Predominant Localization of the LIS Family of Gene Products to Cajal-Retzius Cells and Ventricular Neuroepithelium in the Developing Human Cortex

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Abstract. Mutations that perturb neuronal migration provide important biological clues that can lead to an understanding of the role of specific cells and molecules in the formation of the cortex. The human neuronal migration disorder, Miller-Dieker lissencephaly, results from a hemideletion of LIS-1, which encodes a subunit of a brain platelet-activating factor acetylhydrolase. The cellular localization of the LIS-1 gene product in human fetal brain and its normal role in neuronal migration have yet to be determined. LIS-1 belongs to a family of genes that have identical coding sequences (LIS-1 [chromosome 17] and LIS-2 [chromosome 2]). In the brain, LIS-1 is the more abundant gene as determined by Northern blot analysis. Using antibodies raised against 2 epitopes of the LIS-1/LIS-2 protein sequence, we have localized the LIS family of gene products in the developing human brain to the Cajal-Retzius cells, some subplate neurons, thalamic neurons, the ventricular neuroepithelium, and at later gestational ages, to the ependyma. Therefore, LIS-1 bears some resemblance to reelin, the gene product involved in the cortical mouse mutant reeler, in that Cajal-Retzius cells demonstrate immunolocalization. However, unlike reelin, LIS proteins are expressed not only in the Cajal Retzius cells, but also in the ventricular neuroepithelium, suggesting a potential role for this structure in neuronal migration.

Key Words: Cajal-Retzius cells; Cortical plate; Ependyma; LIS-1; LIS-2; Lissencephaly; Neuronal migration.

INTRODUCTION

Lissencephaly, or smooth brain, refers to the configuration of the cortical mantle in human disorders in which the cortex is malformed. In Miller-Dieker lissencephaly (MDL), the cortical malformation results from the arrest of migrating neurons in the formation of the cortical plate (1-4). Miller-Dieker lissencephaly and some cases of isolated lissencephaly sequence (ILS) result from the deletion of one copy of a gene on the short arm of the 17th chromosome, LIS-1 (5-7).

This gene encodes a protein that is a subunit of a brain platelet-activating factor (PAF) acetylhydrolase, an enzyme that degrades the lipid messenger, PAF. This enzyme complex is a novel serine esterase that is perhaps best classified as a calcium-independent phospholipase A_2; it is pharmacologically related to brain acetylcholinesterase (8-10). It is composed of 3 subunits, the LIS-1 protein (a 45 KD non-catalytic subunit), a 29 KD catalytic subunit, and a 30 KD catalytic subunit. In addition, this form of brain PAF acetylhydrolase may serve as a signaling G-protein-like complex (11). However, it is not known how LIS-1 functions in the development of the cortical plate, nor is it known how a hemideletion of LIS-1 and the resulting alteration in PAF degradation (or signaling) cause a neuronal migration disorder.

LIS-1 is one of two LIS genes in the human genome. The proteins encoded by the LIS genes have a considerable homology in animal species (12). The human gene LIS-2, located on chromosome 2, has an identical coded protein sequence to LIS-1, but it is less abundant than LIS-1 in the brain (13). It has not been associated with a brain malformation.

In order to study the function of the LIS proteins in cortical plate formation, we have immunolocalized these proteins in the developing human cerebrum. We have generated antibodies against 2 epitopes of the LIS proteins and have examined the immunoreactivity of cells and structures involved in cortical formation: the Cajal-Retzius neurons, the subplate neurons, radial glia, germinal matrix, and the ependyma. Because of the homology between LIS-1 and LIS-2, the antibodies do not distinguish between the LIS gene products. However, because the major expression of these genes in brain is LIS-1, we consider that our observations localize the gene product of LIS-1, the gene for MDL and some cases of ILS, in the developing human brain at 14 through 40 weeks gestation.

MATERIALS AND METHODS

Antibodies were derived against amino acid residues 1 to 13 (designated as peptide N1) and amino acid residues 297 to 309 (designated peptide I2) of the LIS protein sequence. The peptides were chosen to be outside of the WD-40 repeats in the LIS proteins in order to prevent cross-reactivity to other proteins with these sequences (14). The peptides were coupled to keyhole limpet hemocyanin with m-maleimidobenzoyl-N-hydroxysuccinamide ester, placed in 50% phosphate-buffered saline/50% Freund's complete adjuvant and injected into Japanese white rabbits. Although the anti-I2 antibody occasionally labeled a 60-KD protein in Western blots of human brain homogenates, the only protein stained by both antibodies was a...
45-KD protein, consistent with specific staining of the LIS proteins (14). These antibodies were used to isolate the LIS family of gene products in human fetal cortices, gestational ages 14, 17, 20, 23, 26, 30, 34, 37, and 40 weeks, periods when cortical plate formation is occurring or has just been completed.

Human brains were fixed (within 1 to 10 hours [h] of death) in formaldehyde, dehydrated, cleared in CHCl₃, and then embedded in paraffin. Five-micrometer sections for microscopy were mounted and dried at 58°C for 10 minutes (min). Subsequently, the tissue was dewaxed in xylene and hydrated from absolute ethanol to distilled water.

An antigen retrieval protocol was utilized. Tissue sections were incubated in a 0.01 M citric acid solution of pH 6.0 for three 5-min periods in a microwave on high setting. The tissue was allowed to cool and then transferred to PBS. Tissue was then treated with 3% H₂O₂ in phosphate buffer solution (PBS) for 8 min in order to block endogenous peroxidase. After rinsing in PBS, the tissue was transferred to normal goat serum (1:20) for 30 min in order to block background antibody binding. The tissue was transferred to PBS containing the primary antibody for 48 h (anti-N1, anti-12, anti-calretinin, anti-MAP-HM-2, anti-MAP-2) or for 6 days (anti-calbinden-D) at 4°C. The slides were washed in PBS and transferred to a 1:400 goat antirabbit or 1:200 goat antimouse antibody for 30 min at room temperature. After a wash in PBS, the tissue was treated with AB complex for 45 min at room temperature. The slide was then washed and the chromogen diaminobenzidine tetrahydrochloride was applied (5 mg/10 ml with 33 μL of 30% H₂O₂). The slides were then stained with hematoxylin for nuclear detail.

The LIS antibodies, rabbit anti-N1 or anti-12, were used at dilutions of 1:300. Anti-calbinden-D (Sigma, St. Louis, Missouri), a mouse monoclonal antibody, was used at a dilution of 1:1000. Anti-calretinin (Chemicon, Temecula, California), a rabbit polyclonal antibody, was used at a dilution of 1:3000. Anti-MAP-HM-2 (Sigma), a mouse monoclonal antibody directed against MAP-2a, -2b, and -2c, was used at a 1:2,000 dilution. Anti-MAP-2 (Boebringer Mannheim, Indianapolis, Indiana), a mouse monoclonal antibody directed against the MAP-2a and -2b (phosphorylated forms), was used at a dilution of 1:800.

RESULTS

In the formation of the human cerebral cortex, neuronal migration occurs between 8 and 20 weeks gestation. We have studied the developing human neocortex at the gestational ages of 14–40 weeks using antibodies derived against the LIS protein. These examinations of human fetal tissue showed strong immunoreactivity at 2 sites: the cytoplasm of the Cajal–Retzius cells and the ventricular neuroepithelium (see Fig. 1). Weaker immunoreactivity was noted in the neuropile of layer 1, some subplate neurons, and thalamic neurons. Human fetal tissue prepared with the same antigen retrieval protocol showed no reactivity to preimmune serum (data not shown).

As previously noted, a faint neuropile staining with both antibodies was seen in the gray and white matter of human brain (14). This staining was particularly apparent in layer 1 (see Fig. 1). With longer exposures to the primary antibodies, this neuropile staining could be made more intense. The significance of this staining was not certain, but preimmune sera did not show a similar pattern (14).

These antibodies and the preabsorbed antiserum stained endothelial cells, which served as an internal positive control for our studies. In our study (as was noted previously [14]), there was a difference in the intensity of immunoreactivity for the two antibodies. Anti-N1 showed a consistently better definition of the blood endothelial cells, the background, the cytoplasm of the Cajal–Retzius cells, and the ventricular neuroepithelium. However, both antibodies appeared to selectively and consistently define the Cajal–Retzius cells and the ventricular neuroepithelium (see Figures, Tables 1, 2).

The large neurons of layer 1, Cajal–Retzius cells, were all noted to stain with LIS antibodies at all gestational ages tested (see Figures, Tables 1, 2). The immunoreactivity in these cells was consistent with a nuclear cytoplasmic localization of the LIS proteins, and some processes could be visualized with the immunocytochemistry techniques used. These cells had the typical pleomorphic configuration of the human Cajal–Retzius cells and were all located in the marginal zone (layer one). These same cells were found to contain calretinin, a protein known to be localized to the Cajal–Retzius cells at the gestational ages tested (see Fig. 2, Tables). Small granule cells of layer 1 did not stain with LIS antibodies (see Figures).

LIS antibody staining of the cortex was apparent at 14 weeks gestation, but the precise cellular localization of the staining was difficult to determine because of the dense cellularity of the forming cortical plate and the prominence of newly formed blood vessels at this gestational age. It was felt that the majority of the staining was in the recently formed blood vessels. At 17 and 20 weeks gestation, occasional neurons were noted to stain within the cortical plate with LIS antibodies. The pattern of immunoreactivity was consistent with a nuclear protein expression, and the neurons that stained were the more mature cells in the cortical plate (see Figures, Table 1).

Large cells below the forming cortical plate were also occasionally seen to stain with both LIS antibodies (see Table 2). These cells had a pyramidal shape and processes typical of mature neurons. It was felt that these cells represented subplate neurons. This was not a consistent observation and it was more prominent in the older fetal brains (20 to 34 weeks gestation). When compared with MAP-2 and MAP-HM-2 staining (which stained an extensive population of cells with a neuronal morphology in the region between the germinai matrix and the cortical plate, the subplate), the LIS-positive cells represented a minor subpopulation of probable subplate neurons.

Fig. 1. Immunolocalization of LIS proteins (at 20 weeks gestation) to layer 1 and the ventricular neuroepithelium. Immunopositive cells and structures are reddish-brown. Hematoxylin-stained nuclei are blue or purple. Slides of representative specimens were scanned, digitized, and labeled using Photoshop (Adobe). A. Anti-LIS (N1 antibody) staining of layer 1 (designated by a line on the right side of the panel) and the superficial cortical plate is shown. An immunopositive Cajal-Retzius cell is designated by an arrow and the letters CR. Immunostaining was also noted in the neuropile of layer 1. B. A low-power view of anti-LIS (N1 antibody) immunostaining of layer 1 is shown. Three Cajal-Retzius cells, designated by arrows, are immunopositive. The Cajal-Retzius cell to the right has a long immunopositive process in the same plane as the section. Neuroplee staining of layer 1 was also noted. C. Immunostaining of the ventricular neuroepithelium with the N1 antibody is displayed. Cells lining the ventricle typically stained for LIS proteins; these cells had short apical processes. D. GFAP immunolocalization in the ventricular neuroepithelium differed from that of LIS. In contrast to the anti-LIS immunostaining of cell bodies of the ventricular lining, GFAP stained long processes that terminated in a thin ventricular lining. These processes were probably those of radial glia. The width of panels A and C was 300 μ. The width of panel B was 533 μ and of panel C, 320 μ. Cajal-Retzius cells were approximately 20 μ in diameter.
Fig. 2. Localization of proteins in developing human brain by immunocytochemistry. For consistency and comparison, all panels show representative examples from human gestational age 20 weeks. The antibody used for each column is displayed on top of the column: LIS refers to the anti-N1 antibody, CalR refers to anticalretinin, MAP2 refers to anti-MAP-2, and CB refers to anti-calbindin. The region of fetal cortex is listed to the left of the corresponding row. Row designations are CR for Cajal Retzius cells, and this row shows the immunostaining for layer 1. CX refers to the developing cortical plate. SP refers to the subplate region. EP refers to the ventricular neuroepithelium and the germinal matrix. Immunopositive cells are brown, and the nuclear staining is blue. Arrows in the top row point to the large neurons of layer 1, the Cajal-Retzius cells. Arrowheads point to the ventricular neuroepithelium. Asterisks denote blood vessels. Each panel is approximately 264 μ in width; the nuclei of the cortical plate cells typically measure 6 μ in diameter.
### TABLE 1

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Tabulated results from immunocytochemical examination of human fetal brains from gestational ages 14 weeks (1 specimen), 17 weeks (1 specimen), and 20 weeks (2 specimens) are displayed. The antibodies (L2 is anti-L1 [LJS], N1 is anti-N1 [LJS], CR is anti-calretinin, MAP-2 is anti-MAP-2 [MAP-2a and -2b], MAP-HM is anti-MAP-2-HM [MAP-2a, -2b, and -2c] and CB is anti-calbindin-D) used are displayed above each column of results. Each row represents a cell group or an area of developing cortex (subpl refers to the subplate region and vent neo refers to the ventricular neuroepithelium). Strong positive reactions (deep brown staining) are designated as ++, moderate positive reactions are designated as +++, weakly positive reactions or equivocal reactions (light brown) are designated as ±, and negative reactions are designated as –. Only weak nuclear staining is designated as N, and only weak cytoplasmic staining is designated as C.

### TABLE 2

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Tabulated results from immunocytochemical examination of human fetal brains from gestational ages (one of each) 23, 26, 30, 34, 37, and 40 weeks. Rows and columns are the same as in Table 1. The result designation is the same as in Table 1; + designates immunoreactivity between ++ and ±.
Both LIS antibodies stained the cytoplasm of the neurons of the thalamus and basal ganglia at gestational ages of greater than 23 weeks. It appeared that all neurons in these structures stained with the LIS antibodies, with an intensity of staining less than that of the ventricular neuroepithelium and Cajal-Retzius cells. The staining was more prominent in the older brains. The thalamus is thought to play a role in cortical specification, a process that takes place at late gestational ages and after birth.

At 14 to 20 weeks gestation, the main period of cortical neuronal migration, cells in the ventricular neuroepithelium reacted strongly with LIS-1 antibodies. Cilia were noted on some of the immunoreactive cells at 20 weeks gestation, leading to the conclusions that these cells represented immature ependyma (see Figures) and that the elements in the ventricular neuroepithelium of less than 23 weeks gestation possibly represented tanyocytes, precursors to ependyma (15). In addition to the ventricular neuroepithelium, some elements of the ventricular germinal matrix at 14–20 weeks gestation also stained with the LIS antibodies. These elements were felt to represent either the processes of ventricular neuroepithelium or those of tanyocytes (see Figures). From 23 to 40 weeks gestation, staining of more mature ependymal cells was noted (see Table 2). No other antibody tested stained the ventricular neuroepithelium or the ependymal cells, suggesting that LIS antibody reactivity in these structures was specific (see Figure 2, Tables 1, 2). Glial fibrillary acidic protein (GFAP) and vimentin immunostaining distinguished these LIS-positive cells from radial glia, since radial glia had long processes in contrast to the short apical processes seen in the LIS-positive cells. Furthermore, LIS-positive cell bodies were noted to line the ventricles, whereas GFAP immunoreactivity was not present in the same cell bodies, but in the long processes directed towards the ventricular lining.

The immunoreactivity of the developing neocortex was also examined with antibodies to other proteins that are expressed in a regulated sequence during maturation. Each of these exhibited patterns of reactivity that differed from those of the LIS proteins. For example, calretinin immunoreactivity was examined in one fetal cortex of 14 weeks gestation and in two fetal cortices of 20 weeks gestation. These cortices demonstrated positive immunoreactivity in the same layer 1 cells that were stained with the LIS antibodies, the Cajal-Retzius cells, but demonstrated no immunoreactivity in the periventricular cells. At 20 weeks, one brain showed a trace of staining for calretinin in the subplate neurons.

At all ages examined, the Cajal-Retzius cells, the subplate neurons, and the mature cortical neurons demonstrated strong immunoreactivity to MAP-2 antibodies (see Tables 1, 2). These antibodies did not stain the cells of the ventricular neuroepithelium, but did stain a minority of the cells of the neighboring germinal matrix.

Antibodies to calbinden revealed no immunoreactivity in the youngest fetal brains. At 30 weeks gestation, immunoreactivity appeared in the neurons of the subplate and of the cortex. The Cajal-Retzius and ventricular neuroepithelial cells remained negative.

DISCUSSION

Mutants in which cortical malformations occur have offered clues to the mechanisms underlying cortex formation (16, 17). This has certainly been true of the reeler mouse. The isolation, sequencing, and localization of reelin, the gene product responsible for this mutant, have given neurobiologists the foundation of the understanding of one of the genes involved in proper cortical plate organization. Reelin is only expressed in the Cajal-Retzius cells, the early pioneering neurons of the molecular layer (18, 19, 20). This observation not only points to the importance of the Cajal-Retzius cells in the process of cortex formation, but also gives neuroscientists a clue to the function of the reelin gene in the process of cortical plate organization.

The human brain malformation disorder MDL offers another opportunity for neurobiologists to gain an understanding of the process of cortex formation. This disorder is one involving neuronal migration since the cortical plate is malformed and neurons appear to be arrested in the process of neuronal migration. The lack of complexity of the rudimentary cortical plate in MDL results in a smooth cortical mantle; this is the pattern of classic lissencephaly.

Humans with MDL have a haploinsufficiency of the LIS-1 gene, whose only described function is that of a component of a PAF acetylhydrolase (7, 21, 22). The normal role of the LIS-1-encoded protein and of PAF acetylhydrolase in brain formation has not been determined. In the present study, we have used antibodies raised against the protein sequences encoded by the LIS genes in order to determine the possible sites of LIS protein expression during cortical development. The LIS-1-encoded protein is presumably haploinsufficient in MDL and ILS, and this deficiency results in aberrant cortical plate formation such that the cells that are destined to form the cortical plate are arrested in the process of radial migration. We have therefore hypothesized that the normal expression of the LIS family of gene products would occur in structures critical to the neuronal migration leading to the formation of the cortical plate. The present study seems to support this hypothesis.

At the stage of human fetal development when the majority of cortical migration is occurring (14 through 20 weeks gestation), LIS proteins appear to localize predominantly to the Cajal-Retzius cells and to the ventricular neuroepithelium of the developing human cerebrum. Although in previous studies, no LIS-1 message was noted in the marginal zone of one human cortex of 19 weeks.
gestation (23) and only punctate areas of Lis-1 (mouse homolog of LIS-1) expression were seen in the marginal zone of the E15.5 mouse brain (24), the data presented here could be consistent with these previous observations, since the Lis-1 expression in those studies was in isolated large cells of mouse layer 1 that probably were Cajal-Retzius cells.

The advantages of the protein expression studies reported here are that they are less likely to be confounded by post-transcriptional changes in message and by post-translational changes in protein than are message expression examinations of tissues. The disadvantages of the immunolocalization approach in the present study are the inability to distinguish the LIS family of gene products and the limited availability of early gestational age fetal brains. Given the limitations of all approaches to the localization of the LIS gene products, the consistency of immunolocalization reported here points to important and specific cellular localizations of the protein encoded by a gene that is abnormal in human lissencephaly. We feel that the present report augments previous message expression studies and together, may lead to a better understanding of the role of the LIS proteins in cortex formation.

Although the observed neurite staining with LIS-1 may eventually be shown to be an important expression of LIS-1 protein in human neuronal migration, we have concentrated upon the localization of the more apparent LIS protein expression. It is likely that this predominant protein expression in Cajal-Retzius cells and ventricular neuroepithelium predates the onset of migration and therefore is important in setting the stage for the mass movement of cells to the cortical plate.

**LIS-1 Localization to Cajal-Retzius Cells**

The predominant localization of the LIS proteins to the early pioneering neurons, the Cajal-Retzius cells, implicates them in other essential developmental roles. Martin-Padilla (25, 26) has illustrated the significance of the Cajal-Retzius cells in layer 1 of the mammalian cortex, where they organize early and possibly late cortical development. The subplate neurons share in this organizational role, marking the limits of the cortex and providing temporary synaptic sites for growing axons. Some of the neurons of the subplate in our fetal specimens also contain the LIS proteins.

In the reeler mouse, the cortical plate is deposited in an inverted manner, with the first migrating neurons residing in the most superficial layers rather than in the deepest layers of the cortex. Reelin, the abnormal gene product in reeler, is immunolocalized solely to the Cajal-Retzius cells (19, 20). It is intriguing that in the 2 mutants in which cortical abnormalities have been characterized and in which the gene has been identified, reeler mice and humans with MDL, both have gene products that localize to the pioneering neurons of the cortical plate. Whereas reelin is exclusively localized to the Cajal-Retzius cells, LIS is additionally localized to other structures important in cortical development. In a manner similar to LIS protein expression, L1, an important cell adhesion molecule involved in brain development, also has been found to predominantly localize to the Cajal-Retzius cells (27), thus pointing to the critical role that these cells play in proper cortical plate formation. Recently, elegant studies of in vitro neuronal migration using explants of Cajal-Retzius cells demonstrate that these important pioneering neurons produce a soluble signal that induces the formation of radial glia and promote the unidirectional migration of neurons toward the Cajal-Retzius cell explant (28). The nature of that soluble signal is unknown at present.

**LIS-1 Localization to Ventricular Neuroepithelium**

The cells and processes that were immunoreactive to LIS-1 in the ventricular neuroepithelium appeared to be consistent in location and morphology with previous descriptions of tanyocytes, cells that are felt to represent the precursors to ependyma (15, 29). The staining of ependyma at later gestations by LIS antibodies supports this impression. The cellular morphology of the LIS-positive cells in the ventricular lining differed significantly from the GFAP-positive structures in the same area (see Fig. 1). LIS antibodies stained the soma of cells with short apical processes, whereas GFAP-positive elements appeared to be processes situated between ventricular cells.

The functions of the ventricular neuroepithelium in cortex formation are not often emphasized, yet this structure has essential roles in neurogenesis, axonal guidance, and in the retraction of radial glia upon the termination of neuronal migration (30, 31). These functions are regulated by diverse molecules such as retinoic acid, keratin sulfate, neural cell adhesion molecule, and nerve growth factor (29). Retinoic acid and the closely related 3,4-didehydroretinol are lipids that are secreted by the floor plate, a specialized ventricular neuroepithelium of the ventral midline of the developing central nervous system (32). These lipids are involved in the development of dorsoventral polarity of the developing central nervous system, perhaps by promoting differentiation of neuroepithelial cells (32). In light of the results presented here, it is possible that PAF may play a similar role to retinoic acid in neural development, since the expression of a component of the enzyme that degrades PAF, LIS-1 protein, is found in ependyma. This suggests that regulation of PAF in this structure during neurodevelopment is important for proper cortex formation. Our demonstration of LIS expression in ventricular neuroepithelium and fetal ependyma suggests that there is a role for PAF in peripheral structures, and because ependymal abnormalities have been observed in lissencephaly (33), that role may be related to neuronal migration.
CONCLUSIONS

At this time, it is not clear what role the LIS-1 protein has in cortical plate formation, but the cellular localization of this protein should provide some clues. The only known function of the LIS proteins is that of a PAF acetylhydrolyase subunit. Therefore, it is possible that a deficiency in the degradation of the lipid messenger, PAF, leads to MDL and ILS. It is also possible that a PAF-related signaling system may be dysfunctional in this disorder (11).

A definitive role for PAF in neuronal migration has not been elucidated, but PAF does appear to participate in a number of important developmental mechanisms. PAF has been shown to participate in neuronal differentiation (34) and synaptic plasticity (35, 36); it causes neuronal growth cone collapse and neurite withdrawal (37). Perhaps PAF serves as a neurite guidance molecule in the development of the cortex. The leading process of migrating neurons has a cytoskeletal arrangement similar to that of neurites (38), and therefore may be affected by PAF in a similar manner.

We have predominantly localized the LIS proteins to the Cajal-Retzius cells and periventricular neuroepithelium of the developing human brain. We therefore believe that the function of this important family of genes involves the Cajal-Retzius cells and the periventricular neuroepithelium, and when haplo-insufficient, an LIS-1 defect in these structures leads to an orderly disruption of neuronal migration that results in the classic lissencephaly phenotype. Based upon these results, we believe that investigators should turn their attention to the function of LIS-1 in these structures. Because the only known function of LIS-1 is that of a PAF acetylhydrolyase, the regulation of this lipid messenger in these cells may be critical for proper cortical plate formation, and when haplo-insufficient, a disordered PAF-related function in these cells leads to the neuronal migration defects MDL and ILS.

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