Primary and Secondary Elastin-Binding Protein Defect Leads to Impaired Elastogenesis in Fibroblasts from G_{M1}-Gangliosidosis Patients

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G_{M1}-gangliosidosis is a lysosomal storage disorder caused by acid β -galactosidase deficiency. Aside from the lysosomal β -galactosidase enzyme, the β -galactosidase gene also encodes the elastin-binding protein (EBP), deficiency in which impairs elastogenesis. Using expression studies and Western blots of COS-1 cells, we identified and characterized four new and two known β -galactosidase gene mutations detected in G_{M1}-gangliosidosis patients with infantile, juvenile, or adult forms of disease. We then focused on impaired elastogenesis detected in fibroblasts from patients with infantile and juvenile disease. The juvenile patient showed connective-tissue abnormalities, unusual urinary keratan sulfate excretion, and an EBP reduction, despite mutations affecting only β -galactosidase. Because galactosugar-bearing moieties may alter EBP function and impair elastogenesis, we assessed infantile and juvenile patients for the source of altered elastogenesis. We confirmed that the infantile patient's impaired elastogenesis arose from a primary EBP defect, according to molecular analysis. We examined the juvenile's fibroblasts by immunohistochemistry, addition of keratanase, soluble/insoluble elastin assay, and radiolabeling of tropoelastin. These experiments revealed that the juvenile's impaired elastogenesis likely arose from secondary EBP deficiency caused by keratan sulfate accumulation. Thus, impaired elastogenesis in G_{M1}-gangliosidosis can arise from primary or secondary EBP defects in fibroblasts from infantile and juvenile patients, respectively. (*Am J Pathol* 2005, 167:1689–1698)

A deficiency of the human acid β -galactosidase (GLB1) results in the lysosomal storage disorders G_{M1}-gangliosidosis and Morquio type B disease (MIM 230500). The catalytic activity of GLB1 toward G_{M1}-ganglioside, glycosaminoglycans, and glycopeptides is reduced or absent in G_{M1}-gangliosidosis patients whereas keratan sulfate is the major storage product in Morquio type B and type A diseases.¹

The *GLB1* gene gives rise to two alternatively spliced mRNAs that encode the lysosomal GLB1 enzyme and the elastin-binding protein (EBP).^{2–4} The GLB1 precursor (70 kDa) is proteolytically processed in lysosomes into the 64-kDa mature form.^{5–7} The active enzyme can be present in a high molecular weight lysosomal complex with protective protein/cathepsin A [PPCA (MIM 256540)], neuraminidase [NEU1 (MIM 608272)], and *N*-acetyl galactosamine-6-sulfate sulfatase [GALNS (MIM 25300)].⁸ The alternative spliced EBP is involved in elastic fiber assembly on the cell surface, where it is postulated to be present in complex with PPCA and NEU1.⁸ EBP acts as a molecular chaperone, protecting tropoelastin from premature degradation and facilitating its assembly into a microfibrillar scaffold that consists of several glycoproteins such as fibrillins and microfibril-associated glyco-proteins (MAGP).^{9–11} Covalently cross-linked polypeptide chains of soluble tropoelastin are assembled along the microfibrils, providing the extracellular elastic fibers of the tissues.¹⁰

Impaired elastogenesis has been previously associated with primary EBP deficiency in patients with either G_{M1}-gangliosidosis or Morquio type B disease.⁹ EBP contains a galactose domain that binds microfibrillar acceptors in the glycosylated microfibrillar scaffold of elastic fibers.^{9–11} This EBP domain may also interact with galactosugar-bearing moieties, such as chondroitin sulfate and dermatan

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Table 1. Clinical Features of G_{M1} -Gangliosidosis Patients in This Study

Patient	Infantile with cardiac involvement	Juvenile	Adult
Onset	4 months	3 years	5 years
Facial dysmorphisms	Course facies	Course facies, hypomimia	Absent
Connective/bone tissues alterations	Dysostosis multiplex	At 3 years: inguinal hernia, thoracolumbar scoliosis, L2 deformity; at 6 years: vertebral ovoid deformities with increased anterior-posterior diameter, L1 to L2 and L2 to L3 reduced spaces, L2 thinning and beaking; at 8 years: lumbar gibbous, platyspondylia of cervical and lumbar vertebra	Short stature
Muscle	Marked axial hypotonia with lower limb hypertonia, hyperactive deep tendon reflex, nystagmus	Hypertonia, muscle weakness, spastic tetraplegia, hyperactive deep tendon reflex, bilateral clubfoot	Ataxia, speech deterioration bilateral clubfoot, corneal opacity
Brain magnetic resonance imaging	Cerebral and cerebellar atrophy	Cerebral and cerebellar atrophy and enlarged ventricular system	Mild hyperintensity in the posterior periventricular white matter and mild alteration in the lenticular nuclei
Psychomotor development	Psychomotor delay	Psychomotor delay	Normal intelligence (Q.I. 99)
Urinary oligosaccharides	Abnormal	Abnormal	Normal
Urinary glycosaminoglycans	Normal	Elevated keratan sulfate excretion	Normal
Course	Death at 24 months	Alive at 18 years	Alive at 27 years

sulfate, that are pericellularly accumulated in Costello syndrome and Hurler disease. In those cases, EBP undergoes a premature shedding from the cell surface and tropoelastin is dissociated far away from its proper microfibrillar template.⁹⁻¹¹ Thus, it has been previously demonstrated that impaired elastogenesis in Costello syndrome and Hurler disease is related to a secondary EBP deficiency caused by chondroitin sulfate and dermatan sulfate accumulation, respectively.^{10,11} These data have been supported by transduction of skin fibroblasts from Costello syndrome with versican V3 (which lacks chondroitin sulfate). This retrovirally mediated overexpression completely restores normal elastogenesis, suggesting the rescue of EBP to be responsible of the phenotypic reversal.¹²

Here we report the biochemical and molecular characterization of four new and two known mutations detected in the *GLB1* gene of three patients with infantile, juvenile, or adult form of G_{M1} -gangliosidosis phenotypes. This study also focuses on impaired elastogenesis in those patients. In particular, we show that impaired elastogenesis arises from primary and secondary EBP defects, respectively, in the infantile and juvenile patients' fibroblasts here reported.

Materials and Methods

Case Reports

Patients' clinical presentations are summarized in Table 1.

RNA Isolation and cDNA Analysis

All reagents for mRNA extraction from patients' fibroblasts and lymphocytes were purchased from Eppendorf AG (Hamburg, Germany). RNA integrity was verified by 1% agarose gel electrophoresis. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *GLB1* cDNA was performed by a set of six overlapping amplifications encompassing the entire coding region of the mRNA as previously reported.¹³ The RT-PCR products were checked on a 1.5% agarose gel, excised, and purified using Nucleospin Extract kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using the ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. The nomenclature of *GLB1* genetic lesions is as designed previously.¹⁴ Nucleotide numbering starts at the ATG translation initiation codon.

Analysis of Genomic DNA

The *GLB1* mutations were confirmed in DNA samples of patients and their relatives. The oligonucleotides and the amplifying conditions have been described previously.^{13,15} The PCR products were directly sequenced as described above.

Multiplex Fluorescent RT-PCR of *GLB1* mRNA

Multiplex RT-PCR and quantitation of fluorescent PCR products of insulin receptor and *GLB1* total mRNA were

performed as previously described.¹⁶ Primer sets for cDNA synthesis and amplification of human mRNA corresponding to insulin receptor (INSR) were designed as previously reported,¹⁶ whereas the *GLB1* primers used were the following: 1035 cDNA 5'-CACAGTGAGGTC-CCCAGCCTCACTCA-3' (1035/1009 nucleotides exon 10); 378 forward 5'-CTGTGCAGAGTGGGAAATGGG-AGGA 3'-(378/402 nucleotides exon 3/4); and 565 reverse 5'-ATTCATTTTCAACCTGCACTGTTAT-3' (565/541 nucleotides exon 6/5). Fluorescent RT-PCR products were amplified and loaded on an ABI Prism 310 genetic analyzer (Applied Biosystems) as previously described.¹⁶

RT-PCR of *EBP*

EBP cDNA synthesis was performed by a commercial kit purchased from Eppendorf AG. Primer sets for cDNA synthesis and amplification of human mRNA corresponding to INSR were designed as previously reported,¹⁶ whereas the *EBP* primers used were the following: cDNA 5'-CACAGTGAGGTCCCCAGCCTCACTCA-3' (644/618 nucleotides exon 10); 185 *EBP* for 5'-GCTTCTACTG-GAAGGACCGGC-3' (185/205 nucleotides exon 2); and 744 *EBP* Rev 5'-GATGTTGCTGCCTGCACTGTTA-3' (353/342 nucleotides exon 5/7). The amplifying conditions have been previously described.¹⁶

Expression Studies

Site-directed mutagenesis and fragment replacement were used to introduce the genetic lesions c161G>A (S54N), c175C>T (R59C), c602G>A (R201H), c689G>A (C230Y), c985A>G (T329A), and c1325G>A (R442Q), detected in the patients, in transient *GLB1* expression vectors as described previously.¹⁵ The integrity of the DNA and the exclusive presence of the expected mutations in the *GLB1* cDNA inserts, were verified by sequencing on both strands. The oligonucleotide primers for site-directed mutagenesis have been previously reported¹⁵ except for the following: S54N forward 5'-CATCTCAGGAAACATTCCTACTAC-3' (150/171 nucleotides exon 2); S54N reverse 5'-GTAGTGAAT-GTTTCCTGAGATG-3' (171/150 nucleotides exon 2); R59C forward 5'-CACTACTCC7GTGTGCCCC-3' (166/184 nucleotides exon 2); R59C reverse 5'-GGGGCACACAGGAG-TAGTG-3' (184/166 nucleotides exon 2); R201H forward 5'-GACTACCTGCACTTCTCTGC-3' (592/610 nucleotides exon 6); R201H reverse 5'-GCAGGAAGTGCAGG-TAGTC-3' (610/592 nucleotides exon 6); C230Y forward 5'-CATTCCTGAAATATGGGGCCC-3' (677/697 nucleotides exon 6); C230Y reverse 5'-GGGCCCCATATTCAG-GAATG-3' (697/677 nucleotides exon 6); T329A forward 5'-GCACAGCCCGCCAGCTACG-3' (976/994 nucleotides exon 10); T329A reverse 5'-CGTAGCTGGCGGGCT-GTGC-3' (976/994 nucleotides exon 10); R442Q forward 5'-CCACGATCAAGCATATGTTG-3' (1317/1336 nucleotides exon 13); and R442Q reverse 5'-CAACATATGCTT-GATCGTGG-3' (336/1317 nucleotides exon 13). The italicized bases correspond to a mispairing with the normal sequence. The mutated transient expression vectors were

used to transform the *Escherichia coli* strain Solo pack gold cells (Stratagene, Amsterdam Zuidoost, The Netherlands), obtaining a great quantity of each recombinant vector.

Transfection into COS-1 Cells

Normal and mutant vectors were transiently overexpressed into African green monkey kidney cells (COS-1) as described previously.¹⁵ In each experiment, the value of *GLB1* activity in nontransfected COS-1 cells was used to set up the *GLB1* intrinsic activity of the expression system.

Cell Cultures and Biochemical Enzymatic Assay

COS-1 cells and patients' fibroblasts were cultured in Dulbecco's modified Eagles-Hams F10 medium (1:1, v/v) with fetal bovine serum (10%) and antibiotics. The protein content of transfected COS-1 cells and of patients' fibroblasts was determined in triplicate by the method of Lowry and colleagues.¹⁷ *GLB1* enzyme assay in fibroblasts and transfected cells was performed in triplicate in three independent experiments by fluorescence measurement using 4-methylumbelliferyl β -galactopyranoside artificial substrate.¹⁸

Western Blots

Fibroblasts from patients and normal control and transfected COS-1 cells were harvested by scraping in phosphate-buffered saline and sonicated as described previously.¹⁹ Approximately 20 μ g of total COS-1 cells were used in the blots. Western blots were prepared from 12.5% polyacrylamide gels and probed as described.²⁰ After electrophoresis, proteins were transferred to nitrocellulose (Bio-Rad, Hercules, CA) and immunostaining was performed with the following antibodies: α 85, anti-*GLB1* antibody, previously characterized;^{7,15} Alf1, anti-*EBP* antibody [the identification of *EBP* antigenic and hydrophilic peptides was used to obtain the following *EBP* epitope: NH₂-VGSPSAQDEASPLS-COOH (91 to 104 amino acids) that was also previously used.⁹ This anti-*EBP* peptide, conjugated with bovine serum albumin and immunized into rabbits by Igtech (Perdifumo, Italy), has been previously reported in a study on a galactosialidosis patient, who showed a reduced *EBP* amount and in a study on G_{M1}-gangliosidosis.^{21,22}]; polyclonal human anti-tropoelastin/elastin (Elastin Products Company, Owensville, MO); anti-actin antibody (Sigma, Milan, Italy), used to assay total protein content loaded in the blots. All of the blotted proteins were visualized by reaction with the secondary antibody anti-rabbit IgG (whole molecule) alkaline-phosphatase conjugate (Sigma), using the AP conjugate substrate kit (Bio-Rad).

Restriction-Site Analysis

The *GLB1* gene of 100 normal control DNA samples was analyzed using the *PvuI* enzyme (Roche, Mannheim, Ger-

Table 2. Biochemical Characterization, Mutation Analysis, and Expression Studies on COS-1 Cells

Patient's phenotype	GLB1 nucleotide change	GLB1 amino acid change	Exon	GLB1 activity in patients' leukocytes (% $\pm$ c.r.)*	GLB1 activity in COS-1 cells (% $\pm$ c.r.)*
Infantile	c161G>A	S54N	2	0.1 $\pm$ 0.2	0.1 $\pm$ 0.4
	c175C>T	R59C	2		0 $\pm$ 0.2
Juvenile	c602G>A	R201H	6	2.5 $\pm$ 0.8	33.5 $\pm$ 3.6
	c689G>A	C230Y	6		0.1 $\pm$ 0.5
Adult	c985A>G	T329A	10	4.1 $\pm$ 0.8	1 $\pm$ 0.4
	c1325G>A	R442Q	13		5.7 $\pm$ 1

*Percentages refer to GLB1 activity measured in normal control fibroblasts or COS-1 cells transfected with wild-type *GLB1* vectors. The values are the average of three independent experiments. c.r., confidential range.

many) to screen the R442Q mutation. The PCR fragments were amplified by the genomic primers reported earlier.¹⁵ A 10 μ l aliquot of PCR product was incubated for 1 hour as recommended by the manufacturer in a reaction mixture containing 2 μ l of 10 $\times$ reaction buffer and 1 U of the restriction enzyme. The total volume of the sample was brought up to 20 μ l.

Immunostaining

Parallel 10-day-old cultures of normal and patients' fibroblasts were immunostained as previously reported.⁴ All fibroblast cultures were confluent and derive from early passage cells. Normal control fibroblasts were cultured with or without keratan sulfate (1 μ g/ml). The amount of keratan sulfate added to the medium was based on pathological concentrations detected in the blood of patients affected by Morquio A disease.²³ Juvenile patient's fibroblasts were cultured with or without keratanase (0.1 U/ml/day) as described.¹⁰ Normal control fibroblasts were also cultured with or without keratan sulfate (1 μ g/ml) and keratanase (0.1 U/ml/day). Polyclonal human anti-tropoelastin/elastin (see above), and anti-fibronectin (Sigma) antibodies, developed in rabbits, were used as primary antibodies as previously described.⁴ For immunohistochemical assessment of microfibrillar scaffold, normal and juvenile patients' fibroblasts were incubated with polyclonal primary antibody to fibrillin-1 (Chemicon, Temecula, CA) developed in rabbits, as previously described.⁴ The only difference with respect to the published method⁴ concerns the experiment with the fibrillin antibody in which cell lines were directly fixed in methanol 100% instead of paraformaldehyde.

Fibroblast cultures (48 hours old) from juvenile patient and normal control were incubated with a human monoclonal keratan sulfate antibody (Seikagaku Corp., Tokyo, Japan), developed in mouse. Before adding the primary antibody, fibroblasts were permeabilized as described.¹⁰ Subconfluent 48-hour cultures of normal fibroblasts and juvenile patient's fibroblasts were also incubated with Alf1,^{21,22} anti-EBP antibody. All direct immunofluorescence studies were then performed by secondary fluorescein isothiocyanate-conjugated antibodies purchased from Sigma. To stain nucleic acids, fibroblasts were also incubated with propidium iodide (10 μ g/ml), as described previously.^{21,22}

Quantitative Estimate of Keratan Sulfate

Fibroblasts cultures (48 hours and 10 days old) from juvenile patient and normal control were assessed for a chemical quantitative estimate of keratan sulfate by using a keratan sulfate enzyme-linked immunosorbent assay kit (Seikagaku Corp.). Values on normal and juvenile patient's fibroblasts were normalized by starting from the same amount of protein contents (30 μ g).

Insoluble Elastin Assay

To quantify the elastic fiber content in fibroblasts of the juvenile patient, insoluble elastin was measured in confluent fibroblasts obtained from the patient and normal controls as previously described.⁹ The only difference with respect to the published article⁹ concerns the metabolic radiolabeling with [³H]-leucine instead of [³H]-valine. The experiments were performed in triplicate and the values are the average of three independent experiments.

Statistical Analysis

GLB1 enzyme activities, measured on patients' leukocytes and fibroblasts and transfected COS-1 cells, are presented as percentages (Table 2) coupled with SDs performed in Microsoft Excel 97 SR-2. *P* values less than 0.05 were considered statistically significant to generate the confidence limits (*n* = 3, *t* = 3.18). SDs of keratan sulfate estimate and insoluble elastin assays were also performed by Microsoft Excel 97 SR-2.

Results

Clinical Presentation

The phenotypes of the three G_{M1}-gangliosidosis patients here reported have been summarized in Table 1. Connective/bone tissue alterations can be noticed in patients with both the infantile and juvenile form of G_{M1}-gangliosidosis. The infantile patient showed cardiac involvement and dysostosis multiplex. Moreover, based on the connective/bone tissue alterations, and on abnormal uri-

Figure 1. RT-PCR of *EBP* and *INSR* mRNA. The PCR fragments amplified with specific primers for *EBP* and *INSR* mRNA are 165 bp and 133 bp, respectively. PCR analysis was performed on mRNA from infantile patient's fibroblasts (I), juvenile patient's fibroblasts (J), adult patient's fibroblasts (A), and normal control fibroblasts (N).

nary keratan sulfate excretion, the juvenile patient was first diagnosed as suffering from Morquio type B disease.

Biochemical and Genetic Analysis

The diagnosis of G_{M1}-gangliosidosis was confirmed by the absence or reduction of GLB1 enzyme activity in patients' leukocytes and fibroblasts (Table 2) coupled with normal NEU1 activity. Quantitative analysis of RNA by multiplex fluorescent RT-PCR showed similar levels of GLB1 total mRNA in all control and patient samples. Normal amounts of EBP mRNA were present in all patients, compared with normal controls (Figure 1). Thus, synthesis of mRNA corresponding to *GLB1* and to *EBP* was unaltered in all patients reported here. The full-length *GLB1* cDNA of the patients and the exon/intron boundaries of the corresponding *GLB1* genes were amplified and directly sequenced on both strands. Four new and two known missense mutations were identified in the patients' samples (Table 2, Figure 2).

The known R59C²² and the new S54N amino acid substitutions were detected in the patient affected by the infantile form (Figure 2A). The juvenile patient was found to be compound heterozygous for the novel C230Y and the known R201H²⁴ amino acid changes; both mutations affecting only the GLB1 protein and not the EBP (Figure 2B). The adult patient was found to be compound heterozygous for the new T329A and R442Q mutations (Figure 2C), mapping to the central region of the protein. The mutations detected in the patients' cDNA were confirmed in the patients' and parents' genomic DNA. A possible benign polymorphism for each new point mutation was excluded by transient expression studies and/or restriction site analysis (Table 2, Figure 3).

Expression Studies

Expression vectors with mutated *GLB1* cDNA containing the individual point mutations were created by *in vitro* site-directed mutagenesis and fragment replacement. The pcD-*GLB1*-mutated vectors were transiently expressed in COS-1 cells. As predicted from the clinical phenotype, no residual GLB1 activity was detected for the S54N and R59C amino acid substitutions present in the infantile patient (Table 2). Absence of GLB1 activity was also observed in COS-1 cells expressing the GLB1

Figure 2. Partial nucleotide sequences. **A:** Infantile patient's *GLB1* exon 2 showing the c161G>A (S54N) and c175C>T (R59C) new genetic lesions. **B:** Juvenile patient's *GLB1* exon 6 showing the new c689G>A (C230Y) genetic lesion. **C:** Adult patient's *GLB1* exons 10 and 13 showing, respectively, the c985A>G (T329A) and c1325G>A (R442Q) new genetic lesions.

variants with the new C230Y and T329A amino acid substitutions identified in the juvenile and adult patients, respectively (Table 2). By contrast, the R201H and R442Q mutations, expressed in COS-1 cells resulted in 33% and 6% of residual GLB1 activity, respectively, compared with normal levels. These values indicate that these mutations are responsible for the residual enzyme activity measured in these patients' fibroblasts (Table 2). Given the high residual activity of the new R442Q GLB1 variant, the exclusion of its eventual polymorphic nature was performed by restriction site analysis of 100 control DNAs (Figure 3).

Figure 3. Restriction analysis by *PvuII*. Genomic DNA from the adult patient and her relatives were amplified in the region of 218 bp encompassing the *GLB1* gene exon 13. The transition c1325G>A abolishes the unique *PvuII* restriction site in the PCR fragment. Thus, the normal pattern shows two bands of 156 bp and 62 bp while the mutated heterozygous pattern shows the additional band of 218 bp corresponding to the nondigested PCR fragment. N.D., nondigested; P, patient; M, mother; F, father.

Western Blot Analysis

Immunoblot analysis performed on mutant fibroblasts showed the presence of GLB1 and EBP proteins in all three patients. However, the amount of EBP was significantly reduced in the juvenile patient's fibroblasts (Figure 4A). Western blot analysis of transfected COS-1 cells, demonstrated that the R442Q/T329A mutations did not significantly affect the levels of the GLB1 precursor protein of 85 kDa. Rather, a partial reduction of the precursor was detected in cells expressing the R201H and R59C mutant proteins, while a complete absence of the GLB1 protein was detected in cells expressing the S54N mutation (Figure 4B).

Impaired Elastogenesis

To exclude the variability of elastin deposition and cell vitality on cell numbers and confluence, each cell culture was assayed at the same passage number. The amount of fibronectin detected in the patients' fibroblasts was equal to that produced by normal fibroblasts (data not shown); these data are similar to those produced previously in G_{M1} -gangliosidosis patients.⁹ Condensed cytoplasmic accumulation of keratan sulfate was detected in

the juvenile patient's fibroblasts compared with normal controls (Figure 5, A and B). Direct immunofluorescence showed a substantial reduction of extracellular elastin deposition in the infantile and juvenile patients' fibroblasts compared with normal fibroblasts (Figure 5; C, F, and H). The addition of keratanase (an enzyme capable of keratan sulfate degradation) to the culture medium of the patient's fibroblasts restored the assembly of elastic fibers in juvenile patients' fibroblasts while impaired elastic fiber assembly was still observed in infantile patient's fibroblasts (Figure 5, G and I). Also, the addition of keratan sulfate to the culture medium of normal controls led to a remarkable decrease in the assembly of elastic fibers, as revealed by reduced elastin content (Figure 5D). A second control established by combined addition of keratan sulfate and keratanase in the culture medium of normal control fibroblasts resulted in a normal elastogenesis (Figure 5E). In contrast to the decreased elastin immunoreactivity of the juvenile patient's fibroblasts, the microfibrillar scaffold, represented by fibrillin-1 protein, was normal in that patient (Figure 6, A and B). Moreover, the juvenile patient's fibroblasts immunostained with antibody to EBP showed irregular and punctate immunogenic materials (Figure 6D), in contrast to the EBP widespread distribution on cell surface expressed in normal controls (Figure 6C).

Keratan Sulfate Chemical Estimate

Because qualitative estimate by immunohistochemistry in the fibroblasts from the patient with the juvenile form showed abnormal accumulation of keratan sulfate, quantitative estimate of this mucopolysaccharide has been performed in the 48-hour-old cultured fibroblasts of normal ($2 \text{ ng/ml} \pm \text{SD } 0.66$) and juvenile patient ($4 \text{ ng/ml} \pm \text{SD } 0.7$) and in the 10-day culture medium of normal ($24 \text{ ng/ml} \pm \text{SD } 0.71$) and juvenile patient ($28 \text{ ng/ml} \pm \text{SD } 1.19$).

Tropoelastin Production and Assembly

To further endorse the nature of the elastic fiber defect detected in the juvenile patient's fibroblasts, Western blot analysis with antibody against tropoelastin, and insoluble elastin estimates were also performed. Western blot showed that the ratio of soluble undegraded tropoelastin/degraded tropoelastin products is greater in the normal control compared with the juvenile patient's cell lysate (Figure 7). On the other hand insoluble elastin, the major component of elastic fibers, was significantly reduced in the juvenile patient's fibroblasts compared with normal controls (Figure 8).

Discussion

G_{M1} -gangliosidosis is a rare lysosomal storage disease, and very few patients, especially those affected by juvenile and adult form of the disease, have been previously reported in the Caucasian population.¹ We identified four

Figure 4. **A:** Immunoblot analysis of patients' fibroblasts. The pictures are representative of different independent experiments. Approximately $20 \mu\text{g}$ of proteins were analyzed by Western blots. Total cellular proteins were probed using αEBP and anti-actin antibodies that react with EBP and $\beta\text{-actin}$ (control protein), respectively. A, Adult patient's fibroblasts; J, juvenile patient's fibroblasts; I, infantile patient's fibroblasts; C, normal control fibroblasts. **B:** Immunoblot analysis of COS-1 cells with $\alpha\text{-85 GLB1}$ antibody. Approximately $20 \mu\text{g}$ of proteins were analyzed by Western blots. Each lane represents protein lysate of COS-1 cells transfected with pcDGLB1 vectors containing the *GLB1* mutations indicated directly in the figure. -Control, no vector; +control, pcDGLB1 wild type.

Figure 5. **A** and **B:** Representative photomicrographs of fibroblasts immunostained with anti-keratan sulfate antibody. Fibroblasts are from normal control (**A**) and juvenile patient (**B**). **C–I:** Representative photomicrographs of patients' fibroblasts immunostained with anti-elastin antibodies. Fibroblasts derive from normal control without any addition in cultured media (**C**); normal control with addition of keratan sulfate (1 μ g/ml) in cultured media (**D**); and normal control with addition of keratan sulfate (1 μ g/ml) and keratanase (0.1 U/ml/day) in cultured media (**E**). **F:** Infantile patient without any addition in cultured media. **G:** Infantile patient with addition of keratanase (0.1 U/ml/day) in cultured media. **H:** Juvenile patient without any addition in cultured media. **I:** Juvenile patient with addition of keratanase (0.1 U/ml/day) in cultured media.

novel (S54N, C230Y, T329A, R442Q) and two known (R59C, R201H) mutations in the *GLB1* gene of three G_{M1}-gangliosidosis patients representing a clinical range of the disorder that included infantile, juvenile, and adult phenotypes. It has been suggested that hot spot regions for *GLB1* mutations close to the amino terminus of the enzyme exist in adult.²⁵ However, the adult patient was found to be a compound heterozygous for two amino acid substitutions (T329A and R442Q) mapping to the central region of both *GLB1* and *EBP* proteins. On the other hand, R59C, T329A, and R442Q, found in the infantile and in the adult patients, occur at CpG sites, confirming that these regions are hot spots for mutations, as suggested for the R59 amino acid.²⁶

Expression studies and enzyme assays were performed with the aim of assigning a putative role of these mutations in the *GLB1* stability or catalytic activity. Because S54N and R59C, identified in the infantile patient, and C230Y and T329A, identified in the juvenile and in the adult patients, respectively, abolished the enzyme activity, they can be considered as severe mutations.

The T329 amino acid is found in a conserved cluster region of five amino acids including the catalytic D332.²⁷ At present, only E268, D332, and W273 are predicted to be involved in the catalytic site, even if many other residues are likely to play a role in the enzyme activity.²⁷ It

has been suggested that some enzyme defects may be due to the alteration of binding interaction of the catalytic residues or other residues in the active site.²⁸ Thus, a comprehensive correlation between the mutations and the enzyme defects will be available only when *GLB1* is completely characterized by identification of its crystallographic structure. Nevertheless, our expression studies showed that the T329A mutation abolishes the *GLB1* activity but does not alter the synthesis of the precursor that was still detected on Western blots. This finding confirms the importance of the conserved T329 residue in the formation of the catalytic site. On the other hand, the *GLB1* enzyme carrying the S54N mutation is predicted to be a misfolded and rapidly degraded protein because its presence was not detected.

The juvenile and adult patients also carry *GLB1* mutations associated with residual enzyme activity, which is in agreement with their clinical phenotype. It has been suggested that the R201C mutation affects the enzyme folding process.²⁷ *In vitro* expression studies and enzyme assay confirmed that the R201H mutation does not greatly alter the *GLB1* catalytic activity. These data are in keeping with Western blot analysis that revealed a reduced amount of the enzyme carrying the R201H mutation. The relatively high activity of the R201H *GLB1* variant may arise from the high affinity of this mutated protein

Figure 6. Representative photomicrographs of fibroblasts immunostained with anti-fibrillin 1 and anti-EBP antibodies. **A:** Normal control fibroblasts immunostained with antibody to fibrillin 1. **B:** Juvenile patient fibroblasts immunostained with antibody to fibrillin 1. **C:** Normal control fibroblasts immunostained with anti-EBP antibody. **D:** Juvenile patient fibroblasts immunostained with anti-EBP antibody.

for the synthetic fluorogenic substrate, in agreement with previous expression studies on that mutation.^{29,30}

Defects in the *GLB1* gene are very rare, and most of the genetic lesions reported up to now affect both *GLB1* enzyme and EBP. The detailed clinical evaluation of those *G_{M1}*-gangliosidosis patients with mutations affecting either the *GLB1* coding region alone or both *GLB1* and EBP, could identify specific clinical manifestations associated with defects on either of the two proteins or both. Our juvenile patient showed very peculiar mutations (R201H and C230Y) located in a region of pre-mRNA

Figure 7. Immunoblot analysis of patients' fibroblasts with tropoelastin/elastin antibody. The pictures are representative of different independent experiments. Approximately 20 μ g of juvenile and normal control cell-layer lysates were immunized with a polyclonal antibody to tropoelastin/elastin. J, Juvenile patient; C, normal control. The 70-kDa band represents tropoelastin, and lower bands correspond to degraded tropoelastin products.

Figure 8. Representative graphic of insoluble elastin analysis. Quantitative estimate of [³H]-leucine radiolabeled NaOH-insoluble pellets from normal control and juvenile patient's fibroblasts.

encoding only the lysosomal enzyme. Thus, molecular and cellular studies of this patient became extremely useful in shedding light on the pathogenesis of *G_{M1}*-gangliosidosis.

Some *GLB1* mutations, giving rise to a deficiency in the degradation of keratan sulfate, have been previously hypothesized to cause lesions in connective tissue and bones.²⁵ It has been also predicted that EBP mutations are involved in the extracellular deposition of keratan sulfate, stored in Morquio type B disease.²⁷ Thus, the disruption of both *GLB1* and EBP functions may give rise to clinical features typical of both *G_{M1}*-gangliosidosis and Morquio type B disease.²⁷

Decreased elastin deposition has been previously linked to the lack of EBP in infantile *G_{M1}*-gangliosidosis patients, who carried nonsense mutations, and in Morquio type B patients, due to mutations that cause deficiency in both *GLB1* and EBP.⁹ Interestingly, the EBP amount of the fibroblasts from the juvenile patient here reported is reduced even though the mutational analysis has excluded its involvement. However, it has been demonstrated that the abnormal accumulation of some galactosugar-bearing moieties such as chondroitin sulfate and dermatan sulfate causes EBP functional inactivation in Hurler disease and Costello syndrome, respectively.^{10,11} Our juvenile *G_{M1}*-gangliosidosis patient was initially diagnosed as suffering from Morquio type B disease, because of an elevated urine excretion of keratan sulfate and his skeletal abnormalities. Keratan sulfate accumulation was also detected in his cultured fibroblasts by immunohistochemistry. Based on these data and taking into consideration that even keratan sulfate has galactosugar-bearing moieties, the relationship between keratan sulfate and EBP reduction in this patient was investigated. Severely impaired elastogenesis of the patient's fibroblasts was detected by immunostaining, but this was restored by incubation of the juvenile patient's fibroblasts with keratanase. On the other hand, normal control fibroblasts did not assemble any elastic fibers after the addition of keratan sulfate in the culture medium.

The relationship between keratan sulfate accumulation and impaired elastic fiber assembly was further investigated by testing elastin and microfibrillar synthesis and deposition. The normal amount of *EBP* and elastin mRNA in all our patients, as detected by RT-PCR analysis, excludes a defect in mRNA synthesis. It has been previously shown that tropoelastin content is reduced in fibroblasts from Costello syndrome due to accumulation of chondroitin sulfate that causes the degradation of tropoelastin products.¹⁰ A reduction of tropoelastin in the juvenile patient's fibroblasts, detected by Western blot analysis, suggested a partial decrease of this protein. Also, insoluble elastin assay, that estimates the only integral elastin content present in the mature elastic fibers, was reduced in the patient's cell-layer extracts. These data confirmed that tropoelastin did not properly aggregate on the cell-surface of the juvenile patient's fibroblasts and that tropoelastin, even if normally synthesized, was likely to be quickly shipped to degradation.

Together with tropoelastin, a key molecule in the process of elastic fibers formation is fibrillin-1.³¹ The first step in elastogenesis is marked by the deposition of the microfibrillar components into the extracellular space. The amount and distribution of fibrillin-1 in the juvenile patient's fibroblasts revealed by immunohistochemistry did not differ from these detected in normal controls. Thus, the elastic fibers assembly defect observed in the juvenile G_{M1} gangliosidosis patient is likely to be independent from microfibril secretion. Rather, an EBP depletion of that patient's cell-layer extracts was visualized by Western blot analysis and immunostaining. Keratan sulfate was abnormally elevated in the patient's urine, in his fibroblasts, and culture medium. Monoclonal antibody also revealed a remarkable focal and punctate cytoplasmic amount of keratan sulfate in 48-hour cell cultures of that patient compared with normal control.

In summary, taking into account that the fibroblasts from the G_{M1}-gangliosidosis juvenile patient showed an EBP protein reduction, impaired elastic fibers assembly, and large keratan sulfate accumulation, we hypothesized that his fibroblasts' EBP depletion is of secondary origin caused by keratan sulfate. Additional studies of the role of the single amino acid substitutions on the GLB1 crystallographic structure, would probably clarify the nature of the GLB1 active site involved in the degradation of keratan sulfate.

Lack of EBP of primary origin, has been linked to impaired elastogenesis in infantile G_{M1}-gangliosidosis patients bearing nonsense mutations of the *GLB1* gene.⁹ Interestingly, decreased elastin assembly may also result from a primary defect of EBP function in which a normal amount of the protein is present, as demonstrated in our infantile patient's fibroblasts.

In summary, GLB1 and EBP proteins, altered in function and/or distribution, contribute differently to the specific clinical manifestations of patients with mutations in the *GLB1* gene. The missense mutations detected in these patients are responsible for the characteristic clinical manifestations and they confirm the genetic heterogeneity of G_{M1}-gangliosidosis. In addition, impaired elastogenesis, due to a secondary deficiency of EBP and to

keratan sulfate storage, has been shown in the fibroblasts from the juvenile G_{M1}-gangliosidosis patient here reported. Understanding the role of EBP is crucial for the diagnosis and the pathogenesis of G_{M1}-gangliosidosis and Morquio type B, and should be investigated in all diseases in which the EBP-protein and receptor are involved.

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