〔Original〕

Brain pathology of Lafora disease : fine structural changes of Lafora (polyglucosan) bodies in the developing phase of the formation

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Abstract

Lafora disease（LD）is an autosomal recessively inheritable neurodegeneration due to glycogen metabolic disorder, characterized pathologically by the presence of Lafora bodies（LB）（called polyglucosan bodies; malformed insoluble glucose polymers）in CNS neurons and cells in many other organs. Recently, it has become evident that about 90% of cases of LD are caused by mutations in either the EPM2A（laforin）or the EPM2B（malin）gene. Few previous EM studies have observed LB in the developing phase in detail. This study aimed at obtaining key findings that may reveal molecular events involved in the disease mechanisms underlying the morphology, by EM observations on LB in brain tissue from a previously reported case of LD. The observations revealed that the two main components of LB, i.e. poorly branched, irregular fine filaments and amorphous small dense granules, were distributed sparsely in the LB in the early phase, whereas in the developing phase the fine filaments were innumerable together with a substantial number of dense granules and polysomes along with some lysosomes and no apparent autophagosomes. They intermingled and formed LB. In the developed phase, cored LB consisted of aggregates of dense granules forming the core and radially arranged filaments forming the outer rim. Recently, it has been reported that the lack of laforin-malin complexes causes the dysfunction of autophagy, which plays a primary role in the LB formation. The lack of Laforin-malin complexes and impaired autophagy for aggresome clearance were discussed. In conclusion, the fine structural changes of LB in the developing phase could be the key findings that link to the molecular mechanisms of not only LB but also LD.

Key words: Lafora bodies in the developing phase, fine structural changes, polyglucosan,
　　　　　　aggresome and autophagy, laforin and malin

Introduction

Lafora disease（LD）is an autosomal recessively inheritable neurodegenerative disorder, characterized clinically by myoclonus, epileptic convulsions and progressive mental deterioration. The disease manifests with stimulus-sensitive tonic-clonic, grand mal, visual and myoclonic seizures which rapidly progress severe myoclonic epilepsy and status epilepticus, resulting in dementia, muscle wasting and respiratory failure. The disease mostly occurs between the age of 10 and 17 and death follows about 10 years after the onset（Anraku and Hakusui 1989; DiMauro,1996; Inenaga and Anraku1974; Lafora and Glueck, 1911; Naito and Oyanagi,1989; Seitelberger, 1968）. The disease is also characterized histologically by the presence of intracytoplasmic periodic acid Schiff-positive inclusions known as Lafora bodies（LB）in many tissues, such as the brain, liver, myocardium, skeletal muscle and skin（Adams and Lee, 1982; DiMauro, 1996; Inanaga and Anraku 1974; Thom et al, 2008）. Their histological appearance differs depending on tissues affected（Adams and Lee, 1982; Seitelberger, 1968; Thom et al, 2008）: LB in the brain are seen predominantly in perikarya and dendrites of neurons. The majority of the LB stained strongly with periodic acid-Schiff（PAS）have a darkly stained core and lightly stained radiating shells. The larger LB are invariably perikaryonal in a distribution similar to the endoplasmic reticulum（ER）, and the sandgrain-like granules which

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are also revealed by PAS staining are concentrated mostly in small neuronal dendritic processes and on rare occasion in axons（Adams and Lee, 1982; Iwata 1986; Seitelberger, 1968; Van Heycop Ten Ham, 1974;Yagishita, 1994; Yoshimura et al, 1999）. LB are found in other organs, most prominently in the liver, heart, skeletal muscle, and moderately in smooth muscle of the alimentary tract（Yoshimura et al, 1999）, in addition to the sweat gland duct cells in the skin （Carpenter and Karpati 1981）.

Cytochemical studies indicated that LB contained a substantial amount of a complex carbohydrate, only a small portion of which was consistent with acid mucopolysuccharides（Seitelberger, 1968; Adams and Lee 1982）. In addition, a protein moiety was demonstrated, which appeared to be sensitive to chymotripsin and pepsin, but more specifically to the latter（Gambetti et al, 1971; Nikaido et al, 1971）. LB contain 80-93% polyglucosan and 6% proteins depending on tissues, although the natures of the proteins are mostly unknown （Yokoi et al, 1968; Sakai et al, 1970）. Polyglucosans are malformed glycogen molecules（glucose polymers） that appear as peculiar linear strands defective of normal branching（DePaoli-Roach et al, 2010）. Resembling amylopectin, polyglucosans are poorly soluble hence precipitate inside cells.

LD is caused by mutations in either EPM2A gene present on 6q24, encoding laforin（Minassian et al, 1998; Serratosa et al, 1999）, or EPM2B gene located on 6p22.3 which encodes malin（Chan et al, 2003; Chan et al, 2003）, although there is evidence for a third locus （Chan et al, 2004）. Laforin is a glycogen phosphatase （in detail, a dual specificity protein phosphatase with a functional carbohydrate-binding domain）. Malin is an E3-ubiquitin ligase having RING finger domain at the N-terminus. However, the disease mechanisms that are brought about by mutations in these two genes are poorly understood. Therefore, it is necessary to elucidate what the structural and functional changes are expressed on cell and tissue structures *in vivo*, due to the defective gene product. The fine structural changes of LB have been reported by many authors so far（Anraku and Hakusui, 1989; Collins et al, 1968; Namba, 1971; Kotorii et al, 1974; Van Heycop Ten Ham, 1974; Vanderhaeghen, 1971; Cajal et al, 1974; Gambetti et al, 1971; Ishihara et al, 1987; Oyanagi, 1992; Van Hoof and Hageman-Bal, 1967; Yoshimura et al, 1999）. Almost all of them,

however, have reported on those of matured/developed phase of LB rather than their early or developing phase. The purpose of the present study is to obtain key findings that may reveal molecular events involved in the disease mechanisms underlying the morphology.

Materials and Methods

A male patient with Lafora disease with a 17-year clinical course, aged 30 at death was autopsied 8 hours after death. The brain showed no gross abnormality except for a slight increase in weight（1,500g）（Fig. 1a, b）. The autopsy findings of all organs including the CNS, together with fine structural changes and immunoreactivities of LB in brain tissue against ubiquitin and tau antibodies were reported previously （Yoshimura et al. 1999）. A histological reexamination was made by LM using slides stained with H&E, Kluever-Barrera, Bodian, periodic acid-Schiff（PAS）, and PAS after diastase digestion methods. For electron microscopy（EM）, epon-embedded tissue-piece blocks of frontal（F1）and temporal（T2）cortices and those of the globus pallidus and substantia nigra were selected. These epon-embedded blocks had been made immediately after the autopsy. Semi-thin sections stained with 1% toluidine blue solution were made. After observing the toluidin blue stained slides, ultrathin sections which were obtained by using a Porter-Blum ultramicrotome（RMC; MT-6000）were double-stained with uranyl acetate and lead citrate. They were examined using an H-600 electron microscope （Hitachi, Tokyo, Japan）at 80kV.

Results

After histological examinations described above, LB formed in the perikaryon were classified into 3 phases based on the evolution:（1）the early phase （Figs.1c, d, arrowheads）exhibits LB having a small and vague contour, a moderate reactivity to PAS, and no displacement of the nucleus nor enlargement of the cell body,（2）the developing phase（Figs.1c, d arrows） indicates LB that have a somewhat vague contour, a moderate reactivity to PAS, and no distinct core nor marked displacement of the nucleus,（3）the developed phase（Fig.3a, b double-arrows; Figs. 5c and e）shows enlarged cored or coreless LB having a distinct contour,
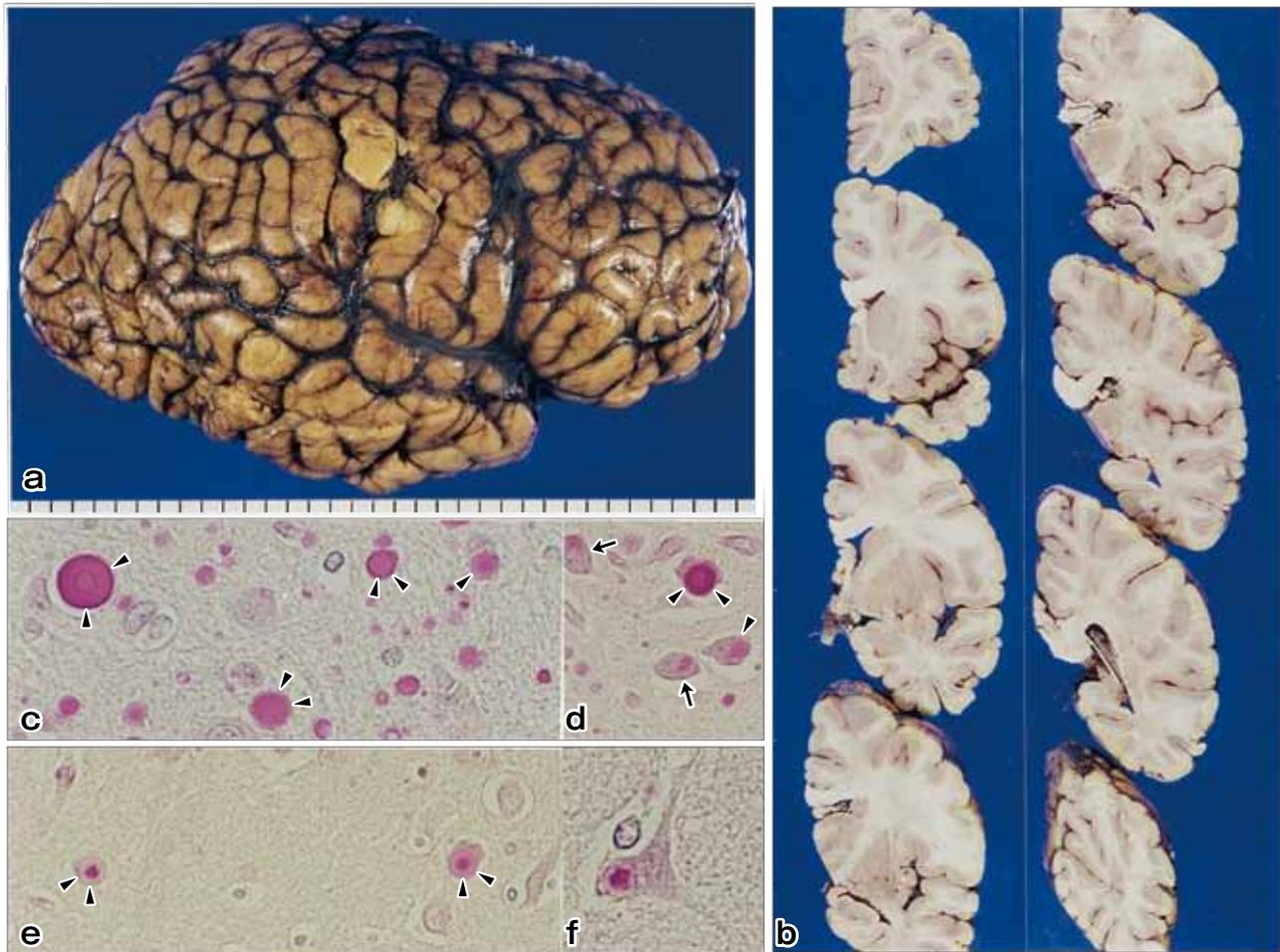
Fig.1   **1a.** Lateral view of the brain showing no gross abnormality.
         1b. Serial coronal sections of the right cerebral hemisphere showing no remarkable change.
         1c & d. Histology of the superior olivary nucleus showing various phases of LB:early phase（arrow）, developing
            phase（arrowhead）, and developed phase（2 arrowheads）.
                                               1c. PAS stain x 530, **1d.** PAS stain x 530.
         1e & f. Histology of LB in the perikaryon of nerve cells in the frontal cortex.
                                         1e. PAS stain x 530, **1f.** PAS stain x 530.

usually a strong reactivity to PAS and marked displacement and deformity of the nucleus with striking distension of the cell body.

EM observations of LB in the early phase

The present study dealt with ultrastructural observations of LB mainly in the developing phase. Before this, however, we first observed LB in the early phase. The ultrastructural changes were very similar to those of LB in the developing phase in that LB appeared as a cytoplasmic mass with no limiting membrane and consisted of two main components in various proportions; fine irregular filaments and amorphous and electron-dense granules showing irregular and rather sparse distribution in the cytoplasm of low density.

EM observations of LB in the developing phase

Electron microscopy of nerve cells bearing LB in the developing phase regularly showed that the perikaryon was occupied by a large mass of cytoplasm of low density containing two main components in various proportions; the fine filaments, approximately 6 nm in diameter, were irregular and occasionally branched, although such branches were short and only traceable up to a short distance. They were frequently continuous with small dense granules which varied in size and shape. The cell nucleus was somewhat displaced toward the cell membrane, along with organella such as rough endoplasmic reticulum（rER）, polysomes, mitochondria and vesicles. At a glance, the cytoplasm consisted of innumerable, short and very fine
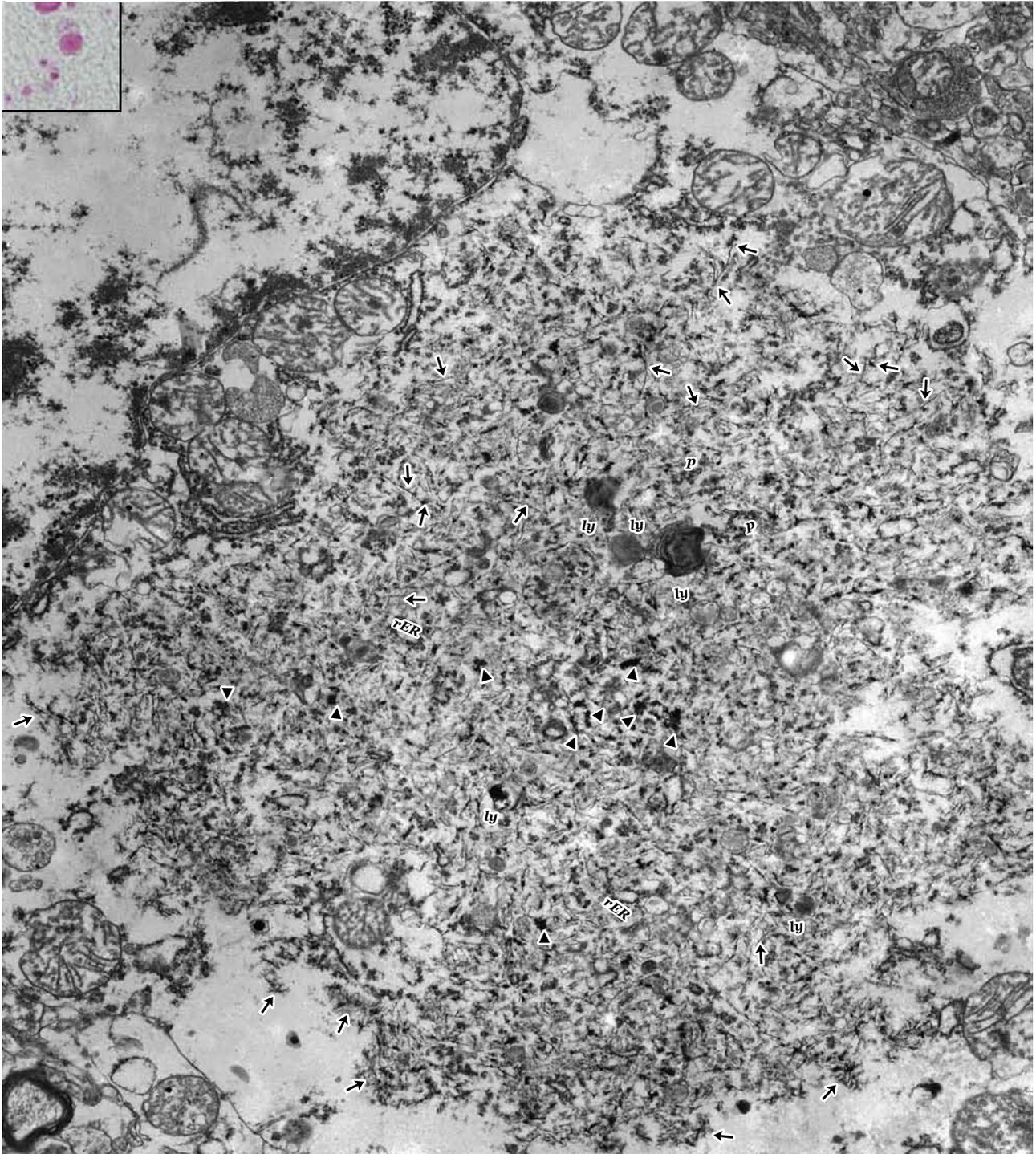
Fig.2   Electron micrograph of a LB in the developing phase illustrating that a newly-formed cytoplasmic mass with no limiting membrane occupies the perikaryon pressing aside the preexisting cytoplasm containing mitochondria, rERs polysomes (p) and lysosomes (ly) toward the cell membrane. The body consists of two main components in various proportions: fine irregular filaments (arrows) and amorphous dense granules (arrowheads) exhibiting irregular distribution in the newly-formed cytoplasm of low density.

x 13,600.   Inset PAS stain x 530.
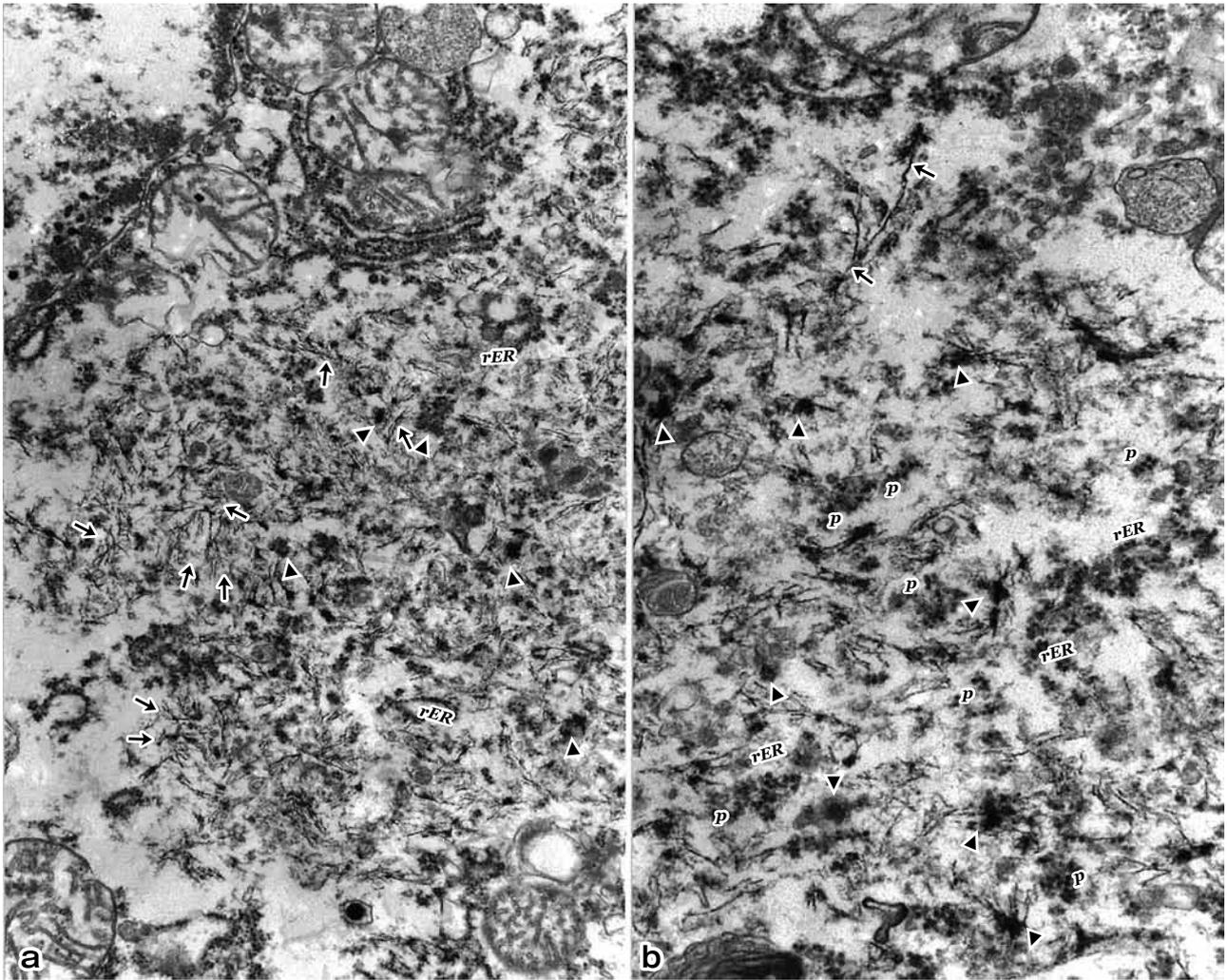
Fig.3   **3a.** Electron micrograph of the left part of the LB in Fig.2, illustrating that the newly-formed cytoplasm consists of fine filaments intermingling with and running in random directions, associated with scarce presence of small dense granules. In addition, note the concurrent presence of many polysomes (p), small rERs, multivesicular bodies (mv), and probable lysosomes (ly).

x   20,000.

     3b. Electron micrograph of the right upper part of the LB in Fig. 2, demonstrating that some of those filaments form a semi-parallel arrangement similar to that of "pine-needles".

x   32,400.

filaments intermingling and running in random directions (Fig.2-4). More precise observations, however, revealed that a substantial number of those filaments tended to form a semi-parallel arrangement similar to that of pine needles. (Figs.3-5a). In the transitional zone between the superficial area of the newly-formed and the pre-existing cytoplasm, there was degradation of rER associated with an increase in their fluffy pattern of short filaments (Figs.2-4). From the surface to the center of LB, there were short fine filaments, two to several of them arranged in a semi-parallel fashion intermingling and running in random

directions. In addition, small dense granules, polysomes, small pieces of rER, lysosomes, and multivesicular bodies were present here and there (Figs.2-4). However, it was difficult to detect autophagosomes, centrioles and mitochondria in the depth of the LB. In the center of the LB there was sometimes a sign of accumulation of the small dense granules which would come to form a central core (Figs.2 and 4b).

EM observations of the LB in the developed phase

The most representative profile of cored LB formed in the perikaryon (Figs.1c-f, Figs.5b and d) often showed perikaryonal distension and marked
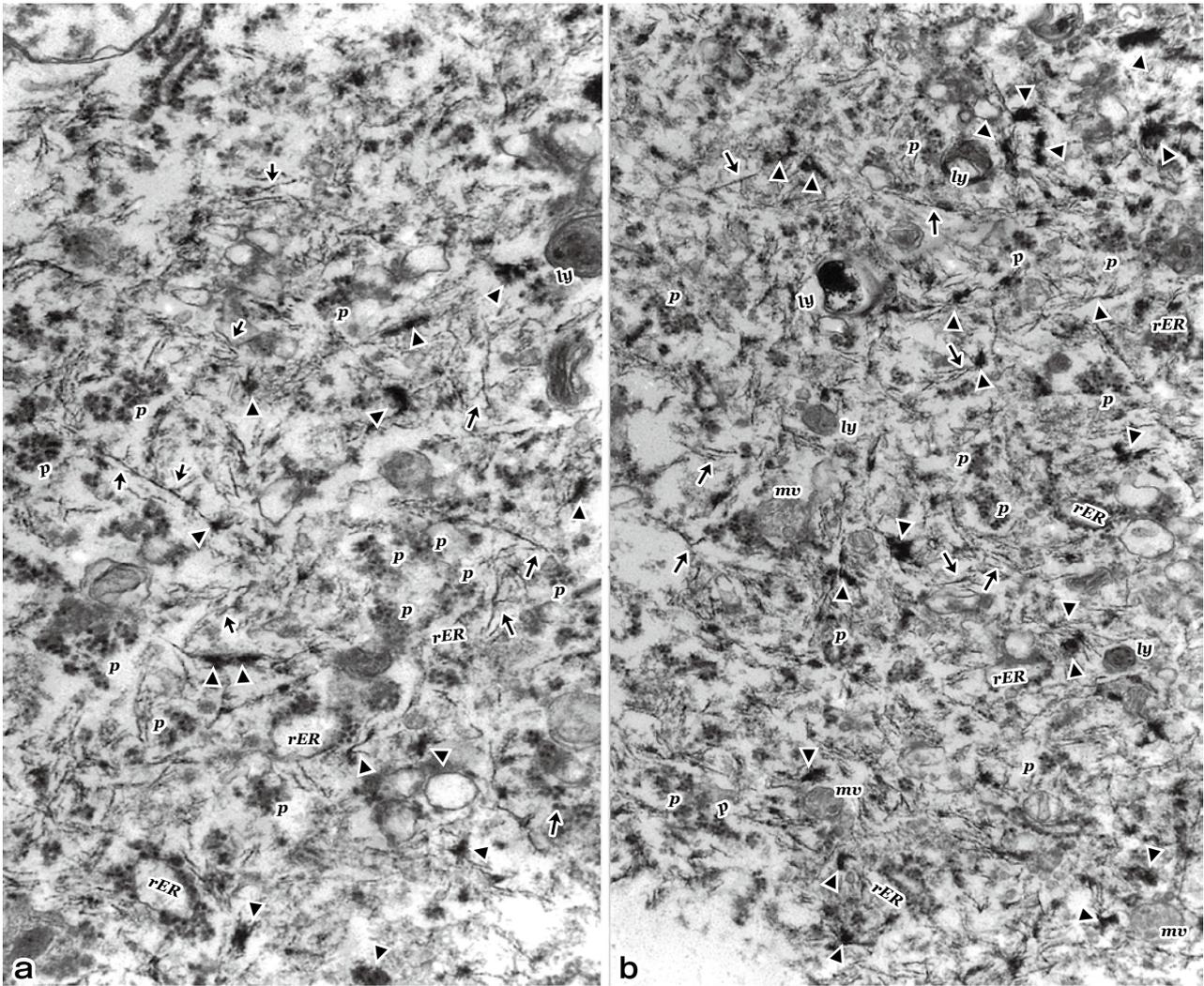
Fig.4   **4a.** Electron micrograph of the left upper part of the LB in Fig.2, depicting that increased fine filaments (arrows) form a semi-parallel arrangement intermingling with cytoplasm which contains many polysomes (p), small pieces of rER and lysosomes (ly).

x   33,400

4b. Electron micrograph of the lower part of the LB in Fig.2, exhibiting innumerably increased fine filaments which intermingle and run in random directions. The filaments (arrows) frequently form a semi-parallel arrangement, which are often continuous with the small dense granules (arrowheads). The cytoplasm containing many polysomes (p), small rER, multivesicular bodies (mv) and lysosomes (ly).

x   25,300

nuclear displacement with deformity, which corresponded to the LB in the developed phase. In the cored LB, the size of the core varied from null or pin-point size to full size appearing to completely occupy the LB. Thus most of the developed/matured LB formed in the perikaryon had a dense core (and/or a ring) and a light outer rim (Figs.1e and f). The bigger and the denser the core became, the more developed/ matured LB seemed to become. Those perikaryonal LB whose cores showed stronger reaction to PAS appeared

to be more developed/matured than those whose cores showed a weak reaction.

(a) The cored LB

Electron microscopy disclosed that cored LB were an increased mass of cytoplasm with no limiting membrane and consisted of two main components in various proportions; irregular filaments and small dense granules. The filaments seemed a little thicker and harder than those seen in the LD in the early and developing phases. The filaments, measuring
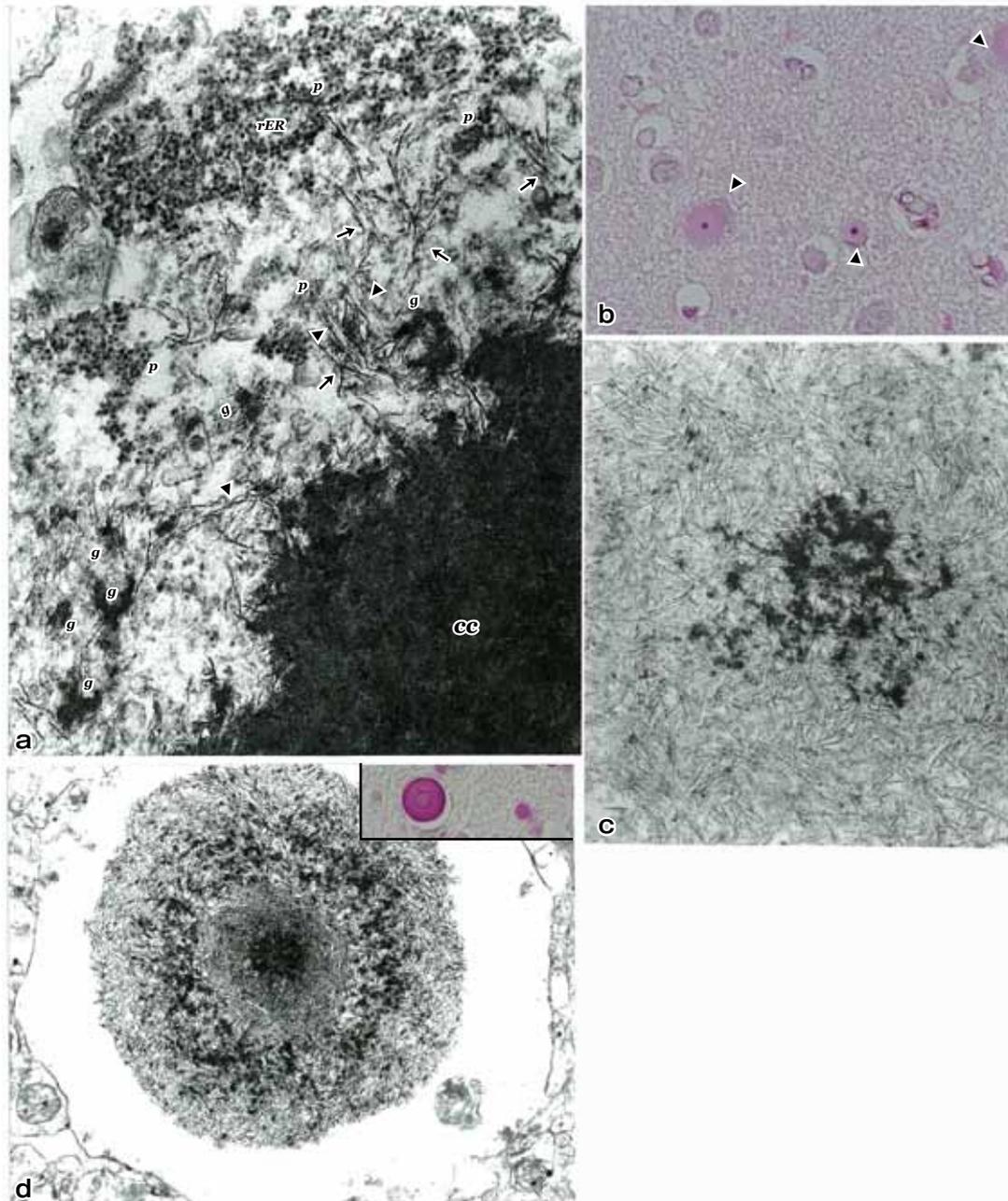
Fig.5   **5a.** Electron micrograph of a cored LB in the developed phase demonstrating that the poorly
branched filaments measured about 8 nm in diameter, frequently form a semi-parallel
arrangement（arrowheads）, some of which resemble that of "pine-needles". Most of the
filaments（arrows）seem to appear from small dense granules（g）and their aggregation
forming a huge central core（cc）, and some of the filaments also have connections with
rER and polysomes（p）.   x 34,000
5b. Histology of the frontal cortex exhibiting a pin-point cored homogeneous LB in the
developed phase. Note 3 nerve cells having marked displacement and deformity of the
nucleus（arrowheads）.   PAS stain x 530
5c. Electron micrograph demonstrating that a light homogeneous LB with a pin-point core in
the developed phase consists of densely increased fine filaments all over the LB, associated
with a focal accumulation of aggregated small dense granules in the center of the body,
which may correspond to a pin-point core under LM.   x 25,000
5d. Electron micrograph of a ring-cored LB in the developed phase demonstrating that light
PAS-reactive ring-zones exclusively consist of densely increased fine filaments and strong
PAS-reactive zones are composed of densely increased filaments plus deposition of
aggregated dense granules in various degrees, which are parallel to the intensity of the
PAS-reaction of the ring.   x 15,000.
Inset shows a ringed LB in the developed phase.   PAS stain x 530.

approximately 8 nm in diameter, were irregular and occasionally branched, although such branches were short and only traceable up to some distance. The filaments were frequently continuous with the small dense granules which varied from small flocculent deposits to large confluent dense granule aggregations, which had gradual transition to a huge dense core of LB (Fig. 5a). Cored LB had a dense core and a light outer rim (Figs.1e, f). Because the filaments intermingled with a large confluent dense granular material in the core of the LB, only in the rim were the filaments recognized as being arranged radially. As were already observed in the LB in the developing phase (Fig.2-4), some of those filaments tended to form a semi-parallel arrangement similar to that of pine needles. On the outer side of those radial arrangements, there were some gatherings of rER whose membranes and polysomes were often continuous with the filaments (Fig. 5a).

(b) Light homogeneous LB

Light homogeneous LB exhibited that the entire area of the pale body consisted of only compactly intermingled filaments innumerably increased in number, associated scarcely with the small dense granules.

The light homogeneous LB with a pin-point core showed that the entire area of the pale body consisted of only compactly intermingled filaments innumerably increased in number, and limited in the central area only there was an aggregation of irregularly fused dense granules forming a micro-core that would correspond to a pin-point core of the LB under LM (Figs.5c).

(c) Dark homogeneous LB

In contrast, the dark homogeneous LB were composed of compactly intermingled filaments innumerably increased in number, associated with substantially increased small dense granules.

The ringed LB exhibited that the presence of the filaments were dominant over that of the small dense granules in the light zone of the ring, and vice versa in the dark zone of the ring.

Discussion

It is known that approximately 48% of the cases of LD result from mutations in the EPM2A gene, which encodes a protein of 331 amino acids, called laforin, with a dual-specificity phosphatase domain and a carbohydrate-binding domain. Laforin binds glycogen and can dephosphorylate both phosphoserine/ phosphothreonine and phosphotyrosine substrates, as well as complex carbohydrates (Minassian et al, 1998; Serratosa et al, 1999; Knecht et al, 2010). About 40% of Lafora cases result from mutations in the EPM2B gene, which encodes a protein called malin, an E3 ubiquitin ligase of 395 amino acids with a RING finger domain at the N-terminus and six NHL domains in the C-terminal region (Chan et al, 2003; Ganesh et al, 2006; Gometz-Abad et al, 2005; Knecht et al, 2010; Delgado-Escueta, 2007).

LB, deposits of abnormally branched, insoluble glycogen-polymers (i.e. polyglucosans) invariably form in neurons, liver, heart and other tissue cells in every patient with LD, so LB are the pathognomonic change for LD, and its hallmark as well. LB are known to consist of 80-93% polyglucosans similar to amylopectin and 6% protein (Yokoi et al, 1968 ; Sakai et al, 1970). LB are decorated by anti-ubiquitin antibodies, suggesting accumulations of undegraded proteins. Thus, LD may be a disorder of both carbohydrate metabolism and protein clearance (Knecht et al, 2010). To elucidate what kind of organella and why they are involved in the formation of LD should lead us to understand the mechanisms of the morphogenesis of LB and some clue to the measures against LB generation.

Most EM studies reported so far are those on LB in the developed phase, while those in the early and developing phases are very limited in number (Cajal et al, 1974; Collins et al, 1968; Oyanagi, 1992; Vanderhaeghen, 1971). In order to investigate the mechanisms of LB morphogenesis, it is necessary to observe LB in the early and developing phases in detail. Therefore, cerebral cortical LB were classified into 3 phases, i.e. early, developing and developed phases, based on the evolution, as has been described above.

Interpretation of the results of the present study

The fine filaments consisting of LB in the early and developing phases measure approximately 6nm in diameter, whereas those of LB in the developed phase

measure about 8nm in diameter. This may be explained that with the lapse of time (ageing of LB) the fine filaments slightly increase in thickness and hardness probably due to an increase in hyperphosphorylation and polymerization of the glycogen. These filaments and the dense granules are known to be digested by amylolytic enzymes (α-amylase and γ-amylase) completely or incompletely, probably depending on the age of LB (Nikaido et al, 1971 Gambetti et al, 1971). This indicates that both the fine filaments and the small dense granules together with their aggregates are of polyglucosans that are abnormal fine filaments made up of insoluble glucose-polymers. The finding that there was a lack of mitochondria in the deep area around the center of LB may imply that the area lacks energy or does not need to use much energy. In addition, intermingling with the filaments and the dense granules there were many small pieces of rER and polysomes in the LB. This may indicate that some protein synthesis is taking place inside LB in the developing phase. Presence of a number of lysosomes and multivesicular bodies may imply that some misfolded proteins and/or insoluble glucose polymers are degraded inside LB in the developing phase.

It may be necessary to consider what factors are involved in the core or ring formation of cored or ringed LB. Most of the LB in the developed phase are cored LB or homogeneous LB (light-pink or dark-pink). EM observations revealed that the parts of LB which consisted of only the filaments stained light-pink with PAS staining. EM of the light-pink homogeneous LB demonstrated that the entire area of the LB consisted only of compactly intermingled filaments innumerably increased in number, associated with a paucity of the small dense granules. In contrast, the dark-pink homogeneous LB were demonstrated that the entire area of the LB consisted of intermingled filaments innumerably increased in number plus substantial amount of small dense granules deposited all over the LB. Generally speaking, the more the dense granules deposited, the darker the homogeneous LB became. It was concluded that if the part of LB stained more intensely, the part had more deposition of the small dense granules or their aggregates.

On the molecular basis for LB generation : mainly on the functions of laforin

The molecular basis for the formation of LB, which has long been believed unknown, is now being elucidated rapidly. Nomally, misfolded and long-lived proteins are targeted by laforin for degradation through the UPS and aggresomes are disposed of via autophagy-lysosome (A-L) pathway. In Lafora cells, however, this will not happen because there is loss of function of the protein laforin or malin due to the gene mutation(s). It is known that the majority of the laforin gene mutations found in LD patients results in lack of phosphatase activity, absence of binding to glycogen and lack of interaction with R5 (Fernandez-Sanchez et al, 2003; Solaz-Fuster, 2008). Normal glycogen is soluble in the cellular environment. Laforin dephosphorylates glycogen and preserves its solubility (Tagliabracci et al, 2007). In tissue cells in LD, however, deficiency of laforin leads to hyperphosphorylation of glycogen and then generation of insoluble glycogen-polymers *in vivo* (Tagliabracci et al, 2007). Mutations of laforin that disable the glycogen binding domain also eliminate its ability to dephosphorylate glycogen (Tagliabracci et al, 2007). Therefore, in cells in LD with laforin or malin gene mutation(s) glycogen is prone to be hyperphosphorylated and become insoluble polymers. Normally such large and insoluble glycogen aggregates are subject to clearance via A-L pathway, whereas in cells in LD the insoluble glycogen aggregates will not be disposed but will remain and accumulate and increase in size to form aggresomes for a long period of time and may become toxic, leading to cell death.

Mainly on the functions of malin

Malin is known to interact with laforin and recruits to aggresomes upon proteasome inhibition and degrades misfolded proteins (Mittal et al, 2007; Rao et al, 2010). So malin interacts with and ubiquitinates laforin, leading to its degradation when laforin is misfolded due to gene mutation(s) (Gentry et al, 2005). Similarly malin and laforin, together with Hsp70 as a functional complex, suppress the cellular toxicity of misfolded proteins and promote their degradation through the UPS (Garyali et al, 2009). Proteasomal dysfunction and cell death frequently occurs in the mutant malin-over-expressed cells (Rao et al, 2010). Malin is unstable, and aggregate-prone protein and co-chaperone CHIP can modulate its stability (Rao et al, 2010) Laforin is a physiologic substrate of malin, an E3-ubiquitin ligase whose activitiy is necessary to prevent neurodegenerative diseases that involve formation of neuroproteinacious

inclusion bodies（Gentry et al, 2005）. Malin-laforin complex is a novel player in the neuronal response to misfolded proteins which could be potential therapeutic targets for neurodegenerative disorders associated with cytotoxic proteins（Garyali et al, 2009）.

On the dysfunction of autophagy and failure of autophagic clearance of aggresomes

The efficient management of misfolded protein aggregates is known to be essential for cell viability and requires 3 interconnected pathways:（1）the molecular chaperone machinery that assists protein folding,（2）the proteasome pathway that degrades misfolded proteins, and（3）the aggresomal pathway that sequesters and delivers toxic protein aggregates to autophagy for clearance（Criado et al, 2011）. As for insoluble glycogen aggregates, the A-L pathway is considered to target them for clearance.

Recently, Criado et al,（2011）demonstrated that the dysfunction of autophagy occurs as a consequence of the lack of laforin-malin complexes in malin-deficient mice. Because the dysfunction of autophagy precedes other pathological manifestations, they propose that decreased autophagy plays a primary role in the formation of LB and it is critical in LD pathogenesis. Decreased autophagy results in the failure of autophagic clearance of aggresomes. Hence LB are a kind of aggresomes formed by hyperproduced polyglucosans and misfolded proteins which are recognized and polyubiquitinated by an E3 ubiquitin-protein ligase such as parkin. Adaptor proteins which may include histone deacetylase 6, ataxin-3, and ubiquilin-1, link the polyubiquitinated proteins to the dynein motor complex for retrograde transport to the aggresome（Olzmann et al, 2008; Aguado et al, 2010）.

Accordingly, we are undertaking the verification of their proposal by means of functional-structural analyses（i.e. functional EM and immunocytochemistry）of the brain tissue and cells in Lafora disease. Individual steps in the aggresome（LB formation）-autophagy pathway of LD may be potentially targeted for therapeutic intervention strategies.

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ラフォラ病の脳病理：形成途上期ラフォラ小体の微細構造変化

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要　　旨

ラフォラ病（LD）は常染色体劣性遺伝性の糖代謝障害による進行性の脳変性症で、病理学的には脳等の広範な実質細胞に出現するポリグルコサン（Lafora小体; LB）を特徴とする。LD患者の約 90%は EPM2A（laforin）または EPM2B（malin）の変異で発症することが最近明らかになった。LBの超微構造変化の発達期別の記載はない。本研究は形態変化の背後にある分子発症機序に関わる所見を捉えることを目的に、既報 1 剖検例の脳組織のLBを発達期別に主に電顕的に観察した。病変形成早期では疎らに、途上期では密に、枝分かれ少ない、不規則な微細線維と小密顆粒が多数出現し、終了期では小密顆粒の集積による芯の形成とその外側にやや放射状に並ぶ細線維が観察された。最近、laforin-malin complex の欠乏がautophagyの障害を引き起こし、それがLB形成に最も重要なを働きをしていることが明らかにされた。laforin-malin complex 異常と autophagy障害による aggresome 除去障害について考察した。結論として、形成途上期LBの微細構造変化はLBのみならずLDの分子機序に連鎖した重要所見とみなせる。

キーワード：発達途上期LB、微細構造変化、ポリグルコサン、ラフォリンとマリン
　　　　　　アグリソームとオートファジー