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Review

Immunohistochemical and microscopic studies on giant cells in tuberous sclerosis

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Summary. Tuberous sclerosis (TSC) is an autosomal dominant disease, caused by mutations in *TSC1* or *TSC2* genes, encoding hamartin and tuberin, respectively. The clinical picture of the disease is connected with the formation of hamartomas, mainly in the heart, kidneys and the brain. In three types of brain lesions: cortical tubers, subependymal nodules and subependymal giant-cell astrocytoma (SEGA) characteristic, so-called "giant cells" are found. In the present review we summarise immunohistochemical findings of two types of studies performed on giant cells aiming at establishing the expression of hamartin and tuberin level and determining the presence of neuron- or astrocyte-specific markers. Moreover, we support our argument with the summary of ultrastructural research done with the purpose of demonstrating structures characteristic of neural and/or glial cells. We conclude that giant cells in cortical tubers and SEGAs are the same undifferentiated cells that, depending on individual determination, can show neural or glial features.

Key words: Giant cells, Tuberous sclerosis, Ultrastructure

Introduction

Tuberous sclerosis (TSC) is an autosomal dominant disease, characterised by skin lesions and formation of tubers in practically all vital organs (Jozwiak et al., 1998, 2000). In particular, the brain is affected, which leads to the characteristic phenotype of mental retardation and epilepsy. It has been found that the disease is caused by the mutation in one of two tumour suppressor genes: *TSC1* on chromosome 9q34 (van Slegtenhorst et al., 1997) or *TSC2* on 16p13 (European Tuberous Sclerosis Consortium, 1993), encoding hamartin and tuberin, respectively. The proteins form an intracellular complex (tuberous sclerosis complex) which in normal cells is responsible for the inhibition of mammalian target of rapamycin (mTOR) kinase. In TSC, lack of mTOR inhibition leads to increased protein translation and proliferation of cells through the activation of S6K kinases, phosphorylating ribosomal S6 protein. S6 is one of 30 distinct ribosomal proteins, which along with 18S rRNA form the smaller ribosomal subunit 40S (Wool et al., 1996).

Brain lesions in TSC are usually divided into three types: cortical tubers, subependymal nodules and subependymal giant cell astrocytoma (SEGA). Cortical tubers are firm, nodular lesions appearing in the cerebral or cerebellar cortex. They show abnormal cortical lamination, are characterised by the presence of dysmorphic neurons and eosinophilic giant cells. SEGAs usually occupy the lateral ventricle, near the foramen of Monro, and contain at least three cell populations: astrocytes, dysmorphic neurons, and giant cells. From our clinical observations we hypothesise that SEGAs may develop from subependymal nodules (Roszkowski et al., 1995). This observation is also confirmed by the fact that SEGAs are very rarely found in neonates, while their incidence increases with age. In all types of TSC brain lesions giant cells are one of cell populations, the one considered to be characteristic for the disease. Because of numerous immunohistochemical and ultrastructural similarities (Bender and Yunis, 1980; Chou and Chou, 1989; Jay et al., 1993; Hirose et al. 1995), it has been hypothesized that giant cells in tubers and SEGAs share the same profile of differentiation and may be of the same cellular lineage (Lee et al., 2003). At the same time, TSC-derived giant cells have been differentiated from similar large cells, appearing e.g. in hemimegalencephaly, a sporadic congenital dysplastic abnormality of the central nervous system, showing that this cell type is unique for TSC (Arai et al., 1999).

Immunohistochemistry of TSC

The first report on giant cells was published by Kapp

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(1967). In 1980, Bender observed under light and electron microscopy "abnormal cells", found in subependymal, cortical, and white matter lesions in ten children with TSC, aged 2 days to 15 years. After detection of the two genes responsible for TSC, immunohistochemical analysis performed on giant cells concentrated on determination of their TSC status, i.e. evaluation of the amount of TSC-related proteins, hamartin and tuberin. According to Knudson's two-hit model of cancerogenesis (Knudson, 1971), both alleles of a tumour suppressor gene have to be mutated in order for the tumour to form. Thus, it would be expected that one of the proteins responsible for the disease would have to be missing in affected cells. Indeed, lack of tuberin or hamartin is often found in bladder transitional cell carcinoma, kidney angiomyolipoma, and cardiac rhabdomyoma occurring in the course of TSC (Henske et al., 1997; Jozwiak et al., 2001; Knowles et al., 2003; Meikle et al., 2005). In our research (Jozwiak et al., 2004) we also confirmed by immunohistochemistry the absence of hamartin and/or tuberin in SEGAs of 9 patients with TSC. However, many other reports (Mizuguchi et al., 2000; Mizuguchi and Takashima, 2001; Tabuchi et al., 2003; Jansen et al., 2004) did not find complete abolishment of hamartin or tuberin production in brain tubers. Also in the Eker rat, the animal model of TSC with spontaneous germline mutation of the *TSC2* gene, giant cells exhibit the expression of tuberin (Wenzel et al., 2004). Thus, we believe that in TSC brain lesions the classic two-hit model of cancerogenesis, although sometimes true, is not sufficient for the explanation of all cases of the disease. It is also interesting to mention that HMB-45 (human melanoma, black) antigen found commonly in uterine and hepatic angiomyolipoma, peritoneal and ovarian epithelioid angiomyolipoma, clear cell epithelioid tumor of the kidney as well as cardiac rhabdomyoma associated with TSC (Weeks et al., 1994; Ribalta et al., 2000; Ji et al., 2001; Anderson et al., 2002; Hino et al., 2002; Cil et al., 2004) suggesting a similar mechanism of their formation, is not found in SEGAs (Gyure and Prayson, 1997; Sharma et al., 2004). This fact seems to be another argument for different pathomechanisms in brain tubers and other hamartomas found in TSC patients.

A new possibility for the explanation of this phenomenon appeared recently. Jansen (2004) in one of the studies evaluating the presence of TSC proteins in a cortical tuber from a TSC patient with confirmed TSC1 mutation, found by immunoblotting that there were no differences in the amount of hamartin or tuberin in tubers and adjacent normal tissues. Interestingly, however, the authors detected increased levels of S6 phosphorylation, being a sign of high mTOR activity. In so far as giant cells showed granular cytoplasmic immunohistochemical staining for tuberin, staining for hamartin showed predominantly nuclear localisation of the protein. Moreover, from among other types of cells present in the tuber, only in giant cells immunoreactivity for phosphorylated form of S6 could be detected. This finding argues for the direct involvement of giant cells in the pathogenesis of TSC, suggesting that this population of cells is primary, and not reactive, in the disease. On top of that, differential localisation of hamartin and tuberin within the cell could explain why S6 phosphorylation is detected in the presence of both proteins (Jansen et al., 2004). Differential cellular localization of both proteins had been reported before in epithelial cells of distal urinary tubules. In this case however, tuberin localized to the cytoplasm as well as apical membrane, while hamartin was detected only at the apical cell membrane (Murthy et al., 2000). At the

Table 1. Different markers found in TSC giant cells.

NEURONAL MARKERS	GLIAL (ASTROCYTIC) MARKERS	LACK OF DIFFERENTIATION MARKERS
SMI 31, SMI 32 (Mizuguchi and Takashima, 2001)	GFAP (Hirose et al., 1995; Mizuguchi and Takashima, 2001; Sharma et al., 2004)	Nestin (Crino et al., 1996; Mizuguchi et al., 2002)
Class IIIb-tubulin (Onda et al., 2002; Hirose et al., 1995; Lopes et al., 1996)	S-100 (Hirose et al., 1995; Sharma et al., 2004)	Vimentin (Hirose et al., 1995)
MAP 2 (Lopes et al., 1996)[2]		MAP 1B (Yamanouchi et al., 1997)
Calbindin D-28k (Hirose et al., 1995)		Doublecortin (Mizuguchi et al., 2002)
Neuron-specific enolase (Sharma et al., 2004)		Ki67 (Crino et al., 1996)
Somatostatin (Hirose et al., 1995)[1]		
Met-enkephalin (Hirose et al., 1995)[1]		
5-Hydroxytryptamine (Hirose et al., 1995)[1]		
Neuropeptide Y (Hirose et al., 1995; Lopes et al., 1996)[1]		
Synaptophysin (Mizuguchi and Takashima, 2001; Sharma et al., 2004)		
NeuN (Lee et al., 2003)		
Serotonin (Lopes et al., 1996)		

[1]: Reactivity found in giant cells from SEGA, but not from tubers. [2]: Reaction in few cells.

same time, in tuberin-transfected Hep G2 cell line, abundant tuberin has been found in the nucleus (Lou et al., 2001). The result is supported by the finding that tuberin can modulate the transcription by binding nuclear transcription factors like ER, VDR and PPAR (Henry et al., 1998).

Immunohistochemical studies on giant cell origin

Another group of studies concentrates on the morphology of giant cells and presence of immunohistochemical markers that could evidence their neuronal or glial origin, as determination of cell origin could supply new information on tuberogenesis in TSC. Neural markers most often used in immunohistochemical examination of giant cells include: class IIIß-tubulin, microtubule-associated protein 2 (MAP 2), neuron-specific enolase (NSE), somatostatin, serotonin, neuropeptide Y and neuronal nuclei protein (NeuN). Mizuguchi (2001) evaluated the presence of phosphorylated and non-phosphorylated neurofilament protein SMI 31 and SMI 32, calbindin D-28k and synaptophysin (SVP-38) in giant cells of cortical tubers of a TSC patient, finding varied immunoreactivity of all three proteins, from negative to strong staining. This result was confirmed later, in the observation by Sharma (2004), who found that synaptophysin is sometimes demonstrated in giant cells that, at the same time, express glial marker proteins. Another neurofilament protein, SMI 33, was found in giant cells in about 30% of TSC brain lesions (Hirose et al., 1995). Class IIIß-tubulin immunoreactivity was demonstrated in 17 of 20 SEGAs, both within the bodies of giant cells and, to varying degrees, within their processes (Lopes et al., 1996). In the same research MAP 2 was detected in a few cells of two tumors, while immunoreactivity for neuropeptide Y and serotonin was found in 6 (30%) and 5 (25%) cases. Also, NeuN, observed in most neuronal cell types throughout the nervous system, seems to be present in some of the giant cells from tubers and SEGAs (Lee et al., 2003). mRNA analysis of giant cells from cortical tubers revealed the presence of KV1 potassium channel, Ca-N, CX26 and CX32, which suggests that giant cells may be capable of synaptic signal transmission (Crino et al., 1996). The presence of CX26 mRNA in TSC-derived giant cells and neurons, but not normal neurons, was one of the premises reasoning for immature phenotype, as CX26 is characteristically found in neuroepithelial progenitor cells.

On the other hand, glial origin of giant cells is often tested with use of specific markers: glial fibrillary acidic protein (GFAP) and S-100 protein. GFAP is a class-III intermediate filament. It is the main constituent of intermediate filaments in astrocytes and serves as a cell-specific marker that distinguishes differentiated astrocytes from other glial cells during the development of the central nervous system. GFAP staining in giant cells varies from weak (Mizuguchi and Takashima, 2001) to strong (Hirose et al., 1995). Similarly, detection of S-100 with use of specific antibodies gives varied results. Sharma (2004) tested the presence of GFAP and S-100 in all populations of SEGA cells in 23 TSC patients, finding reaction in all the cells. In the same research however, reaction for NSE and neurofilament (NF) 68 kDa was present in most SEGAs. Hirose (1995) studied giant cells from 13 cortical tubers and 6 SEGAs, showing reaction for GFAP (in 77% of tubers and 50% of SEGAs) and S-100 (92% and 100%, respectively) not only in cytoplasm, but also within processes. The authors observed that reaction-positive giant cells, whose proportion varied significantly from case to case, were intermixed with negative cells, resulting in a mosaic pattern. Astrocytic origin was also suggested by the results of CD44 antibody staining. CD44 is a cell adhesion molecule, which mediates cell-cell and cell-matrix interactions. In the normal central nervous system, CD44 is expressed in astrocytic processes, suggesting their involvement in the maintenance of a stable central nervous system cytoarchitecture. Arai (2000) observed the presence of CD44 antigen around giant cells in tubers and around tumour cells in subependymal lesions.

Because of such discrepancies in immunostaining, some authors postulated progenitor character of giant cells and their ability to differentiate into cells expressing different markers. To confirm this hypothesis, another set of proteins has been selected and tested against TSC-derived giant cells. Among the most important markers evidencing lack of differentiation are nestin, MAP 1B, doublecortin and Ki67. Nestin is a class VI intermediate filament protein. Although it is expressed predominantly in stem cells of the CNS (Fredriksen and McKay, 1998), its expression is absent from nearly all mature CNS cells (Tohyama et al., 1992). In the report by Crino (1996) approximately 80% of giant cells from cortical tubers stained for nestin. This result was also confirmed on the level of mRNA, by reverse Northern blotting technique. The same technique was also used for identification of cell cycle markers Ki67 and cyclin D1. It has been found previously that expression of Ki67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas during mitosis it is localised on the surface of chromosomes. The fact that the Ki67 protein is present during all active phases of the cell cycle (G1, S, G2, M), but is absent from resting cells (G0), makes it an excellent marker for determining the so-called growth fraction of a given cell population (Scholzen adn Gerdes, 2000). On the other hand, cyclin D1 (PRAD1 oncogene product, Bcl-1 oncogene product), is a member of the cyclin family of proteins that help control transition between phases of the cell cycle. Cyclin D1 is essential for control of the cell cycle at G1/S transitions and interacts with Cdk4 and Cdk6 protein kinases. In the report by Crino (1996) both of these proteins were detected in neurons and giant cells at differing hybridisation intensities. In his immunohistochemical study, Yamanouchi (1997) divided giant cells from cortical tubers into two

populations: of neuronal and indeterminate appearance. Giant cells with neuronal appearance demonstrated consistently strong immuno-reactivity for MAP 1B and occasional immunopositivity for nestin and vimentin and were rarely positive for GFAP. At the same time, indeterminate giant cells, characterized by abundant cytoplasm, absence of Nissl substance, and one or more eccentric nuclei, demonstrated immunoreactivity for nestin, vimentin, and MAP 1B, but more than half displayed immunoreactivity for GFAP. Similarly, Hirose (1995) found that reaction for vimentin is strong, although this author reported reaction in all giant cells.

Also, expression of another lack-of-differentiation-related protein, doublecortin, is interesting. Doublecortin (DCX) is a microtubule-associated protein required for neuronal migration during cortical development. It has been found that DCX mediates fetal migration of neuroblasts from the proliferative ventricular zone toward the pial surface and is transiently expressed in proliferating progenitor cells and newly generated neuroblasts (Brown et al., 2003). As the newly generated cells begin expressing mature neuronal markers, DCX expression decreases sharply. It has been reported that giant cells from the cortical lesions of tuberous sclerosis stained well with anti-doublecortin antibodies, suggesting restricted differentiation of these cells (Mizuguchi et al., 2002). The presence of all of the above markers characteristic for neuronal, glial or undifferentiated cells within tubers of the same morphology lets us hypothesize that giant cells within tubers retain embryonic features normally present in neuroepithelial precursors and not mature neurons/astrocytes.

Other immunohistochemical studies

Still another group of research is devoted to studying proteins, whose expression or localisation could be disturbed in the result of *TSC1* or *TSC2* mutation. Hamartin, for example, interacts with the ezrin-radixin-moesin superfamily of membrane-cytoskeleton-linking proteins which bind to the actin cytoskeleton. It has been demonstrated that inhibition of hamartin function in cells containing focal adhesions results in loss of adhesion to the cell substrate, whereas overexpression of hamartin in cells lacking focal adhesions results in activation of the small GTP-binding protein Rho, assembly of actin stress fibres and formation of focal adhesions (Lamb et al., 2000). Both ezrin and moesin are abundantly co-expressed with hamartin and tuberin within a population of giant cells in cortical tubers. Expression of ezrin and moesin suggests that the proteins are regulated developmentally and expressed in germinal matrix and/or migrating cells during cerebral cortical development. At the same time, in TSC cortical tubers, both proteins appeared to be up-regulated and are co-localized within a population of giant cells. Ezrin-moesin expression and co-localization with TSC1 and TSC2 products may suggest a compensatory up-regulation of expression caused by TSC gene mutation (Johnson et al., 2002).

It is also postulated that mutation of TSC genes influences pro-inflammatory pathways. Immunohisto-chemical studies have been performed on TSC cortical tubers and cortex tissue of TSC1 astrocyte-specific conditional knockout mice (TSC1cKO) (Maldonado et al., 2003). The research demonstrated that the expression of ICAM-1, MAPK, NF-κB and TNF in human tubers and ICAM-1 in the cortex of mice is elevated. As increased expression of ICAM-1 in TSC1cKO mice is probably related to the loss of hamartin, it seems that elevated levels of ICAM-1, MAPK, NF-κB and TNF in human cortical tubers may be a consequence of improper hamartin/tuberin complex activity. ICAM-1 is an important signal transduction protein in neurons and astrocytes. The molecule mediates the binding of leukocytes to astrocytes and initiates activation of pro-inflammatory pathways (Scholzen and Gerdes, 2000), thus, elevation of the expression of this molecule may be used as a marker of tissue inflammation. Indeed, several authors report the presence of mononuclear inflammatory cells gathered among or clustered around giant cells (Maldonado et al., 2003; Sharma et al., 2004).

Light and electron microscopy

Early ultrastructural reports (Sima and Robertson, 1979; Trombley and Mirra, 1981) on giant cells from cortical tubers and SEGAs argued for their astrocytic nature, based on the presence of granules of glycogen, multipolar processes containing glial filaments, membrane-bound dense bodies, formation of hemidesmosomes with pia and vascular basement membranes and numerous glial-glial contacts. Other authors found ependymal similarities, including innumerable microvilli-like projections on the surface of cells and junctional complexes, mostly of the zonula adharens type (Probst and Ohnacker, 1977). Further studies proved however, that the phenotype of giant cells is much more complicated.

Cortical tubers formed in the process of TSC are distinguished from the surrounding brain cortex by disruption of normal laminar cortical architecture, as evidenced by standard haematoxylin staining (Hirose et al., 1995; Crino et al., 1996). Light microscopy examination demonstrates the presence of uni- or multinucleated giant cells, but also dysmorphic neurons and astrocytes, whose appearance is probably reactive to the disease. Single or grouped giant cells dispersed irregularly in both white and grey matter, show consistently eosinophilic staining, they are polygonal or round, and their cytoplasm is homogenous, although sometimes may resemble ground glass. Some of the cells show short processes, and in one case of a tuber, a process of a single large cell was seen to engage in synapse formation with axonal termination and presynaptic vesicles (Hirose et al., 1995). This result is consistent with immunohistochemistry showing the presence of KV1 potassium channel, Ca-N, CX26 and CX32 proteins (Crino et al., 1996). The cells contain

numerous lamellar mitochondria and prominent Golgi apparatus. They form primitive intercellular junctions. Single or multiple nuclei within giant cells are usually eccentric and contain a small nucleolus. Chromatin is aggregated beneath the nuclear membrane. Interestingly, although usually giant cells are clearly distinguished from dysmorphic neurons, sometimes they have enlarged nuclei and peripherally located Nissl-like substance. At the same time, the cytoplasm of giant cells is packed with numerous intermediate filaments (IFs) as well as dense bodies, whose size varies. The abundance of IFs causes displacement of other organelles, like rough endoplasmic reticulum, to the periphery of the cytoplasm and cell processes. Round or elliptical dense bodies, distributed evenly within the cytoplasm, resemble primary lysosomes. Most giant cells also contain rod-like, rectangular or rhomboid crystalloids, not found in dysplastic neurons (Jay et al., 1993). These inclusions measured as much as 8 microns in length and had 7-nm periodicities, often with intersecting lamellae. The membrane-bound dense bodies showed areas of similar periodicities. Based on the periodicity within the substance forming both structures, it is hypothesised that there is a structural transition between dense bodies and crystalloids, which are related to and might originate from the dense bodies. In places where giant cells contacted fibrovascular stroma, basal lamina was present. Although in SEGA giant cells usually arrange in sheets or clusters, their ultrastructure does not show any differences from cortical giant cells. In the case of SEGA, no definite synapse formation was found, but some cells showed structures suggesting neuronal differentiation, including well-developed rough endoplasmic reticulum and microtubules present in the perikaryon and cell processes (Hirose et al., 1995).

Immunohistochemical and ultrastructural similarities suggest that giant cells in cortical tubers and SEGAs belong to the same population of undifferentiated, progenitor neuroepithelial lineage. Depending on localisation and, probably, individual determination, such cells can remain undifferentiated, expressing markers of immaturity, or differentiate into neuron- or astrocyte-like cells, with organelles characteristic for a given tissue. Thus, it seems reasonable to evaluate the presence and appearance of giant cells in TSC-related cortical tubers and SEGAs with a panel of several types of markers, which could distinguish respective subpopulations of cells. This could prove useful for further description and determination of patho-physiology of TSC brain lesions.

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