CORRELATION OF SUBCELLULAR LOCALIZATION OF DISEASE-SPECIFIC INCLUSIONS AND SPHINGOLIPID ACTIVATOR PROTEIN-1 (SAP-1) IN SULFATIDE SULFATASE-DEFICIENT FIBROBLASTS

MASAKO TANIKE¹, KOJI INUI^{1,*}, KOH SHINODA², SHINTARO OKADA¹,
YAHEI SHIOTANI² AND HYAKUJI YABUCHI¹

Department of Pediatrics¹ and Department of Neuroanatomy, Institute of Higher Nervous Activity², Osaka University Medical School, Fukushima-ku, Osaka 553

Received for publication November 18, 1988

Sphingolipid activator protein-1 (SAP-1) stimulates enzymatic hydrolysis of sphingolipids including sulfatide. In this study, subcellular localization of the disease-specific inclusions and SAP-1 in cultured skin fibroblasts from a patient with metachromatic leukodystrophy (MLD), sulfatide storage disease, was examined by immunoelectronmicroscopy. When stained with antibody to SAP-1, acid phosphatase positive membrane-bound vesicles were stained in both the controls and MLD cells, indicating that SAP-1 was localized in lysosomes. After sulfatide loading (20 μ g/ml) for 7 days to MLD cells, 273 lysosomes in a cell were examined regarding the correlation of SAP-1 staining and specific sulfatide inclusions with 6–7 nm periodicity. SAP-1 and specific sulfatide inclusions were simultaneously observed in 59% of total lysosome vesicles examined (164 out of 273) and positive staining of SAP-1 was observed in 92% of lysosomes with specific sulfatide inclusion (164 out of 178). These results demonstrated a positive correlation between SAP-1 staining and specific inclusions and suggested the possible induction of a secondary increase of SAP-1 in lysosomes by the pathological storage of sulfatide.

Sphingolipids are degraded in lysosomes by the sequential hydrolytic actions of acid hydrolases. These reactions require relatively low molecular weight proteins, sphingolipid activator proteins (SAPs), which bind to lipid substrates and activate the reactions by lysosomal hydrolases (5, 11, 13). The studies on one sphingolipid activator protein, SAP-1, have demonstrated that this protein only activates the enzymatic hydrolysis of GM₁ ganglioside by GM₁ β -galactosidase (EC 3.2.1.23), sulfatide by sulfatide sulfatase (EC 3.1.6.8) (18) and globoside by α -galactosidase (EC 3.2.1.22) (12) in spite of its binding affinity to many other sphingolipids, and that its concentration was found to be increased in the tissues from patients with GM₁ gangliosidosis, GM₂ gangliosidosis, Niemann-Pick disease type A and metachromatic leukodystrophy (MLD) (7). Recently Tamuru *et al.* (16) showed intralysosomal localization of SAP-1 in the human liver and colon by the immunocytochemical method. However the exact functional mechanism of SAP-1 and the reason for its increased level in certain lipidoses still remain to be found.

* To whom all correspondences should be addressed.

In the tissues of patients with MLD, sulfatide storage disease due to the defect of sulfatide sulfatase (10) or SAP-1 (8), metachromatic deposits stained with acidified cresyl fast violet were observed under light microscopy (17), and specific "heringbone" and "tuffstone" inclusions were observed under electron microscopy (10). Cultured skin fibroblasts from MLD patients, grown under normal conditions, do not show such inclusions. But metachromatic cytoplasmic granules could be observed in MLD fibroblasts by the addition of sulfatide to the culture medium and these granules are identical to those found in MLD brain (15). In consideration of this phenomenon, we studied the subcellular localization of SAP-1 and searched for the reason for its increase in relation to the intralysosomal sulfatide storage in cultured skin fibroblasts from a patient with MLD.

MATERIALS AND METHODS

Cell Culture

The diagnosis of MLD was based on clinical observations and deficiency of arylsulfatase A activity. Cultured skin fibroblasts were grown in this laboratory from a skin biopsy of a late infantile MLD patient with informed consent, and maintained as described previously (9). For microscopic study, skin fibroblasts were grown on Teflon-sprayed coverglass as described by Chang (1). In the sulfatide loading experiment cells were grown in a medium containing 20 μ g/ml of sulfatide (15).

Purification of SAP-1 and Preparation of Antibody to SAP-1

SAP-1 was purified from human liver and the anti-SAP-1 antibody was prepared in rabbit as described by Inui and Wenger (8). The specificity was checked by crossed immunoelectrophoresis and the monospecificity was confirmed by the absence of the cross-reacting material in fibroblasts from a patient with SAP-1 deficient type MLD. The quantifications of SAP-1 in skin fibroblasts before and after sulfatide loading were carried out by the method of Inui and Wenger (8).

Light Microscopic Study of Metachromasia

The cultured skin fibroblasts grown on the coverglass with or without sulfatide (20 μ g/ml) for 7 days were rinsed in cold saline, immersed into 10% solution of formaldehyde for 30 min and stained for 30 min with 0.1% of acidified cresyl fast violet (Merck, F. R. Germany). After rinsing with distilled water, they were mounted in glycerin and metachromatic deposits were examined with a light microscopy.

Light and Electron Microscopic Studies

The techniques we used for growing cells on coverglass and subsequent epoxy embedding basically followed the method described by Chang (1). In some experiments, cells on coverglass were stained to demonstrate acid phosphatase activity by a modified medium of Novikoff (14). All procedures were carried out at 4°C except when otherwise noted.

Cells grown on coverglass with or without sulfatide for 7 days were rinsed in saline and fixed in 1% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. Fixed cells were washed 3 times in phosphate buffered saline (PBS) and treated with 20% normal goat serum (NGS) in PBS for 3 hr. After washing 3 times with 1% NGS in PBS for 30 min, they were incubated overnight with rabbit anti-SAP-1 serum (1 : 100 dilution in PBS containing 1% NGS). Then they

were rinsed with 3 changes of 1% NGS in PBS and incubated overnight with goat antirabbit IgG serum (Miles Laboratories Inc., USA, diluted 1 : 100 in 1% NGS in PBS). Again after rinsing in 3 changes of PBS, they were treated with rabbit peroxidase-antiperoxidase complex (DAKO Corp., U.S.A.; diluted 1 : 50 in PBS) for 3 hr at room temperature. After washing 2 times with 0.05 M Tris-HCl buffer (pH 7.6) for 30 min, they were preincubated in a 0.05% solution of diaminobenzidine tetrahydrochloride (DAB; Dotite, Japan) in 0.05 M Tris-HCl buffer (pH 7.6) for 1 hr, and then immersed for 6 min in the same buffer containing 0.05% DAB and 0.01% H_2O_2 . After rinsing with 0.1 M phosphate buffer (pH 7.4) and postfixation in 1% osmium tetroxide in the same buffer for 1 hr, the cells on the coverglasses were dehydrated in graded ethanols and propylene oxide and embedded in epoxy resin (Luveac 812, Nakarai Inc., Japan). By immersion in liquid nitrogen, the epoxy blocks were removed from the coverglasses and the thin embedded specimens were examined and photographed under a Nikon universal microscope. The DAB-positive cells were ultrathin-sectioned, stained with uranyl acetate and lead citrate, and examined under a JEM 100CX transmission electron microscope (JEOL, Japan) at 80 kV.

RESULTS

Light Microscopic Observations

When MLD skin fibroblasts were stained with acidified cresyl violet, no metachromasia was seen under the regular culture conditions. However the addition of sulfatide to the culture medium (20 μ g/ml) for 7 days induced metachromatic granules within the cytoplasm of nearly all MLD fibroblasts.

When stained with antibody to SAP-1, brown granules of DAB were positively observed in the cells without sulfatide loading, but the number of granules clearly increased after the loading (Figs. 1a, b). The specificity of staining was confirmed by negative staining by preincubation of anti-SAP-1 serum with purified SAP-1.

Electron Microscopic Observations

When control and MLD cells were cultured under normal conditions, non-specific granular inclusions within the membrane-bound vesicles diameter (0.2–0.4 μ m) were occasionally found. Nearly all of these vesicles had positive acid phosphatase staining, and some of them looked like so-called multivesicular bodies, indicating that these vesicles were lysosomes. After loading sulfatide into MLD cells for 7 days, membrane-bound vesicles had increased in number and size (diameter 0.8–1.0 μ m) and contained various kinds of inclusions, mainly multilayered structures with an average periodicity of 6–7 nm with a “tuffstone” appearance described in MLD.

When stained with the antibody to SAP-1, DAB products were found within the membrane-bound vesicles (diameter 0.2–0.4 μ m) in both the control and MLD cells, indicating that SAP-1 was localized in lysosomes (Fig. 2). After loading sulfatide into MLD cells, immunocytochemically SAP-1 positive lysosomes were increased in number and size (diameter 0.8–1.0 μ m). 273 lysosomes in a cell were examined upon SAP-1 staining and lamellar inclusions of 6–7 nm periodicity were observed which indicated the storage of sulfatide. SAP-1 staining was classified by the staining intensity. The degree of specific sulfatide inclusions in a lysosome was classified into three

FIG. 1a.

FIG. 1b.

FIGS. 1a, b. Light micrograph of immunostaining of SAP-1 of cultured skin fibroblasts from a patient with MLD. MLD skin fibroblasts were grown on coverglasses in the absence (1-a) and presence (1-b) of sulfatide (20 μ g/ml) for 7 days and stained with antibodies to SAP-1 as described in Methods. SAP-1 staining became stronger after sulfatide loading. $\times 90$

TABLE 1. *Correlation of DAB staining of SAP-1 and specific inclusions in MLD cultured skin fibroblasts loaded with sulfatide*

		Intensity of DAB Staining			Total
		—	+	++	
Specific Inclusion	negative	28 (10%)	10 (4%)	4 (2%)	42 (16%)
	probable	18 (7%)	26 (10%)	9 (3%)	53 (20%)
	positive	14 (5%)	47 (16%)	117 (43%)	178 (64%)
	Total	60 (22%)	83 (30%)	130 (48%)	273 (100%)

FIG. 2. Immunoelectron micrograph of SAP-1 staining of cultured skin fibroblasts from a patient with MLD before sulfatide loading. Electron-dense reaction products indicate the intracellular localization of SAP-1. SAP-1 was shown to be localized in lysosomes, some of which contained granular inclusions. $\times 10,000$ Inset: higher magnification. $\times 25,000$

grades: positive; lamellar inclusions of precise 6–7 nm periodicity were clearly observed, probable; positive lamellar inclusions lacking clear periodicity, negative; other inclusion pattern or no inclusion (Figs. 3a, b, c). The results of DAB reactions and specific sulfatide inclusions of 273 lysosomes are summarized in Table 1. SAP-1 (DAB stain) was positive in 78% of the total, and specific sulfatide inclusions appeared in 64% of total lysosomes. SAP-1 and specific inclusions appeared simultaneously in 59% of total lysosome vesicles examined, whereas about 10% of the total were both negative. Moreover, SAP-1 positive lysosomes were found in 92% of the specific sulfatide inclusion (lamellar inclusions with 6–7 nm periodicity) positive lysosomes.

Quantification of SAP-1 by Immunoelectrophoresis

With purified activator protein as the standard, the amount of SAP-1 was

FIG. 3a.

FIGS. 3a-c. Immunoelectron micrograph of SAP-1 staining of cultured skin fibroblasts from a patient with MLD after 7 days of sulfatide loading (20 $\mu\text{g/ml}$). (a) almost all lysosomes were large and DAB positive. ($\times 3,000$); (b) higher magnification of (a): lysosome *1 was classified as SAP-1 (++) and positive specific sulfatide inclusion; *2 classified as SAP-1 (+) and positive specific sulfatide inclusion; *3 classified as negative SAP-1 and probable specific inclusion. ($\times 42,000$); (c) typical "Tuffstone" inclusion in a SAP-1 positive lysosome. $\times 57,000$

estimated to be 1.2 μg cross reacting material (CRM)/mg of protein from MLD cell extracts. This value is compatible to that in the control cells and the values reported previously (6). After the sulfatide loading, about a 30% increase of CRM was recognized in MLD cells.

DISCUSSION

Since the initial observation by Mehl and Jatzkewitz (1964) that a low molecular weight protein was required for *in vitro* degradation of sulfatide by arylsulfatase A, several so-called "sphingolipid activator proteins" have been isolated (5, 11, 13). The roles of the SAP have been disclosed through the studies of SAP deficient-types of GM₂ gangliosidosis (2) and MLD (10). However the mechanism of specific interaction between SAP, enzyme molecules and lipid substrates, and the mechanism of its action, its molecular structure and its biosynthesis are not well known. Recent

FIG. 3b.

FIG. 3c.

studies using the antibody to SAP-1 showed that the concentration of SAP-1 increased in the tissues of several sphingolipidoses, especially GM₁ gangliosidosis, GM₂ gangliosidosis, Niemann-Pick disease type A, and MLD (7). Clarification of this phenomenon is an important step to understanding the pathogenesis of these lysosomal storage diseases. In this study we used cultured skin fibroblasts from a patient with MLD, because it is possible to induce intralysosomal storage by sulfatide loading.

The lysosomal localization of SAP-1 in cultured skin fibroblasts has been suggested by biochemical studies (2, 6, 18). Tamaru *et al.* (16) recently reported the lysosomal localization of SAP-1 in the human liver and colon by the immunohistochemical method. We observed the lysosomal localization in cultured skin fibroblasts (Fig. 2), and in neuronal cells in the human brain (unpublished data). Thus, it is now evident that SAP-1 is present in different cell types (skin fibroblasts, hepatocytes, phagocytic cells of the liver, phagocytic and/or immunoglobulin-producing cells in the colon and neuronal cells in human brain).

To examine the correlation between lysosomal lipid storage and increased amount of SAP-1, we compared specific sulfatide inclusions with the intensity of DAB staining of SAP-1 in skin fibroblasts. By sulfatide loading, SAP-1 staining became stronger as shown in Fig. 1. This immunocytochemical observation was confirmed by rocket immunophoresis, showing a 30% increase in SAP-1 concentration in MLD fibroblast homogenates after sulfatide loading as reported in MLD tissues (7). The intralysosomal presence of characteristic lamellar structure with 6–7 nm periodicity, especially of “herringbone” or “tuffstone” appearance indicated typical sulfatide storage (15). In 92% (164/178) of these specific inclusion positive lysosomes, SAP-1 was positively detected after sulfatide loading in MLD cells (Table 1). This result suggests strong correlation of SAP-1 to lysosomal storage of sulfatide. In an acidic condition SAP-1 binds many sphingolipids including gangliosides and sulfatide (18), and the concentration of SAP-1 was significantly increased in liver and brain from patients with gangliosidoses, Niemann-Pick disease and MLD (7). From these lines of evidence, it is possible to speculate that the intralysosomal storage of sulfatide induces a compensatory increase of SAP-1 in lysosomal vesicles in order to dissolve the stored lipids and it suggests that SAP-1 has important functions in metabolizing sphingolipids in lysosomes.

A gene for SAP-1 has recently been cloned (3) and this will enable us to examine the molecular structure of SAP-1 and mRNA level in tissues by *in situ* hybridization.

ACKNOWLEDGMENTS

The authors are very grateful to Prof. Masaya Tohyama and Dr. Sadao Shiosaka for their kind advice. This study was supported partly by grants from the Ministry of Education, Science and Culture (Grants-in-aid for Scientific Research, for Special Project Research and for Scientific Research on Priority Areas) and the Ministry of Health and Welfare (Grants 62A-11 from NCNP) in Japan.

REFERENCES

1. Chang, J. P.: A new technique for separation of coverglass substrate from epoxy-embedded specimens for electron microscopy. *J. Ultrastructure Res.* 37; 370-377, 1971.
2. Conzelmann, E., Sandhoff, K.: AB variant of infantile GM₂ gangliosidosis. Deficiency of a factor necessary for stimulation of hexosaminidase A-catalyzed degradation of ganglioside GM₂ and glycolipid GA₂. *Proc. Natl. Acad. Sci. USA* 75; 3979-3983, 1978.
3. Dewji, N., Wenger, D. A., Fujibayashi, S., Donoviel, M., Esch, F., Hill, F. and O'Brien, J. S.: Molecular cloning of the sphingolipid activator protein-1 (SAP-1), the sulfatide sulfatase activator. *Biochem. Biophys. Res. Commun.* 134; 989-994, 1986.
4. Fujibayashi, S. and Wenger, D. A.: Biosynthesis of the sulfatide GM₁ activator protein-1 (SAP-1) in control and mutant cultured skin fibroblasts. *Biochim. Biophys. Acta.* 875; 554-562, 1986.
5. Ho, M. W. and O'Brien, J. S.: Gaucher's disease: deficiency of "acid" β -glucosidase and reconstitution of enzyme activity *in vitro*. *Proc. Natl. Acad. Sci. USA* 68; 2810-2813, 1971.
6. Inui, K. and Wenger, D. A.: Biochemical, immunological and structural studies on a sphingolipid activator protein (SAP-1). *Arch. Biochem. Biophys.* 233; 556-564, 1983.
7. Inui, K. and Wenger, D. A.: Concentration of the activator protein for sphingolipid hydrolysis in liver and brain samples from patients with lysosomal storage diseases. *J. Clin. Invest.* 72; 1622-1628, 1983.
8. Inui, K., Emmett, M. and Wenger, D. A.: Immunological evidence for a deficiency in an activator protein for sulfatide sulfatase in a variant form of metachromatic leukodystrophy. *Proc. Natl. Acad. Sci. USA* 80; 3074-3077, 1983.
9. Kato, T., Inui, K., Yutaka, T., Okada, S., Yabuuchi, H., Chiyo, H. and Furuyama, J.: Histochemical demonstration of acid β -galactosidase activity in cultured skin fibroblasts and its application to single cell analyses. *Acta histochem. cytochem.* 14; 343-349, 1981.
10. Kolodny, E. H. and Moser, H. W.: Sulfatide lipidosis: metachromatic leukodystrophy. In *The Metabolic Basis of Inherited Diseases*, ed. by J. B. Stanbury, J. B. Wyngaarden, D. S. Fredricksen, J. L. Goldstein and N. S. Brown, McGraw-Hill, New York, 1983, p. 881.
11. Li, S. C. and Li, Y. T.: An activator stimulating the enzymic hydrolysis of sphingolipid. *J. Biol. Chem.* 251; 1159-1163, 1976.
12. Li, S. C., Kihara, H., Serizawa, S., Li, Y. T., Fluharty, A. L., Mayers, J. S. and Shapiro, L. J.: Activator protein required for the enzymatic hydrolysis of cerebroside sulfatase. *J. Biol. Chem.* 260; 1867-1871, 1985.
13. Mehl, E. and Jatzkewitz, H.: Eine Cerebrosidsulfatase aus Schweineniere. Hoppe-Seyler's Z. *Physiol. Chem.* 339; 260-276, 1964.
14. Novikoff, A. B.: Lysosomes in the physiology and pathology of cells: contribution of staining methods. In *Ciba Foundation Symposium on Lysosomes*, ed. by A. V. S. de Reuck and M. P. Cameron, Little Brown, Boston, 1963, p. 36.
15. Rutsaert, J., Menu, R. and Resibois, A.: Ultrastructure of sulfatide storage in normal and sulfatide-deficient fibroblasts *in vitro*. *Lab. Invest.* 29; 527-535, 1973.
16. Tamaru, T., Fujibayashi, S., Brown, W. R. and Wenger, D. A.: Immunocytochemical localization of sphingolipid activator protein-1, the sulfatide/GM₁ ganglioside activator, to lysosomes in human liver and colon. *Histochem.* 86; 195-200, 1985.
17. Von Hirsch, T. and Pfeiffer, J.: Über histologische Methoden in der Differentialdiagnose von Leukodystrophien und Lipidosen. *Arch. Psychiat. Nervenkr.* 194; 88-104, 1955.
18. Wenger, D. A. and Inui, K.: Studies on the sphingolipid activator protein for the enzymic hydrolysis of GM₁ ganglioside and sulfatide. In *The Molecular Basis of Lysosomal Storage Disorders*, ed. by R. O. Brady, J. A. Barranger, Academic Press, New York, 1984, p. 61.