Am. J. Hum. Genet. 67:23–36, 2000

Impaired Elastic-Fiber Assembly by Fibroblasts from Patients with Either Morquio B Disease or Infantile GM1-Gangliosidosis Is Linked to Deficiency in the 67-kD Spliced Variant of β-Galactosidase

Aleksander Hinek,[1,3] Sunqu Zhang,[2] Adam C. Smith,[1,3] and John W. Callahan[2,4]

Divisions of [1]Cardiovascular Research and [2]Structural Biology and Biochemistry, The Hospital for Sick Children, and the Departments of [3]Laboratory Medicine and Pathobiology and [4]Biochemistry, University of Toronto, Toronto

We have previously shown that intracellular trafficking and extracellular assembly of tropoelastin into elastic fibers is facilitated by the 67-kD elastin-binding protein identical to an enzymatically inactive, alternatively spliced variant of β-galactosidase (S-Gal). In the present study, we investigated elastic-fiber assembly in cultures of dermal fibroblasts from patients with either Morquio B disease or GM1-gangliosidosis who bore different mutations of the β-galactosidase gene. We found that fibroblasts taken from patients with an adult form of GM1-gangliosidosis and from patients with an infantile form, carrying a missense mutations in the β-galactosidase gene—mutations that caused deficiency in lysosomal β-galactosidase but not in S-Gal—assembled normal elastic fibers. In contrast, fibroblasts from two cases of infantile GM1-gangliosidosis that bear nonsense mutations of the β-galactosidase gene, as well as fibroblasts from four patients with Morquio B who had mutations causing deficiency in both forms of β-galactosidase, did not assemble elastic fibers. We also demonstrated that S-Gal–deficient fibroblasts from patients with either GM1-gangliosidosis or Morquio B can acquire the S-Gal protein, produced by coculturing of Chinese hamster ovary cells permanently transected with S-Gal cDNA, resulting in improved deposition of elastic fibers. The present study provides a novel and natural model validating functional roles of S-Gal in elastogenesis and elucidates an association between impaired elastogenesis and the development of connective-tissue disorders in patients with Morquio B disease and in patients with an infantile form of GM1-gangliosidosis.

Introduction

Deficiency in acid lysosomal β-galactosidase (β-Gal [E.C.3.2.1.23]) activity is the primary defect in GM1-gangliosidosis and Morquio disease type B, two clinically different entities. So far, ~30 mutations have been identified in the β-Gal gene (for a review, see Callahan 1999). GM1-gangliosidosis is a progressive neurovisceral disease in which three distinct phenotypic variants are recognized—an infantile (MIM 230500), a juvenile (MIM 230600), and an adult (MIM 230650)—on the basis of both age at onset and severity of the symptoms (Suzuki et al. 1995). All patients with GM1-gangliosidosis store GM1-gangliosides in neurons (Mutoh et al. 1986, 1988), but only patients with the infantile form of this disease display coarse facial features, hepatosplenomegaly, prominent skeletal dysmorphology (Alroy et al. 1992, 1995; Suzuki et al. 1995), diverse connective-tissue abnormalities, and cardiomyopathy. The latter feature is an atypical clinical manifestation that has been reported only in White patients (Hadley and Hagstrom 1971; Benson et al. 1976; Kohlschutter et al. 1982; Rosenberg et al. 1985; Charrow and Hvizd 1986; Morrone et al. 2000). In contrast, patients with Morquio B disease (MIM 253010) do not store GM1-gangliosides in the brain and are neurologically normal but display more-severe connective-tissue and skeletal deformities than are seen in GM1-gangliosidosis (Arbisser et al. 1977; Groebe et al. 1980; van der Horst et al. 1983; Giugliani et al. 1987). They may also display cardiac-valve deformations, aortic stenosis, and intimal thickening in the coronary arteries and in the pulmonary artery (Factor et al. 1978; Haust 1987; Dangel 1998). Development of the connective-tissue and skeletal abnormalities, both in patients with GM1-gangliosidosis and in patients with Morquio B, has been largely attributed to the intra- and extracellular storage of galactose-terminal oligosaccharides derived primarily from the breakdown of glycoproteins and complex glycosaminoglycans. Alternative pathomechanisms that may contribute to abnormalities of the extracellular matrix and development of characteristic clinical phenotypes in these diseases have not been recognized.

We have recently demonstrated that defective for-

Received March 10, 2000; accepted for publication April 12, 2000; electronically published June 6, 2000.

Address for correspondence and reprints: Dr. Aleksander Hinek, Division of Cardiovascular Research, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. E-mail: alek.hinek@sickkids.on.ca

0002-9297/2000/6701-0007$02.00

mation of elastic fibers in tissues of patients with Hurler disease and in patients with Costello syndrome can be directly linked to the development of connective-tissue and cardiovascular disorders, as well as to skeletal deformations observed in these clinical entities, also characterized by accumulation of galactose-containing moieties (Hinek and Wilson 2000; Hinek et al. 2000).

The mature elastic fibers and laminae present in connective tissues and blood vessel walls are complex structures made of polymeric (insoluble) elastin in which polypeptide chains of tropoelastin are covalently cross-linked and placed on a scaffold of 12-nm microfibrils that consists of several glycoproteins—for example, fibrillins and microfibril-associated glycoproteins (Mecham and Hauser 1991; Rosenbloom et al. 1993; Christiano and Uitto 1994; Pasquali-Ronchetti and Baccarani-Contri 1997). Tropoelastin is a soluble 70-kD precursor of extracellular elastin (for reviews, see Vrhovski and Weiss 1998; Debell and Tamburro 1999) synthesized by such cells as fibroblasts, chondrocytes, and smooth-muscle cells, which have to be secreted and properly positioned on the microfibrillar scaffold (Sakai et al. 1986; Zhang et al. 1994; Kielty and Shuttleworth 1995; Gibson et al. 1996) before being cross-linked by lysyl oxidase (Kagan et al. 1986).

Our group has shown elsewhere that early stages of elastogenesis are controlled by the 67-kD elastin-binding protein (EBP), which also has a galactolectin domain that can bind galactosugar-bearing moieties (Hinek et al. 1988, 1991, 1993; Mecham et al. 1989, 1991; Privitera et al. 1998). The EBP acts as a recycling molecular chaperone that protects the highly hydrophobic tropoelastin molecules from intracellular self-aggregation and premature degradation (Hinek and Rabinovitch 1994; Hinek et al. 1995). The EBP also facilitates the orderly assembly of tropoelastin upon the microfibrillar scaffold of growing elastic fibers (Hinek 1994, 1996). The orderly release of tropoelastin from its transportation complex with the EBP occurs at the cell surface, just after the galactolectin domain of the EBP binds to galactosugars residues, protruding from polyglycosylated fibrillin molecules and from fibrillin-associated chondroitin sulfate proteoglycans (Hinek et al. 1995; Hinek 1996; Kielty et al. 1996).

We have established that elastogenesis can be disrupted by pericellular accumulation of galactosugar-bearing moieties, such as chondroitin sulfate or dermatan sulfate, which also can bind to the galactolectin domain of EBP and can induce both its shedding from the cell surface and premature release of tropoelastin, far from microfibrillar acceptors (Hinek et al. 1988, 1991; Hinek and Rabinovitch 1994). Most important, results of our previous studies (Hinek et al. 1993; Privitera et al. 1998) led to the discovery that the 67-kD EBP is identical to the alternatively spliced, catalytically inactive variant of human β-Gal (S-Gal), originally described as a protein with undefined function (Morreau et al. 1989; Yamamoto et al. 1990).

S-Gal arises through the splicing-out of two noncontiguous encoding regions from the mRNA, in such a way that the first deletion introduces a frameshift (exon 5), which is restored by the deletion of a second region. The net result is synthesis of a shortened protein that does not display any enzymatic activity of β-Gal and is not targeted to the lysosomes. We have established that a unique 32-amino-acid-long domain encoded by the frameshifted exon 5 of S-Gal contains a VVGSPSAQDEASPL sequence that binds elastin and laminin (Hinek et al. 1993).

The in vitro transcription/translation of a full-length cDNA clone encoding human S-Gal produced protein that reacted with anti–β-Gal, anti–S-Gal and anti-EBP antibodies and that bound to the elastin- and laminin-affinity columns. S-Gal cDNA transfection of the EBP-deficient smooth-muscle cells (SMC) taken from intimal cushions of the ductus arteriosus also up-regulated their expression of functional 67-kD EBP. In contrast to their untransfected counterparts, the S-Gal–transfected ductus arteriosus SMC attached to elastin-covered plates and displayed normal assembly of elastic fibers in culture (Privitera et al. 1998).

In this study, we tested expression of S-Gal/EBP and assessed elastic-fiber formation in cultures of skin fibroblasts obtained from four patients with Morquio B disease that bear β-Gal gene mutations (G438E, N484K/T500A, and W273L/R482H) localized to the coding region common to lysosomal enzyme and S-Gal/EBP protein. We also tested S-Gal/EBP expression and elastogenesis in (*a*) fibroblasts from two patients with GM1-gangliosidosis that bear a missense R148S mutation localized to the exon 4 of the β-Gal gene that is spliced out of S-Gal and that might not affect synthesis of S-Gal protein and (*b*) fibroblasts from two patients with GM1 that bear either one of two nonsense mutations (R351X and R475X) that likely destabilize both β-Gal and S-Gal mRNAs and that, consequently, lead to deficiency in both protein variants. Our studies, aimed at establishing the connection between the expression of S-Gal and elastogenesis, also included fibroblasts from two patients with an adult form of GM1-gangliosidosis that did not display any connective-tissue problems.

Material and Methods

Material

Chemicals and reagents were obtained as follows. Media, fetal bovine serum, and other tissue-culture reagents were obtained from GIBCO. Polyclonal antibody to bo-

vine tropoelastin (Prosser et al. 1991), polyclonal antibody to fibrillin 1, and BCZ monoclonal antibody raised to bovine EBP (Mecham et al. 1988) were purchased from Elastin Products Co., Inc. Monoclonal antibody to fibronectin (mAB1940) was from Chemicon, and monoclonal anti-laminin antibody was obtained from ICN. Polyclonal antibodies to human collagen type I and to human lysyl oxidase were generous gifts from Drs. Larry W. Fischer (National Institutes of Health) and Katalin Csiszar (University of Hawaii at Manoa), respectively. The polyclonal antibodies anti–S-Gal, raised to VV-GSPSAQDEASPL oligopeptide, corresponding to the elastin-binding domain of the alternatively spliced form of human β-Gal; anti–C-Gal, raised to PPQKNKD-SWLDHV oligopeptide and reflecting the C-terminal sequence (present both in the precursor of β-Gal and in S-Gal variant); and anti–P-Gal, raised to the 88-kD recombinant precursor of human β-Gal (and which recognizes both variants of human β-Gal) were produced and characterized as described elsewhere (Hinek et al. 1993; Zhang et al. 1994; Okamura-Oho et al. 1996). The β-Gal substrate, 4-methylumbelliferyl-β-D-galactopyranoside, was from Koch-Light Laboratories, Ltd. All other chemicals, proteinase inhibitors, enzyme substrates, SDS-PAGE reagents, fluorescein (FITC)- and horseradish peroxidase (HRP)–conjugated secondary antibodies were purchased from Sigma. The enhanced chemiluminescence (ECL) western-blotting detection kit was from Amersham Canada Ltd. Radioactive [^{14}C]-serine and [^{3}H]-valine were supplied by New England Nuclear. The protein-assay kit was from BioRad.

Fibroblasts and Tissue Preparation

Human skin fibroblasts were propagated in the cell repository of The Hospital for Sick Children in Toronto (HSC). Fibroblasts were derived from forearm-skin biopsies of three normal children—patients 3858 (age 4 mo), 4212 (age 7 mo), and 4184 (age 36 mo)—and from two patients clinically diagnosed with the infantile phenotype of GM1-gangliosidosis that bear missense mutations of the β-Gal gene (R148S/D332N in case 4992 and R148S/R482H in case 8981) and nonsense mutations R351X (case 4032) and R475X (case 8982). Both patients with missense mutations (patient 4992, a 12-mo-old male; and patient 8981, a 34-mo-old female) demonstrated a relatively mild clinical phenotype with hepatosplenomegaly but no visible skeletal deformations or cardiac involvement. The residual values of their serum and leukocyte β-Gal activity (4% and 6% of control values, respectively) were consistent with a diagnosis of GM1-gangliosidosis. In contrast, both patients with nonsense mutations of β-Gal gene (patient 4032, an 18-mo-old female; and patient 8982, a 3-mo-old male), in addition to having hepatosplenomegaly and skeletal dysplasia, initially were diagnosed with cardiomegaly and congestive heart failure. Cardiac ultrasound examination demonstrated left-ventricular and septal hypertrophy and diminished contractility in both cases. Their values of serum and leukocyte β-Gal activity were <2% of control values.

In addition, fibroblasts from two patients with an adult form of GM1-gangliosidosis (patients 3633 and 3749) and with β-Gal deficiency reaching, in both cases, 12% of normal value (mutations unknown) also were tested. These patients demonstrated characteristic extrapyramidal neurological symptoms but no skeletal deformities or cardiac involvement.

Morquio B disease fibroblasts were from three newly diagnosed patients with HSC that bear recently described β-Gal gene mutations localized to the coding region common to lysosomal enzyme and S-Gal/EBP protein (Skomorowski et al. 1999) (patient 10213, a G438E homozygote; patient 11983, an N484K/T500A heterozygote; and patient 11984, a N484K/T500A heterozygote) and from one case, described elsewhere (Oshima et al. 1991), who was a W273L/R482H heterozygote obtained from the National Institute of General Medical Sciences cell repository (patient 4080). All fibroblasts originally isolated by collagenase digestion of the skin-biopsy samples were routinely passaged by trypsinization and were maintained in alpha-minimum essential medium (α-MEM) supplemented with 20 mM HEPES, 1% antibiotics/antimycotics, 10% fetal bovine serum, and 1% L-glutamate. Expression of EBP and of lysyl oxidase, as well as deposition of extracellular elastin, collagen type I, laminin, fibronectin, and fibrillin, were compared by immunohistochemistry at passages 2–5.

In a separate series of experiments, we tested whether delivery of exogenous S-Gal/EBP would restore normal deposition of elastic fibers by Morquio B disease (patient 4080) and GM1-gangliosidosis fibroblasts homozygous for the R351X mutation (patient 4032), deficient in S-Gal. These fibroblasts (1×10^6 cells) were mixed with 2×10^5 Chinese hamster ovary (CHO) cells permanently transfected with an S-Gal cDNA clone and cocultured in normal medium. The parallel cocultures of fibroblasts from patients 4080 and 4032, mixed with a comparable number of nontransfected CHO cells, were used for comparison. Both types of cocultures were terminated after 3 and 10 d, and production of elastic fibers was assessed by immunohistochemistry and by insoluble-elastin assay, as described below.

Stable transfection of CHO cells with S-Gal cDNA was performed as described elsewhere (Zhang et al. 1994). In brief, the S-Gal cDNA was subcloned into the mammalian expression vector pRc/RSV (Invitrogen), at the *Hin*dIII site. CHO cells (American Type Culture Collection) were exposed to the DNA-Lipofectin complex

for 5 h, in serum-free α-MEM medium, after which time the cells were incubated in α-MEM medium containing 10% fetal calf serum, for 48–72 h. Transfected cells were selected with geneticin (G418), at a concentration of 400 mg/ml, in the above medium. After 14 d, the clones resistant to geneticin were separately isolated. To assess their ability to produce S-Gal, the cells were lysed in 1% octyl-β-D-glucoside (OBG) and were characterized by western blots. Clone 42 showed the strongest signal of S-Gal and was selected for use. By correlating PCR and RT-PCR primer products reflecting the frameshifted exon 5 (Privitera et al. 1998) and anti–S-Gal antibody reactivity performed as described elsewhere (Hinek et al. 1993), we showed that this DNA fragment was synthesized only in the S-Gal–transfected clones, and not in the nontransfected CHO cells. To test whether delivery of exogenous enzymatically active β-Gal would also reverse faulty elastogenesis, GM1-gangliosidosis (patient 4032) and Morquio B disease fibroblasts (patient 4080) were plated on glass cover slips and were placed (face up) on the top of subconfluent cultures of CHO cells permanently transfected with wild-type β-Gal cDNA and secreting the active enzyme into the conditioned medium (Zhang et al. 1994). Both types of cocultures were terminated after 10 d. Production of elastic fibers, as well as expression of S-Gal, were assessed by immunohistochemistry and by insoluble-elastin assay, as described below. The enzymatically active β-Gal precursor taken up by cultured fibroblasts was histochemically visualized with X-Gal substrate, as described elsewhere (Singh and Knox 1984).

Immunostaining

Subconfluent 2-d-old cultures of normal, GM1-gangliosidosis, and Morquio B disease fibroblasts, as well as 10-d-old dense cultures of these cells, which produce abundant extracellular matrix, were used. The 2-d-old cultures were fixed in cold 100% methanol at −20°C for 30 min and were incubated for 1 h, with either anti-S-Gal antibody (20 μg/ml), an antibody that recognizes the elastin-binding domain of the S-Gal (Hinek et al. 1993), or monoclonal antibody (BCZ) (25 μg/ml), an antibody that recognizes a different epitope on this EBP (Mecham et al. 1989).

The 10-d-old confluent cultures of the normal, GM1-gangliosidosis, and Morquio B disease fibroblasts were fixed for 30 min in cold 100% methanol, and then parallel cultures were immunostained with polyclonal antibody to tropoelastin (20 μg/ml), monoclonal antibody to fibronectin (1 μg/ml), monoclonal antibody to laminin (1 μg/ml), or polyclonal antibody to collagen type I (1 μg/ml), as described elsewhere (Hinek and Wilson 2000). Cultures scheduled for immunohistochemical assessment of microfibrils were fixed in 0.5% paraformaldehyde at room temperature for 15 min, were blocked in PBS containing 0.1 M ammonium chloride, and then were washed in PBS and were treated with polyclonal antibody to fibrillin-1 (20 μg/ml). In addition to this, cultures of all tested fibroblasts also were probed with antibody recognizing lysyl oxidase (1 μg/ml), an enzyme responsible for cross-linking of the secreted tropoelastin. All cultures were incubated with the appropriate FITC-conjugated secondary antibodies (GAR-FITC or GAM-FITC) for an additional hour, and nuclei were counterstained with propidium iodide; as a control for all immunostaining described, the preimmune rabbit IgG or normal mouse ascitic fluid, respectively, was substituted for the primary antibodies. Additional controls included secondary antibody alone. Morphometric analysis of 10-d-old cultures immunostained with antibodies recognizing extracellular matrix components was performed by an Olympus AH-3 microscope attached to a CCD camera (Optronics) and a computer-generated video-analysis system (Image-Pro Plus software; Media Cybernetics). In each analyzed group, 50 low-power fields (20×) from three separate cultures (derived from different patients) were analyzed, and the area occupied by the particular immunodetectable component was quantified. The abundance of each immunodetectable component was then expressed as a percentage of the entire analyzed field.

Assay of β-Galactosidase Proteins and Enzymatic Activity

Confluent dishes of fibroblasts (~5×10^6 cells/dish) maintained in α-MEM containing heat-inactivated 10% fetal calf serum, were washed three times in PBS (10 mM phosphate, 0.15 M NaCl [pH 7.4]), and the cells were harvested by scraping with a rubber policeman. The cells were then briefly centrifuged at 100 *g,* were resuspended in 0.1 M Tris-HCl, pH 7.6, containing 2 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor, and were lysed by three cycles of freezing and thawing. To detect all β-Gal–related proteins, 20-μl aliquots of the cell extracts were mixed with an equal volume of carbon tetrachloride. After the insoluble cell remnants were removed by centrifugation, the extract was subjected to SDS-PAGE, followed by immunoblotting with the polyclonal antibody raised to the β-Gal precursor (anti–P-Gal) that recognizes all forms of β-Gal and S-Gal and their degradation products. The enzymatic activity of β-Gal was determined in supernatants using 4-methylumbelliferyl-β-D-galactopyranoside, as described elsewhere (D'Agrosa et al. 1992), and results were normalized for total protein content (assessed by a BioRad kit).

Isolation of EBP

To compare patterns of EBP expression by the normal, GM1-gangliosidosis, and Morquio B fibroblasts, we car-

ried out metabolic labeling followed by elastin-affinity chromatography. Fibroblasts were initially plated 1×10^6 cells/dish. The quadruplicate cultures were maintained in serum-free Medium 199 for 6 h and then were exposed to 15 μCi [^{14}C]-serine/ml, in serine-free medium, for 3 h. The cultures were then rinsed well, and the cell layers and the media were processed separately. To isolate the EBP from the cell layers, elastin-affinity chromatography was used. Fibroblasts were scraped from each culture dish, suspended in 1 ml of 0.1 M bicarbonate buffer, pH 8, and were extracted, for 1 h at 4°C, with 0.1 M lactose, 0.1 M dithiothreitol (DTT), and 0.25% OBG, in the presence of proteinase inhibitors in the following final concentrations: 2 mM benzamidine, 2 mM epsylon amino capronic acid, 2 mM PMSF, 1 mM EDTA, and 1 mg trasylol/ml. The EBP present in extracts of each culture was then purified by insoluble-elastin–affinity chromatography using minicolumns each made of 1 mg of insoluble elastin slurries, as described elsewhere (Hinek et al. 1993; Privitera et al. 1998). The elastin-bound proteins were released from the insoluble slurries by boiling in 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.001% bromophenol blue with DTT and were resolved by 7.5%–12% SDS-PAGE, followed by autoradiography. The identity of the 67-kD EBP was additionally confirmed by immunoblotting with anti-S-Gal, anti-C-Gal, anti-P-Gal, and anti-EBP (BCZ) antibodies (all in a concentration of 0.2 μg/ml), followed by the appropriate HRP-conjugated secondary antibodies diluted 1:5,000, and signals were developed with the ECL detection system. The β-Gal proteins (precursor, active enzyme, and S-Gal) also were isolated from the cell lysates and were concentrated by affinity chromatography on *p*-aminophenyl-thiogalactose-Sepharose (Hinek et al. 1993).

Tropoelastin and Insoluble Elastin Assays

Normal, GM1-gangliosidosis, and Morquio B fibroblasts were grown to confluency in 10-cm cell-culture dishes, in quadruplicate. To each dish was added 20 μCi of [^{3}H]-valine, along with fresh media. Cultures were then incubated for 72 h, and soluble and insoluble elastin were assessed separately in each culture. First, media were collected and immunoprecipitated with a polyclonal antibody to tropoelastin; then the soluble proteins present in the intracellular compartments were extracted for 6 h, with 0.1 M acetic acid, and the intracellular tropoelastin was immunoprecipitated from each extract, as described elsewhere (Hinek and Rabinovitch 1994), and then was quantitatively assessed after scintillation counting. The remaining cultures, containing cell remnants and deposited insoluble extracellular matrix, were then scraped in 0.1 N NaOH, were sedimented by centrifugation, and were boiled in 0.5 ml of 0.1 N NaOH for 45 min, to solubilize all matrix components except elastin. The resulting pellets, containing the insoluble elastin, then were solubilized by being boiled in 200 μl of 5.7 N HCl for 1 h, and the aliquots were mixed with scintillation fluid and were counted (Hinek and Rabinovitch 1993, 1994).

Analysis of Data

In all biochemical studies, means and SDs were calculated, and statistical analyses were carried out by ANOVA.

Results

Lack of S-Gal in Morquio B Disease and GM1-Gangliosidosis Cells Bearing Nonsense β-Gal Mutations

Assays utilizing the enzyme-specific substrates indicated that all tested fibroblasts derived from patients with either Morquio B disease and or GM1-gangliosidosis displayed levels of hexaminidase similar to those in normal fibroblasts (16,735–22,248 nM/h/mg protein), but they were characterized by only residual levels of enzymatic activity of acid β-Gal (3–47 nM/h/mg protein), compared with normal fibroblasts (2,900–3,730 nM/h/mg protein). Western-blot analysis of cell lysates with anti–P-Gal antibody indicated that Morquio B and GM1-gangliosidosis fibroblasts demonstrated variable amounts of 88-kD β-Gal precursor but very little or none of the mature, 64-kD form of the enzyme. Interestingly, only normal fibroblasts and GM1-gangliosidosis fibroblasts bearing missense mutations of β-Gal displayed the presence of the 67-kD protein immunoreactive with anti–P-Gal antibody (fig. 1). This was consistent with results of another experiment, aimed at isolation of the EBPs metabolically labeled with radioactive serine. SDS-PAGE followed by autoradiography showed that the whole-cell extracts of fibroblasts derived from patients with infantile GM1-gangliosidosis that bear missense mutations of β-Gal gene (patients 4992 and 8981) demonstrated similar amounts of the labeled 67-kD protein isolated by elastin-affinity columns, compared with normal fibroblasts (fig. 2). The 67-kD proteins released from elastin-affinity columns strongly reacted with anti–S-Gal antibody on western blots and also were recognized by anti–C-Gal, anti–P-Gal, and anti-EBP antibodies. The 67-kD species was the only protein retained on elastin-affinity columns; in contrast, fibroblasts derived from an infantile form of GM1-gangliosidosis that bear nonsense Arg351X and R475X mutations of the β-Gal gene (in patients 4032 and 8982, respectively), as well as fibroblasts derived from all four tested cases of Morquio B disease, did not display labeled 67-kD protein that could bind to elastin-affinity column and that could react with anti–S-Gal, anti–P-Gal, anti–C-Gal, or anti-EBP anti-

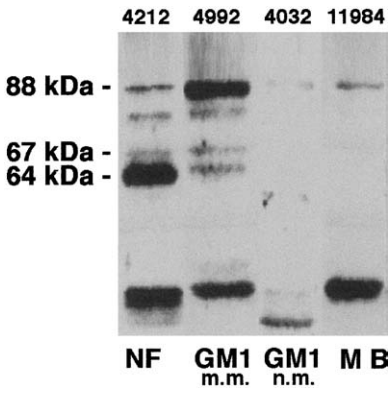

Figure 1 Representative western blotting with anti–P-Gal antibody detecting β–Gal–related species (88-kD precursor, 67-kD S-Gal, and 64-kD mature enzyme—and their degradation products) in lysates of human skin fibroblasts. Morquio B fibroblasts (11984) and GM1-gangliosidosis fibroblasts (4992 and 4032) demonstrate variable amounts of 88-kD β-Gal precursor but little or none of the mature 64-kD enzyme. In contrast to normal (4212) and GM1-gangliosidosis fibroblasts bearing missense (m.m.) mutations of β-Gal (4992), which display the 67-kD immunoreactive protein, neither GM1-gangliosidosis fibroblasts (4032) bearing the nonsense mutation (n.m.) nor cells from Morquio B patient (11984) show any trace of the 67-kD species.

bodies. Interestingly, fibroblasts from patients with an adult form of GM1-gangliosidosis that did not display connective-tissue abnormalities (cases 3749 and 3633) demonstrated similar amounts of the labeled 67-kD protein isolated by elastin-affinity columns, when compared with fibroblasts obtained from normal individuals (data not shown).

Fibroblasts from Morquio B Disease and GM1-Gangliosidosis That Do Not Express S-Gal but Show Impaired Secretion of Tropoelastin and Do Not Assemble Elastic Fibers

Immunocytochemistry with anti–S-Gal antibody demonstrated clear localization of the EBP on the surface of nonpermeabilized cultured fibroblasts obtained from normal controls and from patients with GM1-gangliosidosis with missense β-Gal mutations (fig. 3*A* and *B*). In contrast, neither fibroblasts from a patients with GM1-gangliosidosis that bear a nonsense β-Gal mutation (fig. 3*C*) nor Morquio B fibroblasts (fig. 3*D*) displayed an S-Gal immunodetectable protein. Cultures of fibroblasts from patients with an adult form of GM1-gangliosidosis showed normal production of S-Gal (data not shown). Immunostaining with a BCZ antibody recognizing a different epitope on the S-Gal/EBP showed similar patterns of localization, on all tested fibroblasts.

The lack of S-Gal expression correlated with impaired elastic-fiber production. Comparison of 10-d-old fibroblast cultures immunostained with an anti-tropoelastin antibody revealed that, in contrast to normal fibroblasts (fig. 3*E*) and GM1-fibroblasts with missense mutations (fig. 3*F*) that produced a dense network of elastic fibers, the fibroblasts from the patient with GM1-gangliosidosis that bear the nonsense mutation (fig. 3*G*) and from the patient with Morquio B disease (fig. 3*H*) did not demonstrate immunodetectable extracellular elastin. This was in contrast to cultures of fibroblasts from patients with an adult form of GM1-gangliosidosis (showing normal expression of S-Gal), which produced as many elastic fibers as were produced by fibroblasts obtained from normal individuals (data not shown). Morphometric analysis of 10-d-old cultures immunostained with matrix-specific antibodies indicated that the S-Gal–deficient fibroblasts were selectively afflicted with the inability to deposit elastic fibers, but they demonstrated normally assembled microfibrils (detected by an antibody to fibrillin-1) and normal amounts of immunodetectable col-

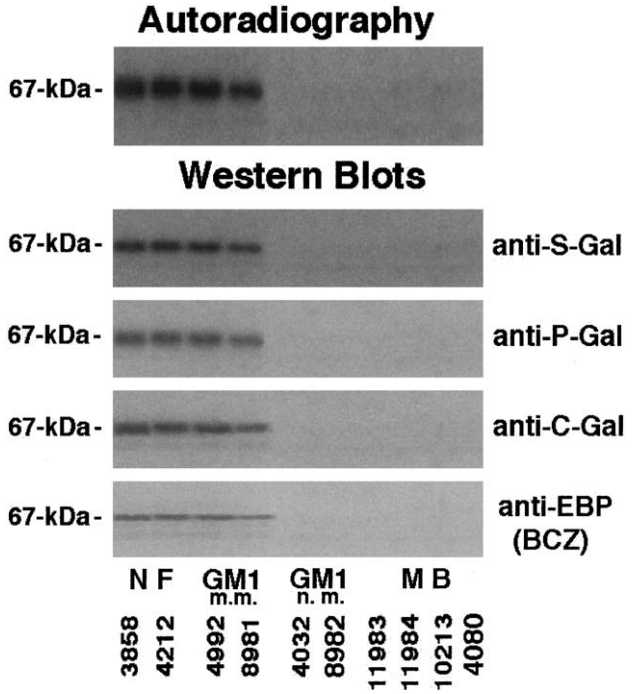

Figure 2 SDS-PAGE of whole-cell extracts, followed by autoradiography. The amount of metabolically labeled, newly produced 67-kD protein isolated by elastin-affinity columns is similar in fibroblasts derived from a patient with infantile GM1-gangliosidosis that bear missense mutations of the β-Gal gene (GM1 m.m. lanes 4992 and 8981) and in fibroblasts obtained from normal individuals (NF lanes 3858 and 4212). The parallel western blots demonstrate that newly synthesized 67-kD protein that binds to the elastin-affinity column and reacts with anti–S-Gal antibody is also recognized by anti–C-Gal, anti–P-Gal, and BCZ antibodies. In contrast, GM1-gangliosidosis fibroblasts bearing nonsense mutations of the β-Gal gene (GM1 n.m. lanes 4032 and 8982), as well as Morquio B fibroblasts (MB lanes 11983, 11984, 10213, and 4080) do not display radiolabeled and immunoreactive S-Gal/EBP.

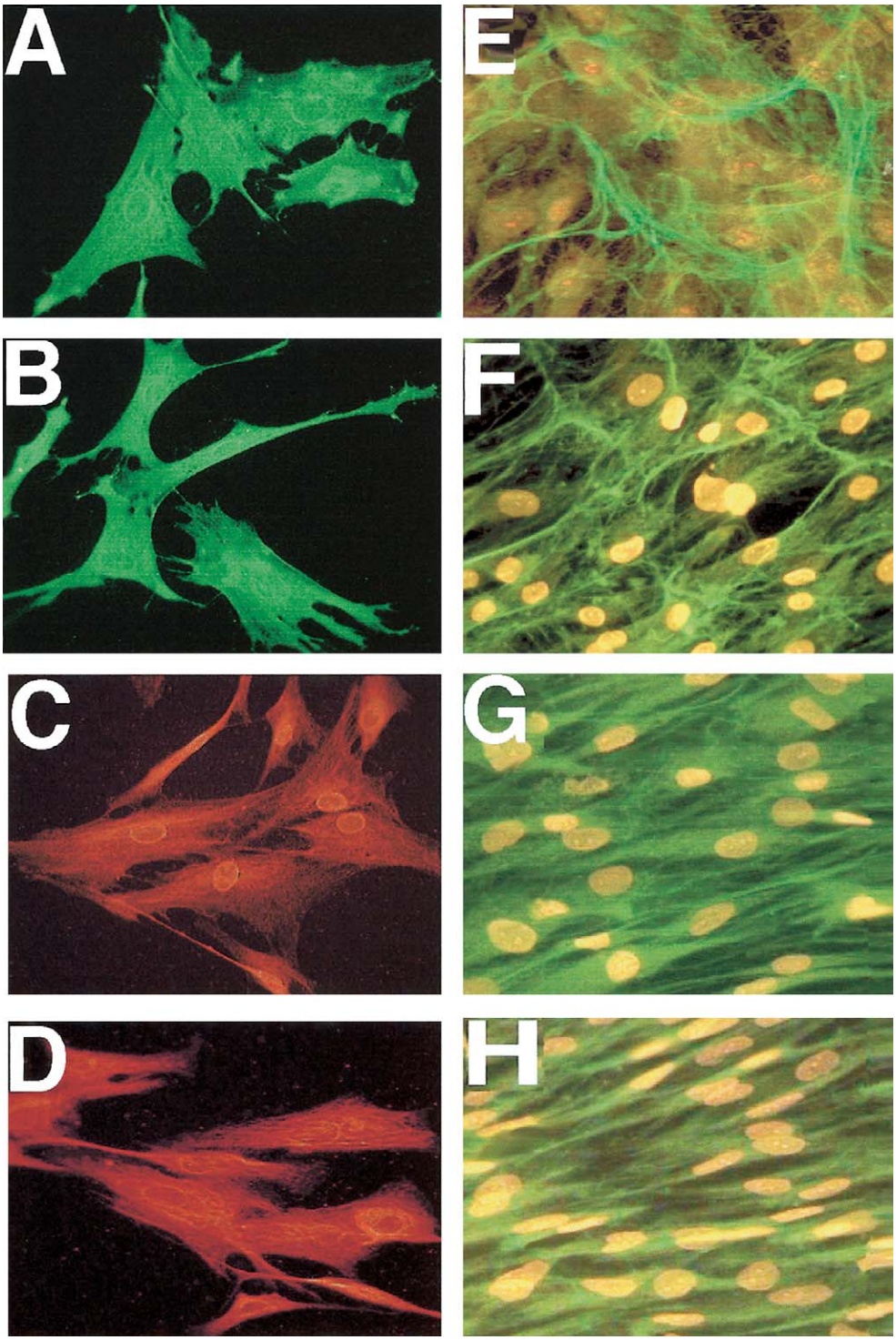

Figure 3 *A–D,* Representative high-power photomicrographs depicting immunostaining with anti–S-Gal antibody, which recognizes EBP. Normal fibroblasts (*A*) and GM1-gangliosidosis fibroblasts bearing missense mutations of the β-Gal gene (*B*) demonstrate strong cell-surface expression of this protein, whereas GM1-gangliosidosis fibroblasts bearing nonsense mutations of the β-Gal gene (*C*) and Morquio B fibroblasts (*D*) show no immunodetectable S-Gal/EBP. *E–H,* Representative photomicrographs of 10-d-old cultures immunostained with anti-tropoelastin antibody, indicating that normal fibroblasts (*E*) and fibroblasts bearing missense mutations of the β-Gal gene (*F*) produce long, branching elastic fibers, whereas fibroblasts from patients with GM1-gangliosidosis bear nonsense mutations of the β-Gal gene (*G*), and Morquio B fibroblasts (*H*) do not deposit any extracellular elastin.

lagen type I, laminin, and fibronectin (fig. 4). It should also be stressed that all tested fibroblasts displayed an equally good immunodetectable expression of lysyl oxidase, an enzyme responsible for tropoelastin cross-linking (data not shown).

Impaired deposition of insoluble elastin by the S-Gal–deficient fibroblasts visualized by immunocytochemistry was additionally confirmed by metabolic tests. Quantitative assessment of [^{3}H]-valine incorporation into immunoprecipitable soluble tropoelastin extracted jointly from the cell layers and from the conditioned media showed that all tested Morquio B disease and GM1-gangliosidosis fibroblasts synthesized amounts of tropoelastin comparable to those seen in normal fibroblasts (fig. 5*A*). Selective immunoprecipitation of metabolically labeled tropoelastin from the cell layers showed that, in contrast to cultures of normal fibroblasts and GM1-gangliosidosis cells that bear missense mutations of β-Gal gene, the cell-layer fractions of fibroblasts from S-Gal–deficient patients retained the majority of the radioactive tropoelastin intracellularly. Cultures of these cells consistently incorporated very little [^{3}H]-valine into NaOH-insoluble elastin. These results indicate that the deficiency in S-Gal did not affect normal tropoelastin synthesis but that it did correlate with impaired tropoelastin secretion and extracellular assembly into insoluble elastin, a major component of elastic fibers.

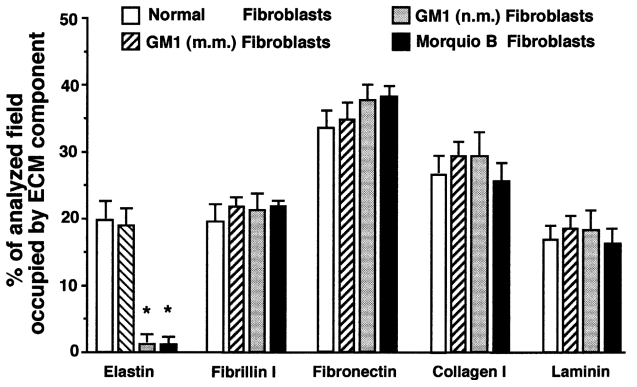

Figure 4 Morphometric analysis of extracellular-matrix components immunostained with specific antibodies in 10-d-old cultures of normal, GM1-gangliosidosis, and Morquio B fibroblasts. Fibroblasts from GM1-gangliosidosis fibroblasts bearing nonsense mutations of the β-Gal gene (n.m.), as well as Morquio B fibroblasts deposit only negligible amounts of immunodetectable extracellular elastin. The amounts of fibrillin 1, fibronectin, collagen type I, and laminin produced by those fibroblasts do not differ from what is produced by either normal fibroblasts or GM1-gangliosidosis fibroblasts bearing missense mutations (m.m.) of the β-Gal gene. In each group, 50 low-power (20 ×) fields from three separate cultures (per independent patient) were analyzed, and the area occupied by the particular immunodetectable component was quantified. The abundance of each component was then expressed as a percentage (mean ± SD) of the entire analyzed field, and results from cultures of GM1-gangliosidosis fibroblasts and Morquio B fibroblasts were statistically compared with those in cultures of normal skin fibroblasts. An asterisk (*) denotes $P < .001$.

Uptake of Exogenous S-Gal—but Not Enzymatically Active β-Galactosidase—by S-Gal–Deficient Morquio B and GM1-Gangliosidosis Fibroblasts: Improvement in Assembly of Elastic Fibers

Both immunocytochemistry and metabolic labeling indicated that S-Gal–deficient fibroblasts (patients 4032 and 4080) restored deposition of elastic fibers when their physical contact was maintained with cocultured CHO cells permanently transfected with the S-Gal cDNA but that this was not the case with untransfected CHO cells (figs. 5*B* and 6*A–D*). Such restoration of elastin deposition by these fibroblasts correlated with the appearance of their immunostaining with anti–S-Gal antibody (fig. 6). S-Gal–deficient fibroblasts did not display any immunostaining with anti–S-Gal antibody when they were cocultured with nontransfected CHO cells. These data indicate that the cell-surface–targeted S-Gal protein produced by CHO cells was translocated to the adjacent fibroblasts, on the close physical contact between both cell types. Interestingly, delivery of active β-Gal, secreted from CHO cells expressing the enzyme and also taken up by tested fibroblasts, did not enhance their ability to deposit insoluble elastin (fig. 6*E–H*).

Discussion

Elastin is often thought to be produced during the late stages of fetal life and during the perinatal period (Cleary et al. 1967; Mecham and Hauser 1991; Rosenbloom et al. 1993; Pasquali-Ronchetti and Baccarani-Contri 1997). In situ hybridization has revealed that tropoelastin mRNA is expressed in the early stages of chick-embryo development (Selmin et al. 1991; Holzenberger et al. 1993). Studies by Hurle et al. (1994) also demonstrated the presence of elastic fibers during early morphogenesis of the limb skeleton and suggested that the elastic-fiber scaffold plays an important role in coordinating the size, shape, and spatial location of the cartilaginous skeletal elements within the limb buds. Hurle et al. also observed precise patterns of elastic-fiber arrangement present in the outflow tract and atrioventricular-cushion tissue of the heart, the early developing lung, the notochord, and the somites. These observations pointed to previously unsuspected functions for elastic matrices during embryonic development and substantiated our hypothesis—that is, that impaired elastogenesis in the developing skeleton, heart, or connective tissue may be a common denominator in the pathophysiology

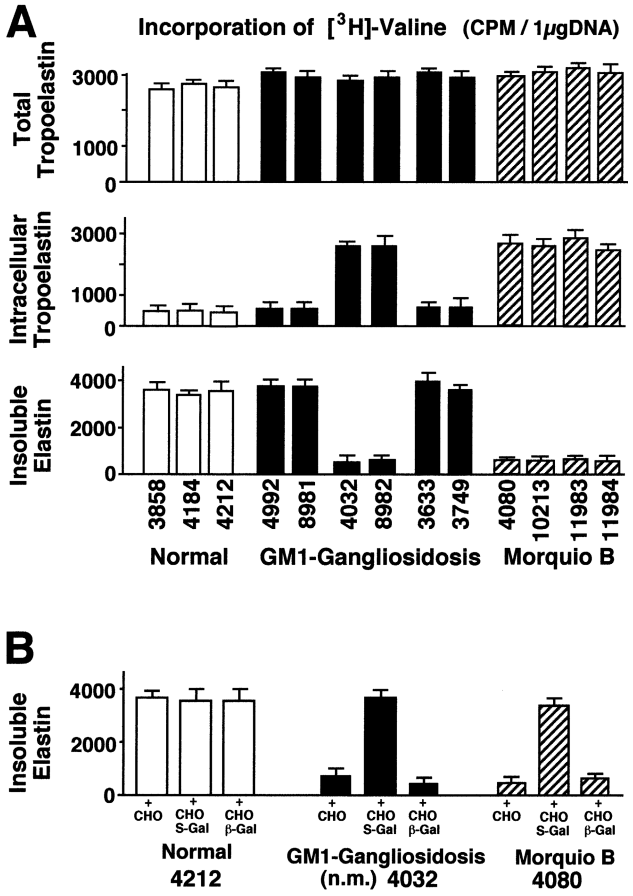

Figure 5 *A, upper graph,* Quantitative analysis of [^{3}H]-valine–labeled immunoprecipitable tropoelastin, indicating that all tested fibroblasts (i.e., normal, GM1-gangliosidosis, and Morquio B) synthesize comparable amounts of total metabolically labeled tropoelastin. *A, middle graph,* Fibroblasts from normal individuals (3858, 4184, and 4212), infantile GM1-gangliosidosis fibroblasts with missense mutations of the β-Gal gene (4992 and 8981), and fibroblasts from the adult form of GM1–gangliosidosis (3633 and 3749), which retained very little newly produced tropoelastin intracellularly. In contrast, fibroblasts from patients with infantile GM1-gangliosidosis that bear nonsense mutations of the β-Gal gene (4032 and 8982), as well as Morquio B fibroblasts (4080, 10213, 11983, and 11984) retain the majority of their metabolically labeled tropoelastin intracellularly. *A, lower graph,* S-Gal–deficient fibroblasts, all of which incorporate very little [^{3}H]-valine into extracellular insoluble elastin. *B,* Metabolic labeling of cultured fibroblasts with [^{3}H]-valine, followed by quantitative analysis of radiolabeled NaOH-insoluble residues, demonstrating that S-Gal–deficient fibroblasts (4032 and 4080) show normal deposition of insoluble elastin when maintained in the mixed culture with CHO cells permanently transfected with the S-Gal cDNA but do not do so with untransfected CHO cells. Coculture of neither GM1-gangliosidosis fibroblasts bearing nonsense mutations of the β-Gal gene (4032) nor Morquio B fibroblasts with β-Gal cDNA-transfected CHO cells secreting enzymatically active precursor (taken up by these fibroblasts deficient in both variants of β-Gal) improved deposition of insoluble elastin.

of different inherited diseases with dysmorphic phenotypes. Moreover, inherited diseases that manifest skeletal deformities are often characterized by such common features as loose skin, ligamentous laxity, hernias, cardiomegaly, and cardiac valvular deformities, which further suggest a common disorder involving impaired elastic-fiber production.

Among all inherited diseases that affect the structure, distribution, and abundance of elastic fibers, only supravalvular aortic stenosis, Williams syndrome, and cutis laxa have been directly linked to primary alterations in the elastin gene (Curran et al. 1993; Ewart et al. 1993, 1994; Tassabehji et al. 1997). Other genetic diseases that severely affect elastic-fiber integrity (e.g., Marfan syndrome, pseudoxanthoma elasticum, and Menkes disease) have been linked either to primary defects in microfibrillar proteins or to errors in copper metabolism and lysyl oxidase (Sandberg et al. 1981; Lee 1991; Mecham and Hauser 1991; Rosenbloom et al. 1993; Christiano and Uitto 1994; Pasquali-Ronchetti and Baccarani-Contri 1997; Vrhovski and Weiss 1998; Debell and Tamburro 1999). Since the 67-kD EBP that plays a crucial role in elastogenesis has been definitively characterized as an inactive spliced variant of β-Gal, we investigated the possibility that either the functional inactivation of S-Gal/EBP or faulty primary expression of the β-Gal gene could result in impaired elastic-fiber assembly. This is in keeping with our recent studies demonstrating that excessive tissue accumulation of dermatan sulfate in Hurler disease, which causes constant shedding of the EBP, leads to faulty elastogenesis (Hinek and Wilson 2000). Disruption of elastic-fiber production was demonstrated also in cultures of normal fibroblasts, chondroblasts, and arterial smooth muscle experimentally depleted of the EBP, after either treatment with exogenous lactose, galactose, chondroitin sulfate, and dermatan sulfate or blocking of this tropoelastin chaperone by anti–S-Gal antibody (Hinek et al. 1988, 1991, 1992, 1993).

In the present study, we have used (*a*) skin fibroblasts from four patients with Morquio B disease that bear β-Gal gene mutations (G438E, N484K/T500A, and W273L/R482H) localized to the coding region common to lysosomal enzyme and S-Gal/EBP protein and (*b*) fibroblasts from two patients with GM1-gangliosidosis that bear nonsense mutations (R351X and R475X), which also destabilize both β-Gal and S-Gal mRNAs and which, consequently, may lead to deficiency in both protein variants. We hypothesized that these cells would not produce S-Gal and that such primary deficiency of this tropoelastin chaperone would be sufficient for impaired elastogenesis. We also tested expression of S-Gal/EBP and elastic-fiber formation in cultures of (*a*) skin fibroblasts obtained from two patients with GM1-gangliosidosis that bear a missense R148S mutation local-

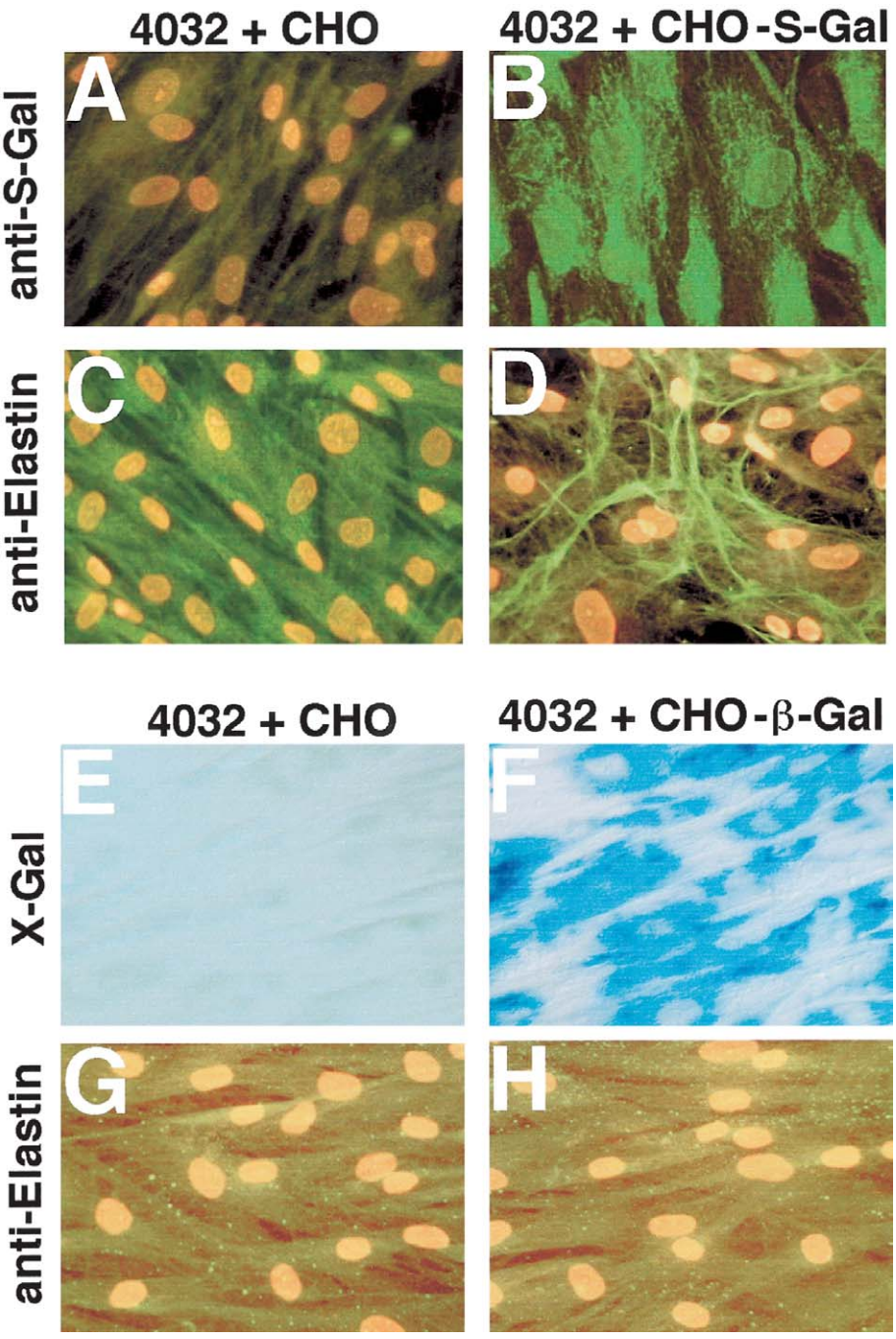

Figure 6 *A–D,* Representative micrographs depicting 10-d-old GM1-gangliosidosis fibroblasts bearing nonsense mutations of the β-Gal gene (4032) maintained in mixed culture, with CHO cells permanently transfected with the S-Gal cDNA clone (*B* and *D*) or with nontransfected CHO cells (*A* and *C*). Immunostaining with anti–S-Gal antibody and FITC-conjugated secondary antibody indicates that cultured 4032 fibroblasts cocultured with untransfected CHO cells do not display any S-Gal antigen (*A*). In contrast, the same fibroblasts cocultured with CHO cells permanently transfected with the S-Gal cDNA demonstrate the presence of green fluorescence–marked S-Gal protein (*B*). Immunostaining of the parallel cultures with anti-elastin antibody and FITC-conjugated secondary antibody indicates that 4032 fibroblasts restored deposition of elastic fibers when cocultured with S-Gal cDNA–transfected CHO cells (*D*) but not with untransfected controls (*C*). Note that CHO cells that were loosely siting on top of the fibroblast layers for the duration of the experiment were washed out just before fixation. *E–H,* Histochemical detection of β-Gal activity, marked by the dark-blue precipitate of X-Gal substrate, indicating that GM1-gangliosidosis fibroblasts bearing nonsense mutations of the β-Gal gene (4032) uptake enzymatically active β-Gal precursor secreted from β-Gal cDNA–transfected CHO cells (*F*) but not from untransfected CHO cells (*E*). Immunostaining of parallel cultures with anti-elastin antibody indicate that 4032 fibroblasts cocultured with either untransfected CHO cells (*G*) or β-Gal cDNA–transfected CHO cells (*H*) were incapable of normal deposition of elastic fibers.

ized to the exon 4 of the β-Gal gene that is spliced out of S-Gal and that might not affect synthesis of S-Gal/EBP protein and (*b*) fibroblasts from two patients with an adult form of GM1-gangliosidosis that did not display any connective-tissue problems.

Both immunocytochemistry (fig. 3*C* and *D*) and metabolic pulse labeling of cultured fibroblasts (fig. 5) clearly demonstrated that tested Morquio B disease fibroblasts and fibroblasts from patients with GM1-gangliosidosis that bear a nonsense mutations of the β-Gal gene did not synthesize S-Gal at all, attesting to their primary deficiency of EBP. Further immunochemical and biochemical studies indicated a strong correlation between deficiency in S-Gal and a selective impairment of elastic-fiber production. We found that neither GM1-gangliosidosis cells bearing nonsense mutations of the β-Gal gene nor Morquio B cells bearing mutations resulting in deficiency in both mature β-Gal and S-Gal produced elastic fibers. This was in contrast to GM1-gangliosidosis fibroblasts bearing missense mutations in the β-Gal gene, which were deficient only in the lysosomal variant of β-Gal but synthesized normal amounts of S-Gal and demonstrated normal deposition of insoluble elastin. Impaired production of elastic fibers by S-Gal–deficient fibroblasts appears to be very distinctive, since deposition of fibronectin, laminin, and collagen type I was not affected. It should also be mentioned that all tested cells derived from the syndromes discussed above synthesized amounts of the [^{3}H]-valine–labeled soluble tropoelastin that were similar to the levels seen in cells taken from healthy individuals, which produced an abundant network of microfibrils (marked by normal distribution of fibrillin-1), which is required for extracellular assembly of secreted tropoelastin into elastic fibers. Moreover, immunocytochemistry with an antibody to lysyl oxidase also tested for normal expression of this enzyme responsible for extracellular cross-linking of newly deposited tropoelastin monomers into an insoluble elastin polymer.

These data excluded the possibility of a primary deficiency in any of the major components that normally build elastic fibers. Our results clearly indicate, however, that newly synthesized tropoelastin could not be properly secreted from the S-Gal–deficient cells and, consequently, assembled into extracellular elastic fibers.

The exquisite importance that the S-Gal/EBP has for the processes of normal elastogenesis was additionally confirmed by results indicating that S-Gal–deficient fibroblasts (patients 4032 and 4080) were capable of normal deposition of the elastic fibers when cocultured with CHO cells permanently transfected with S-Gal cDNA clone. Restoration of elastin deposition by those fibroblasts correlated with the immunodetectable presence of S-Gal protein at their cell surface and indicates that the S-Gal molecules produced by CHO cells were translocated to the adjacent fibroblasts. Since the S-Gal/EBP has been identified as a recyclable and reusable tropoelastin chaperone (Hinek et al. 1995), it is possible that this protein, when taken up to the endosomal compartment of fibroblasts, resumes its basic function, binds tropoelastin, and facilitates its secretion and assembly. That there is specific benefit of such S-Gal delivery is reinforced by the fact that intracellular uptake of enzymatically active wild-type β-Gal by Morquio B and GM1-gangliosidosis fibroblasts did not improve their deposition of insoluble elastin.

Since the presence of elastic fibers in the limb buds and their primitive perichondrial tissue has been suggested as a crucial factor responsible for maintaining the proper shape of embryonal skeleton (Hurle et al. 1994), our results indicate that cellular deficiency, in S-Gal, leading to disruption of elastic-fiber formation may be relevant to the skeletal deformations observed in Morquio B disease and in selected cases of GM1-gangliosidosis. However, further studies, using fetal autopsy tissues, would be required in order to confirm this possibility.

Until recently, no one has been able to correlate the presence of cardiac abnormalities with specific genetic lesions of the β-Gal gene. A recent report by Morrone et al. (2000) has described six newly diagnosed patients with GM1-gangliosidosis with cardiac involvement who were homozygous for either of the following mutations: R59H, Y591C, Y591N, or IVS14−2A→G, occurring in the coding region common to lysosomal enzyme and S-Gal/EBP protein. The authors of that report therefore have concluded that the cardiac involvement in these patients is likely caused by an alteration at the homozygous state of the expression of EBP. This report strongly reinforces a pathophysiological link between S-Gal deficiency and cardiomyopathy, which also has been observed in other genetic diseases characterized by impaired elastogenesis (Haust 1987; Dangel 1998; Hinek and Wilson 2000). The results reported by Morrone et al. (2000) also are consistent with our present data indicating that fibroblasts from two cases of infantile GM1-gangliosidosis that demonstrated skeletal and cardiac abnormalities (fibroblasts bearing a nonsense mutations of β-Gal gene) were deficient in S-Gal protein and did not assemble elastic fibers in vitro. We therefore suggest that S-Gal deficiency resulting in impaired elastogenesis might contribute to lack of properly assembled elastin fibers in the heart, thereby resulting in an aberrant connective-tissue framework allowing for hypertrophic growth of cardiomyocytes.

It has to be mentioned that donors of the S-Gal–deficient fibroblasts (i.e., patients with either Morquio B disease or GM1-gangliosidosis) did not demonstrate any disseminated elastopathy in arteries, lung, or skin. The preservation of elastic fibers in these tissues seems

to be due to unknown compensatory mechanisms, which may derive either from the higher rate of tropoelastin biosynthesis and/or from activation of S-Gal–independent routes of tropoelastin secretion and assembly. Elsewhere, it has been shown that tropoelastin can also bind to other intracellular chaperones, such as BIP and the peptidyl-prolyl cis-trans isomerase (Davis et al. 1998). Moreover, Saunders and Grant (1985) demonstrated that, in arterial SMC, 30%–40% of all synthesized tropoelastin is not transported through the endosomes and Golgi apparatus but can be rapidly secreted through vesicles budding off the rough endoplastic reticulum in close proximity to the plasma membrane. Thus, it is possible that, in certain cell types, this mechanism can compensate for the S-Gal deficiency and can assure production of normal-looking elastic fibers. Nothing is known, however, about the durability of such elastic fibers, which might be compromised over time and cause clinical problems in longer-living patients.

In summary, the present study has provided a novel and natural model for exploration of the functional significance of the nonlysosomal 67-kD alternative spliced variant of β-Gal. Our data demonstrating that fibroblasts bearing natural β-Gal gene mutations that cause deficiency in this protein do not assemble elastic fibers ultimately confirm that the expression of S-Gal is a prerequisite for normal elastogenesis. We suggest that the link between S-Gal deficiency and impaired elastogenesis can be relevant to the pathomechanism of characteristic clinical features observed in patients with Morquio B disease patients and in those patients with infantile GM1-gangliosidosis with connective-tissue disorders, skeletal deformation, and cardiomyopathy.

Acknowledgments

This work was supported by Medical Research Council of Canada grants PG 13920 and MT 13719 and by a grant from the Heart and Stroke Foundation of Canada. A.H. is a Career Investigator of the Heart and Stroke Foundation of Ontario.

Electronic-Database Information

Accession numbers and the URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for infantile [MIM 230500], juvenile [MIM 230600], and adult [MIM 230650]) GM1-gangliosidosis and Morquio B disease [MIM 253010])

References

Alroy J, Knowles K, Schelling SH, Kaye EM, Rosenberg AE (1995) Retarded bone formation in GM1-gangliosidosis: a study of the infantile form and comparison with two canine models. Virchows Arch 426:141–148

Alroy J, Orgad U, DeGasperi R, Richard R, Warren CD, Knowles K, Thalhammer JG, et al (1992) Canine GM1-gangliosidosis: a clinical, morphologic, histochemical, and biochemical comparison of two different models. Am J Pathol 140:675–689

Arbisser Al, Donnelly KA, Scott CI, DiFerrante N, Singh J, Stevenson RE, Aynesworth AS, et al (1977) Morquio-like syndrome with β-galactosidase deficiency and normal hexosamine sulfatase activity: mucopolysaccharidosis IVB. Am J Med Genet 1:195–205

Benson PF, Barbarik A, Brown SP, Mann TP (1976) GM1-generalized gangliosidosis variant with cardiomegaly. Postgrad Med J 52:159–165

Callahan JW (1999) Molecular basis of GM1 gangliosidosis and Morquio disease, type B: structure- function studies of lysosomal beta-galactosidase and the non-lysosomal beta-galactosidase-like protein. Biochim Biophys Acta 1455: 85–103

Charrow J, Hvizd MG (1986) Cardiomyopathy and skeletal myopathy in an unusual variant of GM1 gangliosidosis. J Pediatr 108:729–732

Christiano AM, Uitto J (1994) Molecular pathology of the elastic fibers. J Invest Dermatol Suppl 103:53S–57S

Cleary EG, Sandberg LB, Jackson DS (1967) The changes in chemical composition during development of the bovine nuchal ligament. J Cell Biol 33:469–479

Curran ME, Atkinson DL, Ewart AK, Morris CA, Leppert MF, Keating MT (1993) The elastin gene is disrupted by a translocation associated with supravalvular aortic stenosis. Cell 73:159–168

D'Agrosa RM, Hubbes M, Zhang S, Shankaran R, Callahan JW (1992) Characteristics of the beta-galactosidase-carboxypeptidase complex in GM1-gangliosidosis and beta-galactosialidosis fibroblasts. Biochem J 285:833–838

Dangel JH (1998) Cardiovascular changes in children with mucopolysaccharide storage diseases and related disorders—clinical and echocardiographic findings in 64 patients. Eur J Pediatr 157:534–538

Davis EC, Broekelmann TJ, Ozawa Y, Mecham RP (1998) Identification of tropoelastin as a ligand for the 65-kD FK506-binding protein, FKBP65, in the secretory pathway. J Cell Biol 140:295–303

Debell L, Tamburro AM (1999) Elastin: molecular description and function. Int J Biochem Cell Biol 31:261–272

Ewart AK, Jin W, Atkinson D, Morris CA, Keating MT (1994) Supravalvular aortic stenosis associated with a deletion disrupting the elastin gene. J Clin Invest 93:1071–1077

Ewart AK, Morris CA, Atkinson D, Jin W, Sternes K, Spallone P, Stock AD, et al (1993) Hemizygosity at the elastin locus in a developmental disorder, William's syndrome. Nat Genet 5:11–16

Factor SM, Biempica L, Goldfisher S (1978) Coronary intimal sclerosis in Morquio's syndrome. Virchovs Arch A Pathol Anat Histol 379:1–10

Gibson MA, Hatzinikolas G, Kumaratilake JS, Sandberg LB, Nicholl JK, Sutherland GR, Cleary EG (1996) Further characterization of proteins associated with elastic fiber micro-

fibrils including the molecular cloning of MAGP-2. J Biol Chem 271:1096–1103

Giugliani R, Jackson M, Skinner SJ, Vimal CM, Fensom AH, Fahmy N, Sjovall A, et al (1987) Progressive mental regression in siblings with Morquio disease type B (mucopolysaccharidosis IV B). Clin Genet 32:313–325

Groebe H, Krins M, Schmidberger H, von Figura K, Harzer K, Kresse H, Paschke E, et al (1980) Morquio syndrome (mucopolysaccharidosis IV B) associated with beta-galactosidase deficiency: report of two cases. Am J Hum Genet 32:258–272

Hadley RN, Hagstrom JW (1971) Cardiac lesions in a patient with familial neurovisceral lipidosis (generalized gangliosidosis). Am J Clin Pathol 55:237–240

Haust MD (1987) Arterial involvement in genetic diseases. Am J Cardiovasc Pathol 1:231–285

Hinek A (1994) Nature and multiple functions of the 67-kD elastin-/laminin binding protein. Cell Adhes Commun 2: 185–193

——— (1996) Biological roles of the non-integrin elastin/laminin receptor. Biol Chem 377:471–480

Hinek A, Boyle J, Rabinovitch M (1992) Vascular smooth muscle cell detachment from elastin and migration through elastic laminae is promoted by chondroitin sulfate-induced "shedding" of the 67-kDa cell surface elastin binding protein. Exp Cell Res 203:344–353

Hinek A, Keeley FW, Callahan J (1995) Recycling of the 67-kDa elastin binding protein in arterial myocytes is imperative for secretion of tropoelastin. Exp Cell Res 220: 312–324

Hinek A, Mecham RP, Keeley F, Rabinovitch M (1991) Impaired elastin fiber assembly is related to reduced 67-kDa elastin binding protein in fetal lamb ductus arteriosus and in cultured aortic smooth muscle cells treated with chondroitin sulfate. J Clin Invest 88:2083–2094

Hinek A, Rabinovitch M (1993) Ductus arteriosus smooth muscle cells produce a 52 kDa tropoelastin associated with impaired assembly of elastic laminae. J Biol Chem 268: 1405–1413

——— (1994) 67-kDa Elastin-binding protein is a protective "companion" of extracellular insoluble elastin and intracellular tropoelastin. J Cell Biol 126:563–574

Hinek A, Rabinovitch M, Keeley F, Okamura-Oho Y, Callahan J (1993) The 67-kD elastin/laminin-binding protein is related to an enzymatically inactive, alternatively spliced form of beta-galactosidase. J Clin Invest 91:1198–1205

Hinek A, Smith AC, Cutiongco EM, Callahan JW, Gripp K, Weksberg R (2000) Decreased elastin deposition and high proliferation of fibroblasts from Costello syndrome are related to functional deficiency in the 67-kDa elastin binding protein. Am J Hum Genet 66:859–872

Hinek A, Wilson S (2000) A new insight into the mechanism of connective tissue disorders in Hurler syndrome: accumulation of dermatan sulfate causes inhibition of elastic fiber assembly and increased cell proliferation. Am J Pathol 156:925–938

Hinek A, Wrenn DS, Mecham RP Barondes SH (1988) The elastin receptor: a galactoside-binding protein. Science 239: 1539–1540

Holzenberger M, Lievre CA, Robert L (1993) Tropoelastin gene expression in the developing vascular system of the chicken: an in situ hybridization study. Anat Embryol (Berl) 188:481–492

Hurle JM, Corson G, Daniels K, Reiter RS, Sakai LY, Solursh M (1994) Elastin exhibits a distinctive temporal and spatial pattern of distribution in the developing chick limb in association with the establishment of the cartilaginous skeleton. J Cell Sci 107:2623–2634

Kagan HM, Vaccaro CA, Bronson RE, Tang SS, Brody JS (1986) Ultrastructural immunolocalization of lysyl oxidase in vascular connective tissue. J Cell Biol 103:1121–1128

Kielty CM, Shuttleworth CA (1995) Fibrillin-containing microfibrils: structure and function in health and disease. Int J Biochem Cell Biol 27:747–760

Kielty CM, Whittaker SP, Shuttleworth CA (1996) Fibrillin: evidence that chondroitin sulphate proteoglycans are components of microfibrils and associate with newly synthesised monomers. FEBS Lett 386:169–173

Kohlschutter A, Sieg K, Schulte FJ, Hayek HW, Goebel HH (1982) Infantile cardiomyopathy and neuromyopathy with beta-galactosidase deficiency. Eur J Pediatr 139:75–81

Lee B, Godfrey M, Vitale E, Hori H, Mattei MG, Sarfarazi M, Tsipouras P, et al (1991) Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. Nature 352:330–334

Mecham RP, Hauser J (1991) The elastic fiber in cell biology of extracellular matrix In: Hay E (ed) Cell biology of extracellular matrix, 2d ed. Plenum Press, New York, pp 79–109

Mecham RP, Hinek A, Cleary EG, Kucich U, Rosenbloom J (1988) Development of immunoreagents to ciliary zonules that react with protein components of elastic fibers. Biochem Biophys Res Commun 151:822–826

Mecham RP, Hinek A, Entwistle R, Wrenn DS, Griffin GL, Senior RM (1989) Elastin binds to a multifunctional 67-kilodalton peripheral membrane protein. Biochemistry 28: 3716–3722

Mecham RP, Whitehouse L, Hay M, Hinek A, Sheetz MP (1991) Ligand affinity of the 67 kDa elastin/laminin binding protein is modulated by the protein's lectin domain: visualization of elastin/laminin-receptor complexes with gold-tagged ligands. J Cell Biol 113:187–194

Morreau H, Galjart NJ, Gillemans N, Willemsen R, van der Horst GT, d'Azzo A (1989) Alternative splicing of beta-galactosidase mRNA generates the classic lysosomal enzyme and a beta-galactosidase-related protein. J Biol Chem 264: 20655–20663

Morrone A, Bardelli T, Donati MA, Giorgi M, Di Rocco M, Gatti R, Parini R, et al (2000) β-Galactosidase gene mutations affecting the lysosomal enzyme and the elastin binding protein in GM1-gangliosidosis patients with cardiac involvement. Hum Mutat 15:354–366

Mutoh T, Naoi M, NagatsuT, Takahashi A, Matsuoka Y, Hashizume Y, Fujiki N (1988) Purification and characterization of human liver beta-galactosidase from a patient with the adult form of GM1 gangliosidosis and a normal control. Biochim Biophys Acta 964:244–253

Mutoh T, Naoi M, Takahashi A, Hoshino M, Nagoi Y, Nagatsu T (1986) Atypical adult GM1 gangliosidosis: bio-

chemical comparison with other forms of primary beta-galactosidase deficiency. Neurology 36:1237–1241

Okamura-Oho Y, Zhang S, Hilson W, Hinek A, Callahan JW (1996) Early proteolytic cleavage with loss of a C-terminal fragment underlies altered processing of the beta-galactosidase precursor in galactosialidosis. Biochem J 313:787–794

Oshima A, Yoshida K, Shinimoto M, Fukuhara Y, Sakuraba H, Suzuki Y (1991) Human β-galactosidase gene mutations in Morquio B disease. Am J Hum Genet 49:1091–1093

Pasquali-Ronchetti I, Baccarani-Contri M (1997) Elastic fiber during development and aging. Microsc Res Tech 38:428–435

Privitera S, Prody CA, Callahan JW, Hinek A (1998) The 67-kDa enzymatically inactive alternatively spliced variant of beta-galactosidase is identical to the elastin/laminin-binding protein. J Biol Chem 273:6319–6326

Prosser IW, Whitehouse LA, Parks WC, Stahle-Backdahl M, Hinek A, Park PW, Mecham RP (1991) Polyclonal antibodies to tropoelastin and specific detection and measurement of tropoelastin in vitro. Connect Tissue Res 25:265–279

Rosenberg H, Frewen TC, Li MD, Gordon BL, Jung JH, Finlay JP, Roy PL, et al (1985) Cardiac involvement in diseases characterized by beta-galactosidase deficiency. J Pediatr 106: 78–80

Rosenbloom J, Abrams WR, Mecham RP (1993) Extracellular matrix: the elastic fiber. FASEB J 7:1208–1218

Sakai LY, Keene DR, Engvall E (1986) Fibrillin, a new 350-kDa glycoprotein is a component of extracellular microfibrils. J Cell Biol 103:2499–2509

Sandberg LB, Soskel NT, Leslie JG (1981) Elastin structure, biosynthesis, and relation to disease states. N Engl J Med 304:566–579

Saunders NA, Grant ME (1985) The secretion of tropoelastin by chick-embryo artery cells. Biochem J 230:217–225

Selmin O, Volpin D, Bressan GM (1991) Changes of cellular expression of mRNA for tropoelastin in the intraembryonic arterial vessels of developing chick revealed by in situ hybridization. Matrix 11:347–358

Singh MB, Knox RB (1984) Quantitative cytochemistry of beta-galactosidase in normal and enzyme deficient (gal) pollen of Brassica campestris: application of the indigogenic method. Histochem J 16:1273–1296

Skomorowski MA, Bagshaw R, Zhang S, Whelan D, Clarke JTR, Callahan JW (1999) Novel mutations (Asn484Lys, Thr500Ala, Gly438Glu) in Morquio B disease. J Inherit Metab Dis 22 Suppl 1:131

Suzuki Y, Sakuraba H, Oshima A (1995) β-Galactosidase deficiency (β-galactosidosis): GM1 gangliosidosis and Morquio B disease. In: Scriver CR, Beaudet A, Sly WS, Valle D (eds) Metabolic basis of inherited disease, 2d ed. McGraw-Hill, New York, pp 2785–2823

Tassabehji M, Metcalfe K, Donnai D, Hurst J, Reardon W, Burch M, Read AP (1997) Elastin: genomic structure and point mutations in patients with supravalvular aortic stenosis. Hum Mol Genet 6:1029–1036

van der Horst GT, Kleijer WT, Hoogeveen AT, Huijmans JG, Blom W, van Diggelen OP (1983) Morquio B syndrome: a primary defect inbeta-galactosidase. Am J Med Genet 16: 261–275

Vrhovski B, Weiss AS (1998) Biochemistry of tropoelastin. Eur J Biochem 258:1–18

Yamamoto Y, Hake CA, Martin BM, Kretz KA, Ahem-Rindell AJ, Naylor SL, Mudd M, et al (1990) Isolation, characterization, and mapping of a human acid beta-galactosidase cDNA. DNA Cell Biol 9:119–127

Zhang S, McCarter JD, Okamura-Oho Y, Yaghi F, Hinek A, Withers SG, Callahan JW (1994) Kinetic mechanism and characterization of human β-galactosidase precursor secreted by permanently transfected Chinese hamster ovary cells. Biochem J 304:281–288