunohistochemical protocols were essentially identical for anti-tuberin and anti-hamartin antibodies. Sites of antibody localization were visualized with the Vector Laborato-

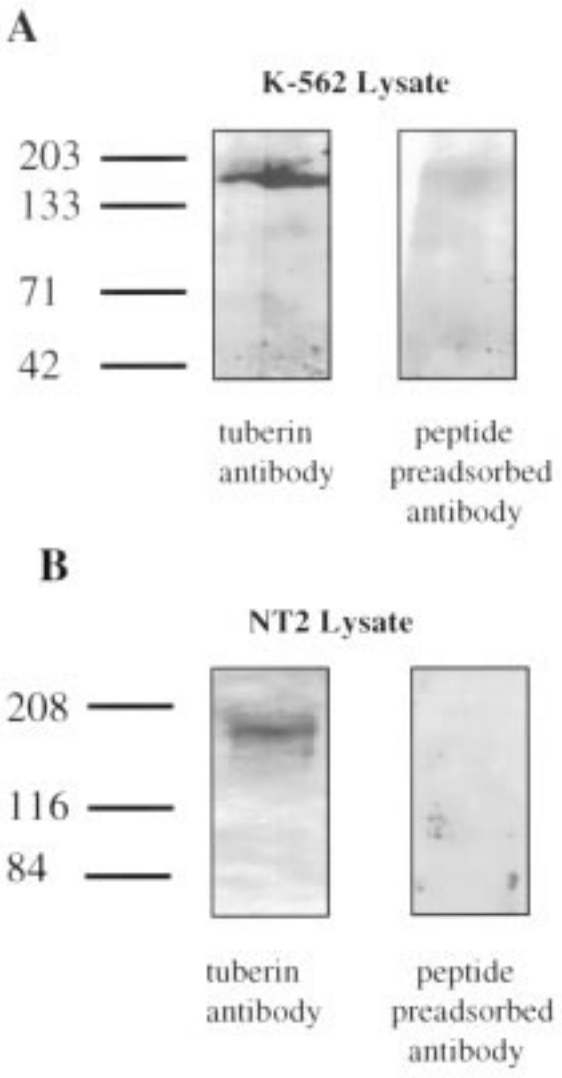

Figure 2. (**A**) Immunoblot of cultured K-562 cell lysate stained with anti-tuberin antibody (left hand lane) and antibody preadsorbed with tuberin peptide used to generate the antibody (right hand panel). Anti-tuberin antibody is to a synthetic peptide representing amino acids #775-794, as described in the 'Materials & Methods'. Note a single band at MW approx. 170-190 kDa, the expected molecular weight of native tuberin. Numbers at left indicate MW size markers. (**B**) Immunoblots of NT2 cell lysate with tuberin antibody (left hand lane) and antibody preadsorbed with peptide (right hand panel).

ries Vectastain Elite ABC kit and the peroxidase substrate diaminobenzidine tetrahydrochloride (DAB). In some cases, antibody was preadsorbed with hamartin or tuberin peptide as described for immunoblotting. In order to optimize assessment of possible co-localization of hamartin and tuberin, immunohistochemical protocols were sometimes carried out on parallel (and sometimes immediately adjacent) tissue sections using antibodies to the two proteins. Sections were counterstained with Hematoxylin.

Double-label Immunohistochemistry. Cortical tubers sectioned at 6 μm were dewaxed in xylene, rehydrated through graded ethanol, and subjected to antigen retrieval as described. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 at room temperature (20 min in PBS). Sections were washed in PBS, treated with 0.2% triton-X in PBS (5 min) and washed again in PBS. After incubating in blocking solution (5% BSA, 3% NGS in PBS) for 1 hour at room temperature, sections were incubated at room temperature with affinity-purified anti-hamartin at a concentration of 1:300 in blocking solution. Sections were vigorously washed, and subsequently incubated in 1:100 HRP-conjugated goat anti-rabbit IgG. Sections were washed and antibody localization was visualized with the peroxidase substrate 3-amino-9-ethylcarbazole (AEC). During this procedure, anti-tuberin (1:100-1:200) was incubated with excess biotinylated goat anti-rabbit IgG (1:200) in blocking solution at 37°C for one hour. Unbound biotinylated anti-rabbit IgG was bound with 10% normal rabbit serum (NRS) at 37°C for an additional hour. The AEC labeled section was subjected to an additional antigen retrieval as described and washed in PBS. The section was blocked in 5% BSA/3% NGS/5% NRS for 1 hour at RT; afterwards, it was incubated with the anti-tuberin/biotinylated anti-rabbit IgG mixture at RT for 1 hour. Anti-tuberin immunoreactivity was detected with the Vector Laboratories Vectastain Alkaline-Phosphatase (AP) kit and the AP substrate nitro-blue tetrazolium chloride (NBT). Because both hamartin and tuberin antisera were made in rabbit, care was taken to ensure that no cross-reactivity occurred. Negative and positive controls for each primary and secondary antibody were performed as previously reported (5).

Results

Immunoblots of human brain (autopsy) homogenates stained with the anti-hamartin (anti-ham) antibody detected a single band only in membrane fractions at an approximate MW of 130-140 kDa, consistent with the predicted MW of hamartin (31) (Figure 1). In immunoblot preparations of K-562 and NT2 cell lysates, antibodies to tuberin (hereafter anti-tub) labelled a single band at a MW of approximately 170-190 kDa, the expected size of tuberin (6) (Figure 2). In both cases, immunoreactivity was effectively blocked by preadsorption of antibody with an excess of peptide (Figures 1, 2).

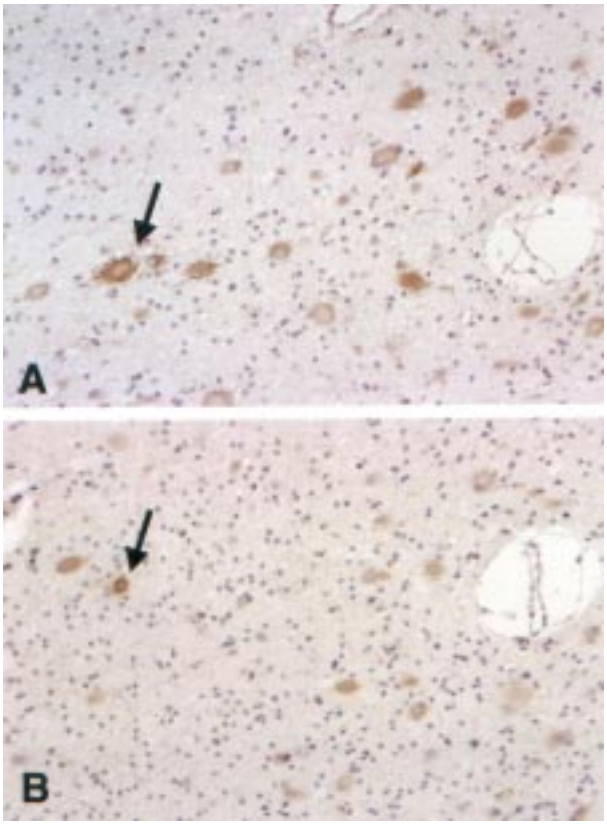

Figure 3. Resected cortical tuber (14-yr-old female), parallel sections, immunostained with anti-hamartin (**A**) and anti-tuberin (**B**) antisera. Similar regions have been selected for photography; for orientation, thin-walled venule at right of panel is the same vessel cut in cross-section. Note immunoreactivity of stellate process-bearing cells (arrows, both panels) using both antibodies, with more numerous stained cells using anti-hamartin. (Mag. X 155)

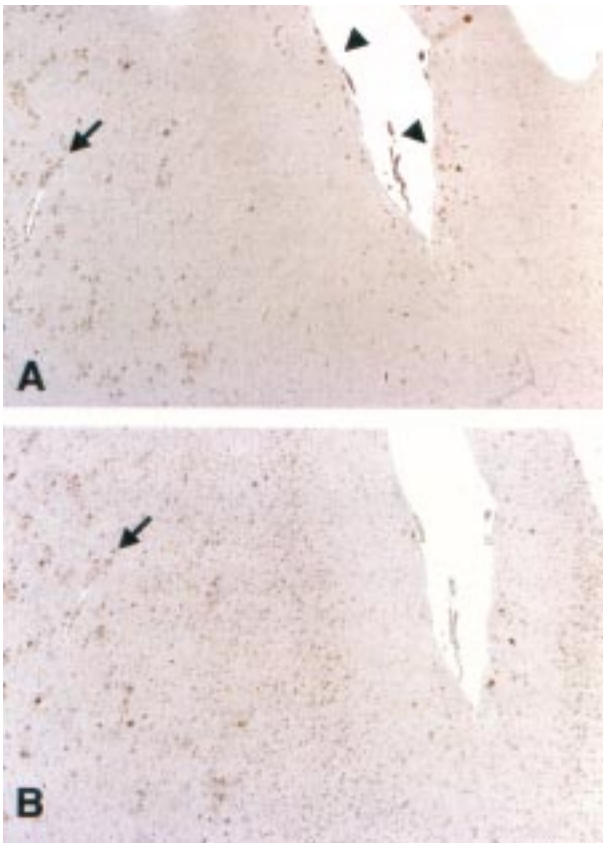

Figure 4. Surgically resected cortical tuber (6-mo-old female); parallel sections immunostained with anti-hamartin (**A**) and anti-tuberin (**B**). Cells in similar locations (left hand side of both panels) are immunopositive in roughly comparable numbers. Anti-hamartin antibody recognizes more cells in a subpial location (arrowheads, panel A), whereas anti-tuberin shows more prominent immunoreactivity of normally arranged mid-layer cortical neurons. Arrows in both panels show a small vessel cut in longitudinal section, which is shown at higher magnification in Figure 5. (Mag X 30).

Cell lysates were used in the characterization of tuberin because of variable autolysis in available frozen autopsy tissue. K-562 is a lymphocytic cell line isolated from leukemic bone marrow. NT2 is a human teratocarcinoma derived neuronal precursor cell line. Both cell lines express abundant tuberin. In addition, the NT2 cells express hamartin in abundance (data not shown).

Immunohistochemical study of several tubers revealed prominent cytoplasmic labelling of neuron-like cells using both antibodies (Figures 3-7). In addition, variable (though usually minimal) nuclear staining resulted with anti-tub. Immunoreactive cells had variable morphology and stained with differing intensity from case to case, but staining of significant numbers of cells was achieved using both antibodies in all four cases. Many cells immunolabelled with both anti-ham and anti-tub had multiple dendrite-like processes emerging from the cytoplasm (Figures 3, 6). In some instances, greater numbers of cells were immunolabelled with anti-ham (Figure 3), whereas in others roughly equal numbers of dysmorphic cells were immunoreactive with both antisera (Figure 4). In one tuber, more superficially located subpial dysmorphic cells labelled with anti-ham than anti-tub (Figure 4). In general, morphologically normal cortical neurons were more consistently immunoreactive for tuberin than hamartin (Figure 4, 5, 7). When cells of neuronal morphology (to the extent this has meaning in the context of tubers (29, 34) were robustly immunolabelled with either antibody, immunoreactivity often extended into cell processes (Figures 3, 6). When staining protocols were carried out using peptide-adsorbed antibodies,

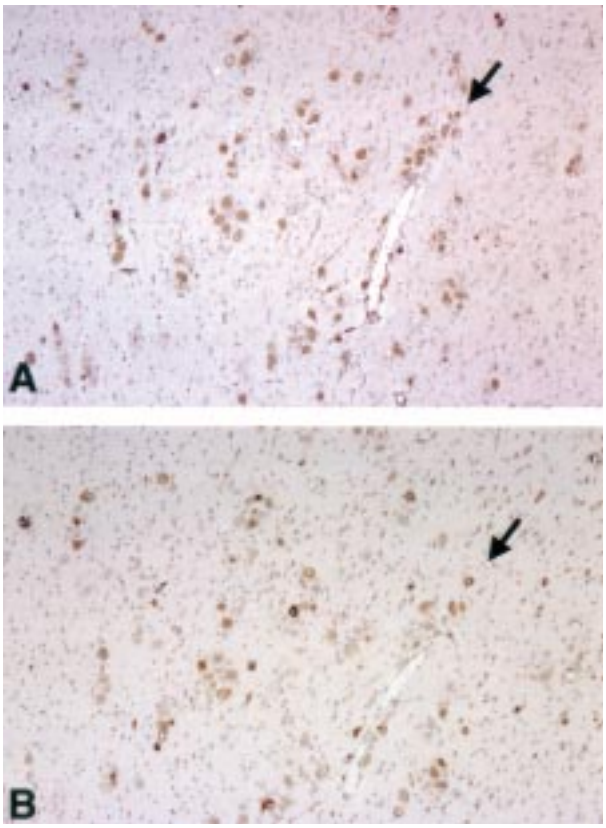

Figure 5. Magnified view of region illustrated by arrows in Figure 4. Panel A, stained with anti-hamartin, panel B with anti-tuberin. Note immunoreactivity of similarly situated neuron-like cells, many with processes. Different stain intensity (e.g. cluster of cells indicated by arrows) suggests differential expression of hamartin/tuberin (Mag X 75).

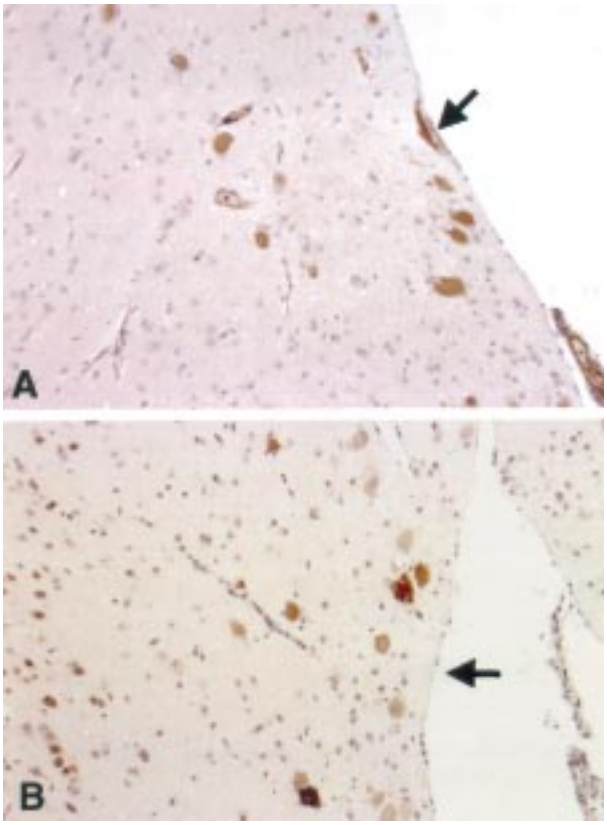

Figure 6. Subpial region of tuber in a section immunostained with anti-hamartin (**A**) and anti-tuberin (**B**). Arrows indicate pial-surfaces in parallel sections. Note variable stain intensity of subpial cells, at least some of which have short dendrite-like processes. (Mag X 150).

negligible background staining was observed in all cases. Blood vessels, including endothelia and smooth muscle (of larger arteries) were variably immunolabelled with both anti-ham and anti-tub antisera, more consistently the former; prominent vessel wall tuberin immunoreactivity has been reported by others (38).

Double-label immunohistochemistry with both anti-tub and anti-ham was completed for all cases. We noted several different types of staining. Double-labelling of many neuroglial cells was noted. Most dramatic was the peripheral tuberin staining on many hamartin immunoreactive cytomegalic neurons (Figure 8). Interestingly, some cells with neuronal morphology showed high immunoreactivity for hamartin but no detectable tuberin. In addition, many other cells with similar morphology were highly immunoreactive for tuberin. However, because the NBT used to detect tuberin is a dark chromogen it was often difficult to determine co-localization in cells highly immunoreactive for tuberin. The heterogeneity of staining suggests that a more informative and quantitative description of the number and types of cells immunoreactive for tuberin, hamartin, or both with fluorescent double-labelling and confocal laser microscopy is warranted.

A preliminary study of the regional expression of tuberin and hamartin in the CNS of non-TSC patients as a function of brain development has produced some data of interest (26). Immunohistochemistry with anti-tub produced granular staining in the cytoplasm of positive cells with strong accentuation of the Nissl substance. Tuberin and hamartin are expressed in cerebral neurons, Cajal-Retzius cells, deep neurons of the basal ganglia and thalamus, hippocampal pyramidal neurons and granule cells, cerebellar Purkinje cells and dentate neurons, neurons in the cranial nerve nuclei of the brainstem and pons, ependyma, and choroid plexus epithelium. In fetus, tuberin and hamartin expression in cerebral cortical neurons is weak until fullterm. Phylogenetically

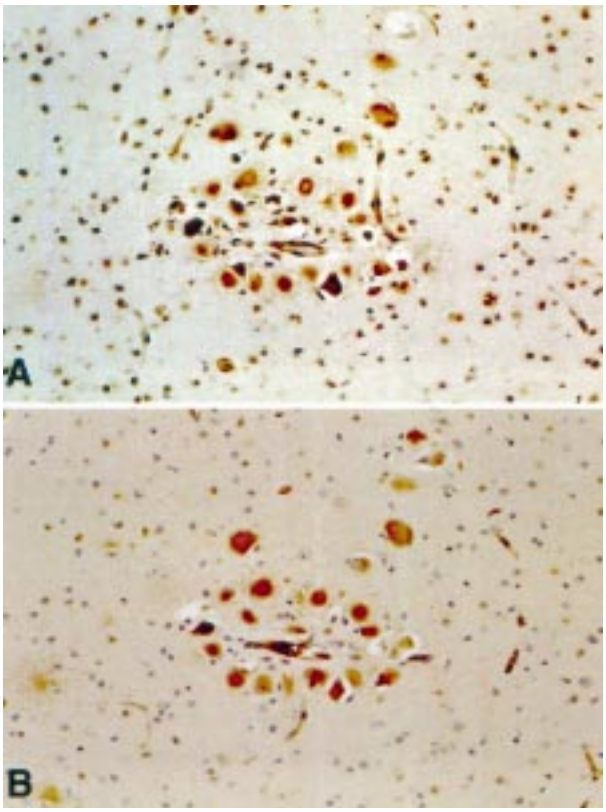

Figure 7. Tuber from a one-year-old female, immediately adjacent sections; (**A**) stained with anti-tuberin, (**B**) with anti-hamartin. Small vessel at center of the field is seen in both sections. Perivascular cells of indeterminate phenotype show variable immunoreactivity for both peptides. Anti-tuberin stains more 'normal'-appearing neurons, as noted in Figure 4.

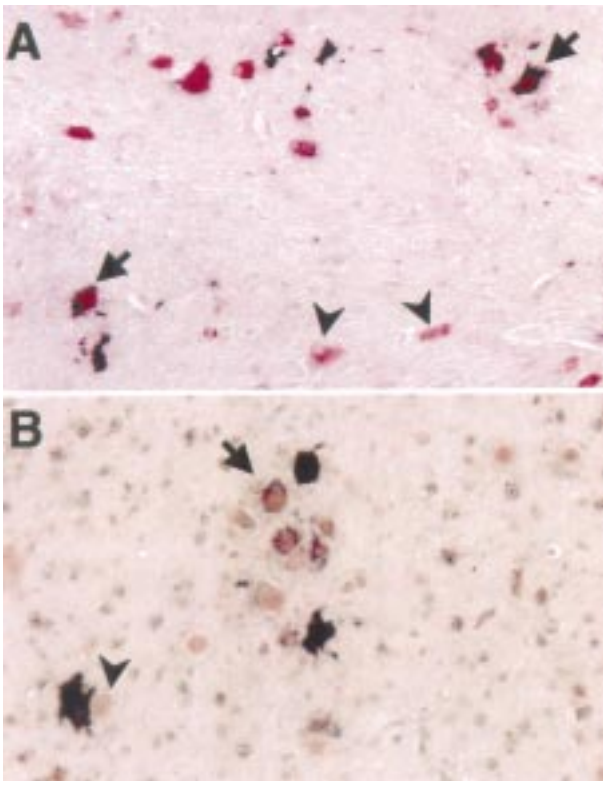

Figure 8. Double-label Immunohistochemical staining of tuber sections from two different cases (**A** and **B**); anti-hamartin is labelled with the peroxidase substrate AEC (pink/red). Anti-tuberin is labelled with the alkaline phosphotase substrate NBT (purple/black). Peripheral tuberin immunoreactivity is evident in several large, abnormal hamartin positive cells (arrows, both panels). Some cells appear to be only hamartin positive (arrowheads, both panels). Note the variable nuclear (perinuclear) staining of surrounding neurons with anti-tuberin. (Mag. X 145)

'old' neurons such as hippocampal pyramidal neurons and granule cells, cerebellar Purkinje cells, dentate neurons, and cranial nerve and pontine neurons express tuberin and hamartin robustly from week 20 gestational age through childhood. Further analysis of the developmental/tissue specific expression of these two proteins is in progress.

Discussion

Tubers, together with subependymal giant cell astrocytoma (SEGA) and subependymal nodules the defining cerebral features of TSC, are multifocal regions of profound neocortical disorganization and dyslamination (29, 34). Tuber counts represent a biomarker predictive of the severity of cerebral dysfunction in TSC patients (13). The cellular constituents of tubers include bizarre, sometimes enlarged and maloriented neuron-like cells and balloon cells that resemble gemistocytic astrocytes (29, 34). The relatively circumscribed nature of tubers, i.e. surrounding cortex may appear morphologically normal, suggests that they originate from a developmental defect which may affect only a circumscribed population of neuronal precursor cells (possibly within the germinal matrix) during formation of the cortex. Tuberin has been demonstrated immunohistochemically in the CNS of a 20-week gestation fetus with TSC (27). Assessment of transcripts within single cells of TSC tubers using novel molecular methodology has shown 'immature phenotypic markers' within them, suggesting disruption of cell cycle regulation and neuronal maturation, processes crucial to normal cortical development (3, 4, 10, 21). The Eker rat, which shows a mutation linked to the Tsc2 gene, develops SEGA-like supendymal nodules but no tuber-like lesions (41). TSC1 or TSC2 knockout mice with tuber-like lesions have not been reported to date. Thus, the present lack of an ani-

mal model with tuber-like cortical lesions necessitates further detailed characterization of TSC (and sporadic CD) in available human tissues.

TSC results from a mutation in either the TSC1 or TSC2 gene (1, 30, 39). Hamartomas found in TSC patients are thought to result from a 'two-hit' mechanism, i.e. a germline mutation followed by a somatic mutation, the latter determining the precise phenotype of an organ-specific lesion. Though the genotypes of the four patients from whom our study tissues were obtained are not known, it is of interest that anti-ham and anti-tub immunolabelled significant numbers of cells in all four patients, sometimes with more robust immunoreactivity or greater numbers of cells labelled with one or the other antibody. Immunoreactive cells included for the most part bizarre neuron-like cells, whereas astroctye-like 'balloon cells' were usually not labelled or were only faintly immunoreactive with both antibodies. Study of sequential sections stained with anti-tuberin or anti-hamartin and anti-tuberin/hamartin double-labelled sections has produced evidence that some of the abnormal cell types in cortical tubers express both proteins abundantly. However, qualitative observation suggests that a substantial group of the dysmorphic cells of neuronal morphology expressing abundant hamartin are not producing detectable tuberin.

Some studies have suggested that tuberin is present within astrocytes and that loss of TSC2 RNA and tuberin is associated with increasingly aggressive behavior of non-TSC-related primary brain tumors (36). Caution must obviously be exercised in assigning a putative phenotype to cells within tubers based upon morphology alone, since bizarre and dysmorphic cells of indeterminate lineage (neurons, astrocytes, intermediate forms) are a defining feature of these lesions, and of sporadic cortical dysplasia (7, 23). Studies aimed at evaluating co-expression of neuronal and astrocytic epitopes with tuberin and hamartin are in progress in our laboratory.

Previous studies have shown tuberin immunoreactivity of cells within TSC tubers (19), though Western blot of homogenized tubers using anti-tub may fail to detect the presence of this protein (24), a result that appears to reflect the relative insensitivity of Western blot in showing infrequent cells (among a large number) with robust expression of tuberin (35). TSC-related subependymal giant cell astrocytomas (SEGAs) show less prominent tuberin immunoreactivity, though single cells within them are often strikingly labelled with anti-tub (18, 20). TSC2 transcript is abundant in most neurons within the brain and spinal cord of human and rodent CNS (8, 9, 19). TSC2 transcript and tuberin are also widely distributed within human viscera (25, 38). Preliminary immunohistochemical study of human CNS tissues and visceral tissues suggests a tissue- and cell type-specific pattern of expression for hamartin that closely mimics the one observed for tuberin (data not shown). This similarity of distribution in non-diseased tissues and the finding of co-localization of tuberin and hamartin in similar regions and, in some cases, in the same cells within cortical tubers are of great interest in view of recent biochemical evidence for interaction between the two proteins (32).

Though the genotype of each of our four patients is currently unknown, they would all be expected to have either TSC1 or TSC2 mutations. Mutations within these genes are of widely differing types, ranging from large deletions to missense mutations, a fact which complicates the process of screening for mutations (1,39). Our findings suggest that TSC1 or TSC2 mutations do not eliminate the capacity of a neural cell to synthesize protein. Indeed, apparent overexpression of protein in dysmorphic cells within tubers suggests a compensatory 'excessive' synthesis of the protein in these cells, whatever germline TSC1/TSC2 mutation they harbor.

The molecular pathogenesis of tubers is currently unknown, but their close similarity to foci of severe CD suggest a profound abnormality of neuronal precursor migration to, and maturation within, the cerebral neocortex (10, 21). Loss of heterozygosity (LOH) studies suggest growth regulating roles for both the TSC1 and TSC2 gene products (2, 14, 15). Such investigations also show allelic loss for TSC1 and TSC2 much more frequently in tumors and hamartomas than in CNS tubers (17), suggesting that visceral and CNS lesions (at least tubers) may have a different molecular pathogenesis. Tuberin co-localizes with Rap1, possibly in the Golgi apparatus (38), and a small region of the carboxy terminus of tuberin shows prominent sequence similarity to a GTPase activating protein (GAP) for Rap1 (37). In addition, tuberin appears to interact with rabaptin-5, an effector of Rab5, a critical component of the endocytotic pathway (40). However, it is not known whether increased endocytosis as a consequence of decreased or defective tuberin function may lead to abnormal neuronal migration and survival that contribute to tuber formation. Recent evidence that nerve growth factor (NGF) induces formation of signaling endosomes suggests a link between endocytotic-mediated mechanisms and aberrant NGF action (16).

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