Tissue-specific Changes in the Hydroxylysine Content and Cross-links of Collagens and Alterations in Fibril Morphology in Lysyl Hydroxylase 1 Knock-out Mice*

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We have generated mice with targeted inactivation of the Plod1 gene for lysyl hydroxylase 1 (LH1). Its human mutations cause Ehlers-Danlos syndrome VIA (EDS VIA) characterized by muscular hypotonia, joint laxity, and kyphoscoliosis. The Plod1 mice are flaccid and have gait abnormalities. About 15% of them died because of aortic rupture and smooth muscle cells in non-ruptured Plod1−/− aortas showed degenerative changes. Collagen fibrils in the Plod1−/− aorta and skin had an abnormal morphology. The LH activity level in the Plod1−/− skin and aorta samples was 35–45% of that in the wild type. The hydroxylysine content was decreased in all the Plod1−/− tissues, ranging from 22% of that in the wild type in the skin to 75 and 86% in the femur and lung. The hydroxylysylpyridinoline cross-links likewise showed decreases in all the Plod1−/− tissues, ranging from 28 and 33% of that in the wild type in the aorta and cornea to 47 and 59% in femur and tendon, while lysylpyridines were increased. The hydroxylysines found in the Plod1−/− collagens and their cross-links were evidently synthesized by the other two LH isoenzymes. Few data are available on abnormalities in EDS VIA tissues other than the skin. Plod1−/− mice offer an in vivo model for systematic analysis of the tissue-specific consequences of the lack of LH1 activity and may also provide a tool for analyzing the roles of connective tissue in muscle function and the complex interactions occurring in the proper assembly of the extracellular matrix.

Lysyl hydroxylase (LH, EC 1.14.11.4) catalyzes the hydroxylation of lysine residues mainly but not exclusively in -X-Lys-Gly- triplets in collagens and proteins with collagen-like sequences (1). The enzyme resides within the endoplasmic reticulum and has three human and mouse isoenzymes, LHs 1–3 (1–7). The hydroxylysine residues formed have two important functions: they are essential for the stability of the intermolecular cross-links that provide the collagen fibrils with their tensile strength and mechanical stability, and they serve as attachment sites for carbohydrate units, either the monosaccharide galactose or the disaccharide glucosylgalactose (1). Collagen cross-link formation occurs in the extracellular matrix and is initiated by the conversion of specific lysine or hydroxylysine residues in the telopeptides, i.e. the short non-triple helical ends of collagen molecules, into the aldehydes allysine or hydroxallysine, respectively, catalyzed by l-lysyl oxidase (8). The telopeptides are connected with the triple helical part of an adjacent molecule by difunctional immature cross-links in the characteristic staggered array of collagen molecules in a fibril. The three main fibril-forming collagens, types I, II, and III, have four cross-linking sites, one in each telopeptide and two in the triple helical region, close to its N- and C-terminal ends (9). If the residue in the telopeptide is hydroxallysine, the difunctional cross-links can mature into trifunctional non-reducible cross-links comprising lysylpyridinoline (LPs), derived from one lysine and two hydroxylysines, and hydroxylysylpyridinoline (HPs) derived from three hydroxylysines. Pyridinoline cross-links are abundant in bone, cartilage, ligaments, and tendons, but much less frequent in the skin (9). The functions of the hydroxylysine-linked carbohydrate units are not fully understood, but they have an important role in the regulation of collagen fibril formation and morphology (10) and the assembly of type IV collagen networks (11, 12).

Mutations in the human PLOD1 gene for LH1 cause subtype VIA, also known as the kyphoscoliotic subtype, of Ehlers-Danlos syndrome (EDS) (13–15). EDS is a heterogeneous group of heritable extracellular matrix disorders in which the typical findings are hyperextensibility of the skin, hypermobility of joints, and a generalized fragility of connective tissues (13, 14). Its current categorization includes six main subtypes and is based on inheritance and diagnostic criteria considered frequent in a certain subtype but infrequent in others, and partly also on genetic analyses. The typical features of EDS VIA are neonatal muscle hypotonia, progressive kyphoscoliosis, abnormal scarring and easy bruising of the skin, increased risk of fatal arterial ruptures and in some cases ocular fragility (13–15).
Over 20 mutations in the \textit{PLOD1} gene have been reported (15–17), but not all EDS VI patients carry \textit{PLOD1} mutations, and EDS VI is therefore divided into forms VIA with \textit{PLOD1} mutations and VIB with unknown defects.

Mutations in the \textit{PLOD2} gene for LH2 have been identified in patients with Bruck syndrome (18, 19), an autosomally recessively inherited disease characterized by fragile bones, congenital joint contractures, scoliosis and osteoporosis. Analysis of bone samples from patients with this syndrome has demonstrated underhydroxylation of lysine residues in collagen telopeptides leading to aberrant cross-linking, while the lysines in the triple helical region are hydroxylated normally (20). No mutations in the human \textit{PLOD3} gene for LH3 have been characterized, but homozygous inactivation of the mouse \textit{Plod3} gene is lethal at E9.5 because of an abnormal aggregation of basement membrane-specific collagen IV (11, 12).

Biochemical data on patients with EDS VIA are currently limited almost entirely to the skin, where the hydroxylsine content is usually only about 5% of normal, whereas its content in the few bone and cartilage samples analyzed so far has been about 50 and 90%, respectively (13). These values suggest that the LH isoenzymes may differ in either expression or substrate specificities between tissues. We report here on the generation of mice with targeted inactivation of the \textit{Plod1} gene. The null mice were viable and fertile. They did not show kyphoscoliosis but were flaccid, a finding that resembles the muscular hypotonia of human EDS VIA. Sudden deaths, mainly due to aortic ruptures, were observed, especially among young null males. Ultrastructural analysis revealed alterations in collagen fibrils, and biochemical analyses demonstrated a deficiency in collagen hydroxylsine content and changes in the collagen cross-linking pattern, the extents of which varied markedly between tissues.

**EXPERIMENTAL PROCEDURES**

\textbf{Construction of the Targeting Vector and Gene Targeting in Embryonic Stem Cells}—A clone containing the \textit{Plod1} gene was isolated from a murine 129SV genomic library (Stratagene) using a human LH1 cDNA fragment (2) as a probe. The 1-kb short arm of the targeting construct (Fig. 1A) was generated by PCR with the genomic clone as a template, the 5’ (forward) primer is the intron 1 sequence CTGGGTACCTCGAGACGTGTTGCCAAGTGTTC with an introduced KpnI restriction site (underlined), and the exon 2 sequence CAGTAAGCTTAGGGTGTGGACAGAGAAG as the 3’ (reverse) primer. The long arm contained \textit{Plod1} exons 7–10 (Fig. 1A) and was isolated from the genomic clone as a 4.5-kb Ncol fragment. The \textit{lacz} neo cassette (21) was ligated into the short arm fragment so that the \textit{lacz} sequence was in-frame with the \textit{Plod1} sequences. The selection gene neo is regulated by the PGK promoter. Homologous recombination leads to deletion of a 5.5-kb \textit{Plod1} region containing exon 2, except for its first five nucleotides, with which the \textit{lacz} gene was fused, and exons 3–6 in their entirety.

The linearized construct was introduced into W4 embryonic stem (ES) cells (Taconic) and colonies that survived G418 selection were screened by PCR using the forward primer CCATCAAGCGAGAAGGT from intron 1 of the \textit{Plod1} gene and the reverse primer CTGGATCTTGTAGTGGAAAGAC from exon 2 for the wild-type allele, and the same forward primer with the reverse primer ACCCTGCCATAAGAAACTGT from the \textit{lacz} cassette for the mutant allele (Fig. 1A). Correct targeting in PCR positive cells was verified by Southern hybridization in which 20 \textmu g of genomic DNA was digested with HindIII and hybridized with a probe generated by PCR from intron 10, a region outside the targeting construct (Fig. 1A).

\textbf{Generation of Mutant Mice}—The correctly targeted clones were expanded and cells of three clones were injected into C57BL/6 blastocysts that were transferred into pseudopregnant recipients. The resulting chimeric males were bred with C57BL/6 females, and the heterozygous offspring were further backcrossed into a C57BL/6 background. Most of the analyses were performed with mice backcrossed for 4–5 generations. Two targeted mouse lines were generated. The genotyping was routinely performed by PCR using the same primer pairs as for the ES cell screening, and the results in the first litters of heterozygote crosses were confirmed by Southern blotting.

An ES Gene Trap cell line LH1: RST531, with a gene trap insertion in intron 11 of the \textit{Plod1} gene, produced by the Research Consortium BayGenomics, was obtained from the Mutant Mouse Resource Center at UC Davis. The cells were expanded, injected into C57BL/6 blastocysts, and heterozygous mice were generated as described above. Primers flanking the gene trap insertion site, a forward primer CCTTGCCGGTTCAGGACC from exon 11 of \textit{Plod1} and a reverse primer CACGACGCCCTGAC from exon 12, producing a 500-bp fragment from the wild-type allele, and the same forward primer and a reverse primer CACTCCAACCTCCGCAAACCTC from the \textit{lacz} gene, producing a 500-bp fragment from the gene trap allele, were used for genotyping. All the animal experiments were performed under an approved license from the Animal Ethical Committee of the University of Oulu.

\textbf{RT-PCR}—For RNA isolation, hearts were quickly dissected, snap-frozen in liquid nitrogen, and stored at −70°C. Total RNA was isolated using the Tripure reagent (Roche Applied Science), dissolved in sterile water, and the concentration was estimated by UV spectroscopy. For RT-PCR, the first strand was synthesized with random hexamer primers using 100 ng of RNA as a template. One-tenth of the volume of the first strand reaction was used in the subsequent PCR with the \textit{Plod1}-specific primers GAAAGCCACCAACAAACTTC from exon 10 and ACAGACGGGCCCCTGAC from exon 12, GCCCAAGTGTGATCTAGCC from exon 18 and AGGGATCGAGAAAAGACA from exon 19, and with the glyceraldehyde-3-phosphate dehydrogenase (\textit{G3PDH})-specific primers TTCACACACCCCTTGGCTGTAG and GACCACAGTCCATGCCACTC.

\textbf{Histological Analysis}—After sacrifice of the mice the tissues were dissected, rinsed briefly in 0.15 M NaCl and 0.02 M phosphate, pH 7.4 (phosphate-buffered saline, PBS) and fixed in 10% formalin in phosphate buffer, pH 7.0. Autopsy samples were fixed in a similar manner. After rinsing in water, the tissues were transferred to 70% ethanol, dehydrated, and embedded in paraffin. Sections of 5–10 \textmu m were cut and stained with hematoxylin-eosin or Masson’s trichrome method. For the detection of \textbeta-galactosidase activity, tissue sections were first rinsed in phosphate-buffered saline followed by fixation in 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl\textsubscript{2} in 0.1 M potassium phos-
phate buffer, pH 7.3, for 1 h. After washes in 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM EGTA, 2 mM MgCl₂, and 0.1 M potassium phosphate buffer, pH 7.3, the tissues were stained overnight at room temperature in 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 10 mM K₃Fe(CN)₆, 10 mM K₄Fe(CN)₆, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM EGTA, 2 mM MgCl₂, and 0.1 M potassium phosphate buffer, pH 7.3, fixed and processed as described above and stained with hematoxylin-eosin.

Transmission Electron Microscopy—Skin samples taken from the lower back and aortic samples from the descending aorta were fixed in a 1% glutaraldehyde and 4% formaldehyde mixture in 0.1 M phosphate buffer, pH 7.4, at room temperature for a minimum of 12 h and postfixed in 1% osmium tetroxide. The eyes were first fixed whole and punctured with a thin needle in the posterior after 1 h to ensure complete fixation. After several hours of fixation the corneas were dissected and processed as above. The samples were dehydrated in acetone and embedded in Epon Embed 812 (Electron Microscopy Sciences, Fort Washington, PA). To select areas for further analysis, semithin sections were stained with toluidine blue and examined by light microscopy. Thin sections (80 nm) were then cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Vienna, Austria), stained with uranyl acetate and lead citrate and examined in a Philips CM100 transmission electron microscope. Images were captured using a CCD camera. Fibril diameters were measured in the region subjacent to the epidermis of the skin and in the media subjacent to the adventitia of the aorta with an original magnification of 24,000 and analyzed with Southern hybridization (Fig. 1A). Homologous recombination led to the deletion of exons 3–6 and fusion of the 129-bp 5′ Plod1 sequence into the bacterial β-galactosidase gene, followed by a stop codon and polyadenylation signal. The message starting from the Plod1 promoter thus encoded the β-galactosidase. Correct targeting was assessed by PCR and confirmed by Southern hybridization (Fig. 1B) and inactivation of the Plod1 gene was verified by RT-PCR using exon 10 and 12-specific primers and heart RNA as a template (Fig. 1C). The absence of any splice variant that might encode a polypeptide with partial LH activity was confirmed further by using exon 18- and 19-specific primers and the same heart RNA (details not shown). These two exons encode the two histidines, one asparagine and one arginine that bind the iron and 2-oxoglutarate substrates that are essential for enzyme activity (25, 26). Heterozygous mice were generated by routine methods and backcrossed into C57BL/6. Two separate mouse lines were generated, both having the same phenotype. Cross-breeding of heterozygous mice produced all three genotypes in the Mendelian ratio (details not shown). These mouse lines were used in all other experiments except those involving X-gal staining (below).

A mouse line carrying a gene trap insertion in the Plod1 gene was generated from the ES cell line LH1:RST531, produced by the research consortium BayGenomics. The gene trap construct contained the Engrailed2 intron 1 and exon 2 sequences (the splice acceptor) as a leader fused to a βgeo reporter selection cassette was fused in-frame into exon 2 (Fig. 1A). Homologous recombination led to the deletion of exons 3–6 and fusion of the 129-bp 5′ Plod1 sequence into the bacterial β-galactosidase gene, followed by a stop codon and polyadenylation signal. The message starting from the Plod1 promoter thus encoded the β-galactosidase. Correct targeting was assessed by PCR and confirmed by Southern hybridization (Fig. 1B) and inactivation of the Plod1 gene was verified by RT-PCR using exon 10 and 12-specific primers and heart RNA as a template (Fig. 1C). The absence of any splice variant that might encode a polypeptide with partial LH activity was confirmed further by using exon 18- and 19-specific primers and the same heart RNA (details not shown). These mouse lines were used in all other experiments except those involving X-gal staining (below).

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Expression of LH1 as Detected by X-Gal Staining in Tissues—X-Gal staining for β-galactosidase reporter activity was weak or lacking in the Plod1⁻/⁻ samples, whereas intense, specific staining was seen in samples of the LH1:RST531 gene trap mouse tissues (Fig. 2). The staining was typically observed in cells producing fibrillar collagens, intense staining seen in fibroblasts of the lungs (Fig. 2A) and the skin, for instance,
In tendons staining was seen in the fibroblasts (Fig. 2). Weaker staining was observed in the heart muscle (not shown). In the auricles, the fibrous extensions of the atria (Fig. 2C), a collagen fibers were stained in the cornea (Fig. 2D). Furthermore, the keratocytes arranged between the lamellae of the surrounding muscle only epimysium and some of the nuclei were stained. In bone, strong staining was detected in the periosteum, chondrocytes, and osteoblasts (Fig. 2F). Where papilla of the hair follicles and arrector pili muscles also showed staining (Fig. 2B). In the heart, strong staining was seen in the auricles, the fibrous extensions of the atria (Fig. 2C), a weaker staining was observed in the heart muscle (not shown). In tendons staining was seen in the fibroblasts (Fig. 2D), while in the surrounding muscle only epimysium and some of the nuclei (not shown) were stained. In bone, strong staining was detected in the periosteum, chondrocytes, and osteoblasts (Fig. 2E). Furthermore, the keratocytes arranged between the lamellae of collagen fibers were stained in the cornea (Fig. 2F).

Hypotonia in Plod1−/− Mice—The LH1-null mice were viable and showed no kyphoscoliosis or other obvious abnormalities when observed in the cage environment, except that they appeared to be somewhat slower than their wild-type or heterozygous littermates. A distinct feature was noticed when the null mice were handled, however, in that they were passive and felt soft and floppy. Experienced persons could identify the null mice simply by lifting them gently by the tail, because even though they tried to struggle, their movements were powerless relative to those of wild-type mice. This feature became more obvious when the mice gained weight. When tested for walking on a rod or on top of the wall of a plastic container, the wild-type mice had no difficulties (Fig. 3, A and C), whereas some of the Plod1−/− mice did not move at all and others were able to proceed without problems only for a short while and tided quickly (Fig. 3, B and D). One Plod1−/− mouse was sacrificed in the animal house because of “paralysis.” According to our observations, the difficulty in locomotion was most likely due to laxity or dislocation of the joints, enhanced by general weakness of the muscles.

Aortic Ruptures in Plod1−/− Mice—Although most of the null mice survived to old age, some sudden deaths were observed. A total of 17% of the Plod1−/− males (21/122) and 9% of the Plod1−/− females (8/88) died before the age of one year, most at 1–4 months of age, whereas all their wild-type litter-

![Figure 1. Targeting the Plod1 gene. A, top shows part of the Plod1 gene, exons 2–12 are depicted as black boxes. Homologous recombination leads to in-frame insertion of the lacZ-neo cassette into exon 2 and deletion of a genomic region containing exons 3–6. The locations of the primers used for genotyping are indicated by thin horizontal bars, and the probe used in Southern analysis is depicted as a thicker bar. The arrow indicates the insertion site of the gene-trap construct in the LH1:RST531 ES cell line. B, Southern analysis of HindIII-digested genomic DNA isolated from mouse tissues. The probe hybridizes to a 12-kb HindIII fragment in the wild-type allele and to a 5.5-kb fragment in the mutant allele. Genotypes are indicated by black boxes. Southern analysis is depicted as a thicker bar. The arrow indicates the insertion site of the gene-trap construct in the LH1:RST531 ES cell line. C, control, no RNA template in the reaction.](http://www.jbc.org/content/282/9/6591)

![Figure 2. X-Gal staining of heterozygote LH1:RST531 mouse tissues. The tissues were stained with X-gal and counterstained with hematoxylin-eosin. A, lung fibroblasts show blue staining. B, in the skin, fibroblasts (open arrow), papilla of hair follicles (closed arrow), and arrector pili muscles (arrowhead) are stained. C, auricle of the heart shows intense staining. D, fibroblasts in the tendon are stained whereas staining is absent in the surrounding muscle. E, strong staining is observed in the bone periosteum (marked with P), chondrocytes (marked with C) and osteoblasts (arrow). F, in the cornea the keratocytes are stained. The tissues were from a 1-month-old male mouse (A and E) or a 2-month-old female (B, C, D, F). Scale bars, 50 μm in A and F, 100 μm in B–E.](http://www.jbc.org/content/282/9/6591)

![Figure 3. Gait abnormalities in Plod1−/− mice. 1-year-old female mice were placed on top of the wall of a plastic container. A and C, wild-type mice were able to grip onto the wall and proceed without problems. B and D, Plod1−/− mice were unable to hold onto the plastic, had difficulties in positioning their hind limbs and tided quickly.](http://www.jbc.org/content/282/9/6591)
mates were alive at that time. The deaths occurred during the night, when mice are most active, without any preceding signs of illness. Autopsies revealed hemorrhages in the thoracic or abdominal cavity in almost all cases (Table 1), and further examination showed aortic dissections (Fig. 4C) and in one case rupture in the media and an accumulation of cells subjacent to the adventitia, presumably as a consequence of a previous injury (Fig. 4D). The ruptures were detected between the external elastic lamellae, whereas the elastic lamellae themselves were not fragmented (Fig. 4, C and D).

No major histological alterations relative to the wild type were found in the structure or thickness of the aortic wall in the healthy Plod1-null mice (Fig. 4A), but the organization of their vascular smooth muscle cells appeared to be less regular. X-Gal staining showed that Plod1 was expressed in the cells of the tunica media and the adventitia of the aorta in the LH1:RST531 mouse line (Fig. 4B). Transmission electron microscopy revealed degenerative changes such as vacuolization and mitochondrial swelling in the smooth muscle cells of the aortic walls of the null mice without ruptures, even some completely degenerated cells being observed (Fig. 4F). Echocardiographic examination revealed no changes in the structure or function of the heart (data not shown).

Abnormal Collagen Fibrils in Plod1−/− Mice—Although one of the characteristic findings in EDS is soft skin with atrophic scars and easy bruising (13) no abnormal scars were found in the Plod1−/− mice, nor any obvious hypextensibility of the skin. The histology of the skin appeared to be comparable to the wild type in light microscopy (not shown). Because ultrastructural abnormalities in collagen fibrils are frequently found in skin biopsies of patients with other EDS subtypes (13), the analysis was continued by electron microscopy. The variation in the collagen fibril diameters was larger in both the aorta and skin samples of the Plod1−/− mice than in the wild type, and the mean fibril diameters were increased, being 67 nm and 91 nm in the Plod1−/− aorta and skin as opposed to 44 nm and 62 nm in the corresponding wild-type tissues (Fig. 5). Some fibrils in the aorta had highly irregular contours (Fig. 5B), and longitudinal sections showed the diameter to vary not only between fibrils but also within a single fibril, whereas the diameters were uniform in the control samples (not shown). Fibrils with irregular contours were also seen in the Plod1−/− skin samples, which additionally contained degrading fibrils (Fig. 5F). No variation in fibril diameter in the cornea was observed between the genotypes (not shown).

TABLE 1

Autopsy findings in 3-week- to 1-year-old Plod1−/− mice found dead without previous signs of illness

<table>
<thead>
<tr>
<th>Autopsy finding</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemorrhage in the thoracic cavity</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Hemorrhage in the abdominal cavity</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Hemorrhage in the colon</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nothing obvious</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Not analyzed*</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

* Discarded in the animal house.

FIGURE 4. Aortic abnormalities in Plod1−/− mice. A, no major histological abnormalities are observed in the aortic wall of a healthy-looking 1-month-old male Plod1−/− mouse. B, blue color upon X-gal staining, indicating high Plod1 expression, is seen in cells of the tunica media (open arrow) and adventitia (closed arrow) of a LH1:RST531 heterozygote mouse. C, aortic dissection in the necropsy sample of a 2-month-old Plod1−/− male mouse. D, scar-like structure (arrow) in the necropsy sample of another 2-month-old Plod1−/− male mouse. Dissection was detected in other sections of the same sample. E, transmission electron micrograph of the aortic wall of a wild-type mouse showing a smooth muscle cell (SMC) and the collagen (marked with C) and elastic layers (marked with E). F, degeneration of the aortic wall in a 1-month-old Plod1−/− male mouse. A degenerated smooth muscle cell (X) and a viable smooth muscle cell (SMC) with dilated endoplasmic reticulum (arrow) are shown. Aortic sections from 1-month-old male mice were stained by Masson’s trichrome method (A, C, D) or with X-gal and hematoxylin-eosin (B). Scale bars, 50 μm in A–C, 100 μm in D, and 2 μm in E and F.

Decreased LH Activity and Collagen Hydroxylysine Levels in Plod1−/− Mice—The level of LH activity was assayed in the soluble fraction of aorta and skin homogenates by a method based on the formation of hydroxy[14C]lysine in protocollagen, a biologically prepared [14C]lysine-labeled substrate consisting of non-hydroxylated polypeptide chains of chick type I procollagen (22). The activity level in the Plod1−/− skin samples was about 35% of that in the wild-type samples and that in the aorta samples about 45%, the Plod1−/− samples having intermediate values (Table 2). The residual activity levels are apparently because of the other two isoenzymes, LH2 and LH3.

The total hydroxylysine content in all the Plod1−/− tissues was lower than in the wild-type tissues. This content in samples of wild-type tissues, expressed per triple helical molecule (defined as 300 4-hydroxyproline residues), ranged from about 21 to 55 residues/triple helix, being lowest in the skin and highest in the lung (Table 3). This variation is evidently because of the presence of different collagen types in various tissues since the hydroxylysine content varies markedly between collagen types, and because the collagen types were not separated in the present analysis. The lowest hydroxylysine content values, 21 and 26 residues/triple helix, as seen in the wild-type skin and
Samples were analyzed for each genotype. These data demonstrate that the two remaining LH isoenzymes were seen in the lung, femur, heart auricle, and aorta (Table 3). Differences in the magnitude of the hydroxylysine deficiency varied markedly between tissues, the lowest relative values, 22, 24, and 34%, being found in the tail tendon and cornea, and highest, 0.7 residues/triple helix, in the aorta, while the femur and lung had intermediate values (Table 4).

The HP content had decreased in all the Plod1−/− tissues analyzed, the lowest values relative to the wild type, 28, 33, and 34%, being found in the aorta, cornea, and lung, and the highest, 59 and 47%, in the tail tendon and femur (Table 4). Interestingly, no correlation was seen between the relative values (i.e., those expressed as percentages of the wild-type values) for total hydroxylysine and HP, so that the relative hydroxylysine and HP values in the tail tendon, for example, were 24 and 59%, respectively, and those in the cornea 30 and 33%, while the corresponding values were 86 and 34% in the lung, 63 and 28% in the aorta, and 75 and 47% in the femur (Tables 3 and 4). These values clearly demonstrate that although the other two isoenzymes were able to hydroxylate lysine residues at the cross-linking sites of the triple helical region of the various collagen chains.

Changes in Collagen Cross-links in Plod1−/− Mice—The amounts of the trifunctional collagen cross-links, HP and LP, were analyzed in five tissues by a reverse-phase high-performance liquid chromatography method without separation of the collagen types. As pyridinoline cross-links are very infrequent in the skin (9), their amount was too low to be detected. The collagen HP content varied markedly between the wild-type tissues, being lowest, 0.05 residues/triple helix, in the tail tendon and cornea, and highest, 0.7 residues/triple helix, in the aorta, where the femur and lung fell intermediate values (Table 4).

The HP content had decreased in all the Plod1−/− tissues analyzed, the lowest values relative to the wild type, 28, 33, and 34%, being found in the aorta, cornea, and lung, and the highest, 59 and 47%, in the tail tendon and femur (Table 4). Interestingly, no correlation was seen between the relative values (i.e., those expressed as percentages of the wild-type values) for total hydroxylysine and HP, so that the relative hydroxylysine and HP values in the tail tendon, for example, were 24 and 59%, respectively, and those in the cornea 30 and 33%, while the corresponding values were 86 and 34% in the lung, 63 and 28% in the aorta, and 75 and 47% in the femur (Tables 3 and 4). These values clearly demonstrate that although the other two isoenzymes were able to hydroxylate lysine residues at the cross-linking sites of the triple helical region, the three LHs were not the same in their ability to hydroxylate these sites.

The LP content was increased in all the Plod1−/− tissues analyzed, the lowest values relative to the wild type, 28, 33, and 34%, being found in the aorta, cornea, and lung, and the highest, 59 and 47%, in the tail tendon and femur (Table 4). These values demonstrate that the two remaining LH isoenzymes were similar to those in type I and III collagens (1), suggesting that these samples did not contain any significant amounts of other collagen types. In samples of the Plod1−/− tissues the magnitude of the hydroxylysine deficiency varied markedly between tissues, the lowest relative values, 22, 24, and 30% of the wild type, being seen in the skin, tail tendon, and cornea, while the highest relative values, 86, 75, 69, and 63% were seen in the lung, femur, heart auricle, and aorta (Table 3). These data demonstrate that the two remaining LH isoenzymes hydroxylated lysine residues quite efficiently in the triple helical regions of the various collagen chains.

**TABLE 3**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Hyl</th>
<th>% Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>Wt</td>
<td>29.01 ± 2.00</td>
</tr>
<tr>
<td>Plod1−/−</td>
<td>18.30 ± 3.23</td>
<td>63</td>
</tr>
<tr>
<td>Femur</td>
<td>Wt</td>
<td>35.94 ± 3.45</td>
</tr>
<tr>
<td>Plod1−/−</td>
<td>26.99 ± 0.97</td>
<td>75</td>
</tr>
<tr>
<td>Lung</td>
<td>Wt</td>
<td>55.43 ± 1.92</td>
</tr>
<tr>
<td>Plod1−/−</td>
<td>47.71 ± 3.37</td>
<td>86</td>
</tr>
<tr>
<td>Tail tendon</td>
<td>Wt</td>
<td>26.33 ± 1.60</td>
</tr>
<tr>
<td>Plod1−/−</td>
<td>6.41 ± 1.82</td>
<td>24</td>
</tr>
<tr>
<td>Cornea</td>
<td>Wt</td>
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</tr>
<tr>
<td>Plod1−/−</td>
<td>12.09 ± 0.92</td>
<td>30</td>
</tr>
<tr>
<td>Skin</td>
<td>Wt</td>
<td>21.13 ± 1.86</td>
</tr>
<tr>
<td>Plod1−/−</td>
<td>4.61 ± 2.39</td>
<td>22</td>
</tr>
<tr>
<td>Heart auricle</td>
<td>Wt</td>
<td>38.70 ± 6.67</td>
</tr>
<tr>
<td>Plod1−/−</td>
<td>26.55 ± 6.25</td>
<td>69</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genotype</th>
<th>Hyl % Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>+/−</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>Skin</td>
<td>+/−</td>
<td>54 ± 9</td>
</tr>
<tr>
<td>Skin</td>
<td>−/−</td>
<td>44 ± 8</td>
</tr>
<tr>
<td>Skin</td>
<td>−/−</td>
<td>36 ± 7</td>
</tr>
</tbody>
</table>

**FIGURE 5.** Transmission EM analysis of collagen fibrils in the aorta and skin. 1-month-old wild-type (A, C, E, G) or Plod1−/− (B, D, F, H) male mice were sacrificed and aorta (A–D) and skin (E–H) sections were analyzed. A, collagen fibrils in the wild-type aorta are round in cross-section and uniform in size. B, irregular contours and variations in fibril diameter in a Plod1−/− aorta. C, diameter distribution of collagen fibrils in a wild-type aorta, and D, in a Plod1−/− aorta, measured from transmission electron micrographs. E, collagen fibrils in the skin of a wild-type mouse are round and uniform in diameter. F, degraded fibrils and variation in fibril diameter are observed in the Plod1−/− mouse skin. G, diameter distribution of collagen fibrils in the wild-type skin, and H, in the Plod1−/− skin measured from transmission electron micrographs. Scale bars, 200 nm.
in the tail tendon (Table 4).

**DISCUSSION**

Comparison of *Plod1* Knock-out Mice and EDS VIA Patients—EDS VIA is characterized by kyphoscoliosis, joint laxity, muscle hypotonia, and in some individuals, ocular problems. Muscle hypotonia with joint laxity is already present in neonates and recurrent joint dislocations are common later in life. Kyphoscoliosis is present during infancy and becomes moderate to severe in childhood. It is thought to be a consequence of muscular hypotonia together with ligamentous laxity as the vertebral bodies are structurally normal, and electromyography and muscle biopsies have shown no abnormalities (13). The lack of kyphoscoliosis in the *Plod1*−/− mice is probably because of the obvious differences in anatomy and size between mice and humans.

Muscle hypotonia is also observed in the *Plod1*−/− mice, although later in life. In situations where muscle strength and coordination was needed, the *Plod1*−/− mice either did not move at all or stopped moving after a short time, whereas the control mice moved with no difficulties. The gait abnormalities became obvious as the null mice became older, most probably due to a combination of muscle weakness and joint laxity. Gait impairment has been reported in biglycan/fibromodulin double knockout mice (28), but there are no reports of hypotonia in EDS VIA patients (13). This feature was also observed in the EDS VIA patients (13). This feature was also observed in the EDS VIA patients (13).

The skin is not as fragile in EDS VIA as in some other forms of EDS, a soft, velvety skin with abnormal scars being typical. The *Plod1*−/− mice have no obvious skin problems, although collagen fibrils in their skin were abnormal with a larger variation in the diameters than in the wild-type skin. The lack of skin findings may be in part because of a less severe reduction in collagen hydroxylsine content in the *Plod1*−/− skin than in that of EDS VIA patients, as the hydroxylsine content of the *Plod1*−/− skin collagen was about 20% of that in the wild type, whereas the corresponding value in EDS VIA patients is typically only about 5% of that in healthy subjects (13). Correspondingly, the level of LH activity in the *Plod1*−/− skin homogenates was about 35% of that in the wild type, whereas the LH activity level in cultured EDS VIA fibroblasts is typically less than 25% of that in control fibroblasts, values as low as 5% having been reported (15). These differences between the *Plod1*−/− mice and EDS VIA patients may be due to differences in the levels of expression of the other two LH isoenzymes in the mouse and human skin.

Aortic dissection and rupture of the medium-sized arteries is the major life-threatening complication encountered in EDS VIA (13). This feature was also observed in the *Plod1*−/− mice. The aortic wall appeared normal at the light microscopy level when samples were taken by sacrificing living mice, but ultrastructural analysis revealed degenerated smooth muscle cells and abnormal collagen fibrils in the null aortas indicating gradual deterioration.

**Decreased Levels of Hydroxylsine and HP Cross-links in *Plod1*−/− Mice**—The tensile strength of most tissues is dependent on the presence of intermolecular cross-links in collagen fibrils. The first step in cross-link formation is catalyzed by lysyl oxidase and involves the formation of lysine and hydroxylsine-derivated aldehydes (8, 9). Two subsequent cross-linking pathways exist, an allysine route and a hydroxylallysine route, initiated by the telopeptide lysine aldehyde and hydroxylsine aldehyde, respectively (8, 9). Only the cross-links that result from the hydroxylallysine route are able to mature into the pyridinoline cross-links HP and LP. These routes predominate to different extents in different tissues, the allysine route occurring primarily in adult skin, cornea, and sclera, while the hydroxylallysine route predominates in bone, cartilage, ligaments, tendons, most internal connective tissues, and embryonic skin (8, 9). It has been suggested that LH1 may be responsible for the hydroxylation of lysine residues in the triple helical regions of various collagens, while LH2 may be a specific telopeptide LH, as its mutations in Bruck syndrome lead to a deficiency in pyridinoline cross-links (18). This suggestion is in agreement with recent data indicating that LH2 is indeed the only LH isoenzyme capable of hydroxylating the N telopeptide in procollagen I, whereas all three isoenzymes hydroxylated peptides corresponding to the cross-linking sites and other sites in the triple helical regions of collagens I and IV, although with
Lysyl Hydroxylase 1 Knock-out Mice

Abnormalities in Collagen Fibrils—Collagen I fibrils often contain small amounts of collagens III and V that regulate the thickness of the fibrils (14, 31). It is not surprising, therefore, that patients with the vascular and classic EDS types, which are caused by mutations in these two collagens, have abnormal collagen fibrils. Such abnormalities have also been demonstrated in Col3a1 knock-out mice (32) and Col5a1 mice (33), murine models for vascular and classic EDS, respectively. Dermosparactic EDS, in which the N propeptide is not cleaved due to mutations in the procollagen N proteinase ADAMTS-2, is characterized by extremely fragile skin (34). Retention of the N propeptide is also found in the arthrochalasia type of EDS (35) and in mouse dermatosparaxis (36), leading to the formation of highly irregular, “hieroglyphic” collagen fibrils. In the case of EDS VIA, studies have focused on characterization of the biochemical abnormalities and PLOD1 mutations. Nevertheless, our analyses show that the Plod1-null mice also have abnormal collagen fibrils, so that abnormalities in collagen fibrils seem to be involved in all forms of EDS, irrespective of the primary defect.

However, abnormalities in collagen fibril morphology are found not only in EDS but also in some other connective tissue diseases, such as Ullrich congenital muscular dystrophy (37) and Marfan syndrome (38). Abnormal collagen fibrils have similarly been found in mouse models with a deficiency in the small leucine-rich proteoglycans biglycan (39), decorin (40), fibromodulin (41), and lumican (42) that show EDS-like symptoms. These small leucine-rich proteoglycans are extracellular matrix proteins that bind to TGFβs, collagens, and other extracellular matrix proteins and have been shown to regulate collagen fibrillogenesis in vitro (43).

The assembly of collagen fibrils is a complex process that is only partly understood (44). It involves linear and lateral growth steps via association and fusion processes operating between preformed intermediates. Collagen fibrils spontaneously attach to each other side-to-side in vitro, and proteoglycans regulate the assembly by preventing side-to-side fusion so that end-to-end fusion can occur. It has been suggested that decorin may become bound to collagen at the time of fibril assembly, prior to cross-link formation (45) and its major binding site has been mapped to the close vicinity of the C-terminal cross-linking site in the triple helical region of collagen I (46), where the extent of lysine hydroxylation was reduced in the Plod1-null mice. Decorin binding to these sites might thus be affected, leading to alterations in fibril formation. Hydroxylysine residues also serve as sites for O-linked glycosylation, which has an impact on the lateral growth of fibrils (10) and may also influence interactions with other extracellular matrix glycoproteins. The deficiency in the hydroxylysine content of Plod1−/− mouse collagens may thus influence collagen fibril morphology by several mechanisms.

The Plod1−/− mice offer the first in vivo model for systematic analysis of the tissue-specific consequences of the lack of LH1 activity. Furthermore, crossing of Plod1−/− mice with various other mouse lines such as those lacking small leucine-rich proteoglycans (see above) will greatly facilitate our understanding of the molecular mechanisms at the level of protein-protein interactions and associated signaling in the proper assembly of the extracellular matrix.

Acknowledgments—We thank Dr. Hanna Leskinen at the Biocenter Oulu Core Facility of Physiological Analyses of Transgenic Animals for performing echocardiographic analysis, Dr. Asta Pirskanen and Dr. Kaia Passoja for initial characterization of the Plod1 genomic clones, Anu Myllymäki, Outi Mänty, Liisa Aijälä, Jessica Snelbl, the personnel of the Biocenter Oulu Transgenic Animal and the Electron Microscopy Core Facilities, and the University of Oulu Laboratory Animal Centre for excellent technical assistance.

3 K. Takaluoma, J. Lanitto, and J. Myllyharju, submitted manuscript.
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Tissue-specific Changes in the Hydroxylysine Content and Cross-links of Collagens and Alterations in Fibril Morphology in Lysyl Hydroxylase 1 Knock-out Mice
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