A genetic defect in the biosynthesis of dermatan sulfate proteoglycan: Galactosyltransferase I deficiency in fibroblasts from a patient with a progeroid syndrome

(galactosyltransferase II/heparan sulfate/p-nitrophenyl β-D-xylotriose)

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ABSTRACT  A small proteoglycan that contains only a single dermatan sulfate chain is the main proteoglycan synthesized by skin fibroblasts. Fibroblasts from a patient with progeroidal appearance and symptoms of the Ehlers-Danlos syndrome have a reduced ability of converting the core protein of this proteoglycan into a mature glycosaminoglycan chain-bearing species. This abnormality is the consequence of a deficiency in galactosyltransferase I (xylosylprotein 4-β-galactosyltransferase; EC 2.4.1.133), which catalyzes the second glycosyl transfer reaction in the assembly of the dermatan sulfate chain. The glycosaminoglycan-free core protein secreted by the patient's fibroblasts bears an unsubstituted xylose residue. The mutant enzyme is abnormally thermostable. Preincubation of fibroblasts at 41°C leads to a further reduction in the production of mature proteoglycan and affects the capacity for glycosaminoglycan synthesis on p-nitrophenyl β-D-xylotriose more strongly in the mutant than in control cells.

The occurrence of inborn errors in the metabolism of connective tissue proteoglycans in humans has long been recognized, and over the past two decades the basic genetic defects in many of these disorders have been elucidated. The majority of the diseases are caused by faulty degradation of the polysaccharide components of the proteoglycans (see ref. 1 for a review). In a few instances, defects in the biosynthesis of proteoglycans have been demonstrated in experimental animals, such as impaired posttranslational processing of the cartilage proteoglycan core protein in nanomelic chicken (2) and a tissue-specific deficiency in the biosynthesis of 3'-phosphoadenylylsulfate in brachymorphic mice (3). Defective biosynthesis of proteoglycans is probably the cause of the connective tissue abnormalities observed in several diseases in humans, including mucarial cornal dystrophy (4) and a form of spondyloepiphyseal dysplasia (5), though the defects in these diseases have not yet been elucidated.

We have previously described a patient who represented a progeroid variant with signs of the Ehlers-Danlos syndrome (6). In addition to aged appearance, developmental delay, dwarfism, craniofacial disproportion, and generalized osteopenia, this patient suffered from defective wound healing, hypermobile joints, hypotonic muscles, and loose but elastic skin. Biochemically, his cultured skin fibroblasts were defective in the biosynthesis of a ubiquitous proteoglycan named small dermatan sulfate proteoglycan II (DS-PG II; refs. 7 and 8) or decorin (9), which consists of an Mr 36,319 core protein (10), a single glycosaminoglycan chain on the serine residue at position 4 (11), and either two or three asparagine-bound oligosaccharides (12). The fibroblasts synthesized normal amounts of this core protein but converted at most 80% and in some experiments as little as 20% into mature proteoglycan molecules. The remainder was secreted in a glycosaminoglycan-free form.

We had discussed previously that the patient could carry a mutant allele yielding a core protein with an absent or buried recognition site for the attachment of the glycosaminoglycan chain. In light of the limited induction of glycosaminoglycan biosynthesis in the presence of low concentrations of p-nitrophenyl β-D-xylotriose, which serves as an artificial stimulator of glycosaminoglycan synthesis, an abnormality in one of the enzymes involved in the synthesis of the polysaccharide-protein linkage region, glucosyl-(3)Gal-(β1-3)Gal(β1-4)Xyl, was also considered. The results presented in this paper provide evidence that the second explanation is the correct one. Fibroblasts from the patient contain reduced activities of an abnormally thermostable galactosyltransferase I (xylosylprotein 4-β-galactosyltransferase; EC 2.4.1.133), which catalyzes the second glycosyl transfer reaction in the assembly of the xylose/serine-linked proteoglycans (see ref. 13 for a review).

MATERIALS AND METHODS

Materials. O-β-D-Xlyopyranosyl-L-serine and O-β-D-galactopyranosyl-(1→4)-O-β-D-xlyopyranosyl-L-serine were synthetized as described (14). UDP[4,5-3H]galactose (specific radioactivity, 1.5 GBq/mol) was obtained from Du Pont. Sodium boron[3H]hydride (929 TBq/mol), L-[4,5-3H]leucine (1.8 TBq/mol), and sodium [35S]sulfate (carrier-free) were from Amersham. A high-performance carbohydrate analysis column was purchased from Millipore Waters; Aminex HPX-87 H and TSK DEAE-SPW Bio-Gel columns were from Bio-Rad.

Assay of Galactosyltransferases I and II. Human skin fibroblasts were cultured as described (15). To minimize the concentration of endogenous galactose acceptors, confluent cultures were incubated for 3 hr with 20 μM cycloheximide prior to harvesting according to Esko et al. (16). During the course of the experiments, however, it became apparent that this pretreatment had been unnecessary. Cells were homogenized by ultrasonication in 50 mM 2-(N-morpholino)ethane-sulfonic acid/200 mM KCl/0.05% Triton X-100/20 μM phenylmethylsulfonyl fluoride/2 μM leupeptin, pH 5.5. Fifty microfilters of the suspensions containing 50–100 μg of cell protein (17) was mixed with 30 μl of 16.25 mM Tris/acetate buffer (pH 7.5) containing 37 kBq (86 pmol) of UDP-[3H]galactose, 16.25 mM KCl, 12.5 mM 2,3-dimercaptopropanol, 31 mM MnCl2, 0.25 mM ATP, 52 mM CDP-choline, and either 13 mM xylosylserine (galactosyltransferase I) or 21.8 mM galactosylxylosylserine (galactosyltransferase II, EC 2.4.1.134). After 1 hr at 37°C, the incubation mixture was processed exactly as described (14). Briefly, proteins were

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precipitated with ethanol, and the soluble material was subjected to cation-exchange chromatography for the purification of the radioactive product. Without exception, the purity of the radioactive products, Gal-Xyl-Ser or Gal-Gal-Xyl-Ser, was subsequently checked by high-voltage electrophoresis (14) and determination of the $^3$H radioactivity in 1-cm paper segments. By-products accounted for up to 10% in the control cell lines and 50% in the patient's fibroblasts, and the ion-exchange data were corrected correspondingly.

**Preparation of DS-PG II and Its Core Protein.** Conditioned medium that contained only insulin and transferrin as exogenously supplied proteins (18) served as the source of unlabelled DS-PG II and core protein. Glycosaminoglycan-free core protein and DS-PG II were separated from each other by anion-exchange chromatography (12). Core protein-containing fractions were dialyzed against 0.1% Triton X-100, lyophilized, and immune precipitated with an affinity-purified antiserum against DS-PG II core protein (12). DS-PG II-containing fractions were similarly dialyzed and lyophilized but were then exhaustively digested with chondroitin ABC lyase (19). Both preparations were subjected to preparative SDS/polyacrylamide gel electrophoresis (20). Proteins were visualized by treating the gels with 1 M KCl (21), and bands of interest were electroeluted. After drying the samples under reduced pressure, salts were removed by washing with methanol. The secretion of $[^3]$H]leucine-labeled DS-PG II and core protein was followed as described (12).

**Identification of Xylose Residues.** Core protein and chondroitin ABC lyase-digested DS-PG II from 240 ml of conditioned medium were dissolved in 20 μl of 0.92 M Na$^+$H$^+$B$^+$A$^+$ (specific activity, 929 GBq/mol) in 0.1 M NaOH and incubated for 21 hr at 50°C. After acidification to pH 5 with acetic acid, each sample was concentrated to dryness, and the residue was evaporated three times with methanol. The samples were then subjected to descending paper chromatography for 14 hr on Schleicher & Schuell paper no. 2043 BMGL in butanol/acetic acid/H$_2$O, 2:7:1. Samples that had been hydrolyzed under nitrogen with 1 M HCl for 4 hr at 105°C were analyzed in parallel. The radioactive peak migrating as authentic $[^3]$H]xylitol was eluted with H$_2$O and applied either to a carbohydrate analysis column or to an Aminex HPX-87 H column. The first column was eluted at ambient temperature with 80% acetonitrile in H$_2$O at a flow rate of 1 ml/min; the second column was operated at 40°C and was eluted with 3 mM H$_2$SO$_4$ in 15% acetonitrile in H$_2$O at a flow rate of 0.5 ml/min.

**Other Methods.** The secretion of glycosaminoglycan chains induced on exogenously added p-nitrophenyl β-D-xyloside was measured as described (6). Briefly, fibroblasts were preincubated for 48 hr at 37°C or 41°C, respectively, and incubated with $[^3]$S)sulfate in the presence of p-nitrophenyl β-D-xyloside for 4 hr at 37°C. Proteoglycans were removed by an (NH$_4$)$_2$SO$_4$ precipitation step, and induced chains were purified by chromatography on Dowex AG 1X2 (200–400 mesh). For the determination of the glycosaminoglycan chain length, DS-PG II was isolated after an incubation period of 4 hr in the presence of 0.37 MBq of $[^3]$S)sulfate per ml. Dermatan sulfate chains were obtained by a β-elimination reaction and chromatographed on a calibrated Sephacryl S-300 column as described (18).

**RESULTS**

**Presence of Unsubstituted Xylose Residues.** Intact DS-PG II and its glycosaminoglycan-free core protein from the secretions of the patient's fibroblasts were separately purified for an analysis of constituents of the polysaccharide protein linkage region. Alkaline boroiodite reduction of both preparations led to the incorporation of similar amounts of radioactivity into glycosaminoglycan-free core protein and chondroitin ABC lyase-digested DS-PG II, the former containing 77% of the radioactivity of the latter. After further purification by descending paper chromatography, material behaving as authentic $[^3]$H]xylitol was found upon chromatography on either an Aminex HPX-87 H or a carbohydrate analysis HPLC column when the glycosaminoglycan-free core protein was analyzed (Fig. 1). Acid hydrolysis prior to the chromatographic separations did not result in an increased recovery of $[^3]$H]xylitol, indicating that the alditol was not substituted with galactose residues. Analogous treatments of enzyme-digested DS-PG II resulted in the formation of $[^3]$H]xylitol after acid hydrolysis only.

**Deficiency of Galactosyltransferase I.** The presence of unsubstituted xylose residues on the glycosaminoglycan-free core protein could result from a deficiency of galactosyltransferase I. Indeed, the activity of this enzyme in homogenates of cultured fibroblasts from the patient was only about 5% of that in normal control cells (Table 1). The low activity was not due to enhanced degradation of the enzyme during the 3-hr preincubation of the cultures with cycloheximide that was carried out routinely, as no decrease in activity occurred during this period in control experiments with normal and deficient cells. Mixing experiments excluded the presence of excessive amounts of an inhibitor of galactosyltransferase I. The cells of the patient contained half the normal activity, in accordance with the assumption that the disorder is inherited in an autosomal recessive manner. It will be shown below that the normal and the mutant enzymes

![Fig. 1. Identification of xylitol by chromatography on a carbohydrate analysis column. Glycosaminoglycan-free core protein (core) and intact DS-PG II (PG), both from the secretions of the patient's fibroblasts, were subjected to alkaline boroiodite reduction, and the products were purified. Similar amounts of radioactivity were applied for each run. The arrow marked 1 indicates the elution maximum of N-acetyl[1-3H]galactosaminitol; the arrow marked 2 indicates the elution maximum of [1-3H]galactitol.](image-url)
Table 1. Activities of galactosyltransferases I and II in cultured fibroblasts

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Galactosyltransferase I, fmol/min per mg of protein</th>
<th>Galactosyltransferase II, fmol/min per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>Deviation</td>
<td>13–53* (9)</td>
<td>2–8* (4)</td>
</tr>
<tr>
<td>Mother</td>
<td>277</td>
<td>10</td>
</tr>
<tr>
<td>Father</td>
<td>274</td>
<td>15</td>
</tr>
<tr>
<td>Controls</td>
<td>559</td>
<td>20</td>
</tr>
<tr>
<td>Range of 1 standard deviation</td>
<td>334–794</td>
<td>16–26</td>
</tr>
<tr>
<td>No. of cell strains tested</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

Formation of 1 fmol of product is represented by the incorporation of 26 dpm of $^3$H radioactivity.

*The number of separate determinations is given in parentheses.

†Calculated for logarithmic normal distribution.

...differ in their thermostability. On incubation at 37°C, product formation by either enzyme did not increase linearly with time (Fig. 2). The low activity of the mutant enzyme made it necessary, however, to choose 1 hr as the standard incubation period.

Surprisingly, the patient’s fibroblasts were also deficient in galactosyltransferase II, the activity being only 20% of the mean normal value. Activities below the range of 1 standard deviation were found in the cells from his parents (Table 1).

Galactosyltransferase I activity was also determined by using p-nitroph enil β-D-xyloside (5 mM) as substrate under otherwise identical conditions. The radioactive product was quantitated by chromatography on a 1 × 7 cm Bio-Gel P2 column in H₂O where its elution volume was 1.75 times that of $^3$H₂O. Two control cell strains exhibited activities of 480 and 600 fmol/min per mg of protein, respectively, whereas in the patient’s cells the activity was below the limit of detection (<50 fmol/min per mg).

Properties of Mutant Galactosyltransferase I. Some differences were noted between the $K_m$ values of normal and mutant galactosyltransferase I. For xylosylserine the $K_m$ value of the patient’s enzyme (6.4 mmol/liter) was about twice as high as the control value (3.4 mmol/liter). With respect to UDP-galactose, $K_m$ values of 0.026 mmol/liter (patient) and 0.17 mmol/liter (control) were calculated and the activity of the mutant was only about 1% at substrate saturation. Similar pH activity profiles with sharp maxima at pH 6.0 were observed for the normal and the mutant enzyme (results not shown).

In a fibroblast homogenate, galactosyltransferase I did not function optimally at 37°C. Instead, the normal enzyme exhibited the highest activity at 30°C, and the mutant enzyme was most active at 25°C, the lowest temperature tested (Fig. 3). There were no differences in the temperature optima of galactosyltransferase II, which were at 30°C in both cases.

The effect of temperature on glycosaminoglycan chain formation in intact cells is illustrated in Fig. 4. After preincubation at different temperatures for 2 days, fibroblast cultures were pulse-labeled with [3H]leucine for 30 min at 37°C and then analyzed by SDS/polyacrylamide gel electrophoresis. Following preincubation at 41°C, 80% of the immunoreactive radioactive products synthesized by the patient’s cells was glycosaminoglycan-free core protein. In...
Table 2. Influence of temperature on biosynthesis of induced glycosaminoglycan chains

<table>
<thead>
<tr>
<th>p-Nitrophenyl β-D-xylidose, mM</th>
<th>35S, cpm × 10^-3 per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient</td>
</tr>
<tr>
<td>0.1</td>
<td>140</td>
</tr>
<tr>
<td>1.0</td>
<td>490</td>
</tr>
</tbody>
</table>

Fibroblasts were preincubated for 48 hr at the temperatures indicated and then incubated with [35S]sulfate (0.37 MBq/ml) for 4 hr at 37°C. ND, not determined.

contrast, mature proteoglycan molecules were the major products after preincubation at 37°C and 30°C, and free core protein accounted for only 18% and 13%, respectively, of the total radioactivity in the immunoreactive material. Furthermore, it should be noted that the total incorporation of [3H]leucine, in control as well as patient cells, was reduced by about 60% when the preincubation was carried out at 41°C rather than at 30°C or 37°C. In addition to the temperature sensitivity, Fig. 4 also indicated that DS-PG II from the patient’s fibroblasts migrated more slowly than the product of normal cells. Labeling with [35S]sulfate at 37°C showed that this abnormality was caused by an increased glycosaminoglycan chain length in the mutant (M, 42,000 versus M, 35,000), as determined by gel filtration on a calibrated Sephacryl S-300 column. Dermatan sulfate chains from the parents were of normal size.

Temperature Dependence of the Synthesis of Induced Glycosaminoglycan Chains. We have shown previously that the patient’s fibroblasts did not respond normally to induction of glycosaminoglycan chain formation by p-nitrophenyl β-D-xylidose, an artificial substrate for galactosyltransferase I (6). Whereas a xylidose concentration of 0.1 mM was sufficient for maximal response in normal cells, the mutant cells produced maximal amounts of glycosaminoglycan chains only at a concentration of 1 mM (6). Examination of the effect of temperature on the induction of glycosaminoglycan chain formation (Table 2) showed that mutant and control cells had a reduced ability to synthesize free chains after preincubation at 41°C rather than 37°C. However, the patient’s fibroblasts were about twice as sensitive as normal cells.

DISCUSSION

This investigation has shown that cultured fibroblasts from a patient with a progeria-like syndrome and signs of the Ehlers-Danlos syndrome are severely deficient in galactosyltransferase I, which catalyzes the second glycosyl transfer step in the biosynthesis of several connective tissue polysaccharides. Additional findings indicate that this deficiency is the primary genetic defect in this syndrome and that it is inherited in an autosomal recessive manner. Thus, the patient’s enzyme was more thermostable than its normal counterpart, a property often observed in mutant enzymes. Furthermore, both parents exhibited only half of the mean normal enzyme activity in their fibroblasts.

Consonant with the profound enzyme deficiency was the accumulation in the culture medium of incomplete proteoglycan molecules without a glycosaminoglycan chain but possessing an unsubstituted xylose residue attached to the core protein of DS-PG II. However, mature proteoglycan molecules with glycosaminoglycan chains of increased length were also present and represented, in several experiments, the majority of the material reacting with an antibody to the DS-PG II core protein. Although we do not know what fraction of the normal galactosyltransferase I activity is sufficient to sustain a normal level of proteoglycan biosynthesis in vivo, it seems likely that the profound deficiency observed in vitro should have reduced substantially the capacity of intact fibroblasts to synthesize mature DS-PG II.

A possible explanation of the fact that this did not occur is that the physiological acceptor—the xylosylated core protein—may be a much better substrate than the two artificial substrates used in this study. Attempts were therefore made to use the core protein secreted by the patient’s fibroblasts as a galactose acceptor for partially purified galactosyltransferase I. Although galactose was indeed incorporated, characterization of the product indicated that the sugar had been transferred only to asparagine-bound oligosaccharides (E.Q., unpublished work).

A second explanation of the high level of proteoglycan synthesis in the intact cells is that the mutant enzyme may be more stable in its native environment than in the cell-free extract. Galactosyltransferase I in normal cells is firmly bound to intracellular membranes, and disruption of the membrane structure—e.g., by digestion with phospholipase C—results in partial inactivation (22). Whereas solubilized, purified preparations of the normal enzyme are fairly stable in the presence of appropriate phospholipids or nonionic detergents, the mutant enzyme may be more sensitive to perturbations of its native environment and may have lost activity during preparation of the cell-free extract.

The possibility should also be considered that a “nonspecific” galactosyltransferase may augment the activity of galactosyltransferase I in the mutant cells in vivo. Lactose synthase is known to catalyze galactose transfer to xylose, albeit with low efficiency. However, the increased thermal sensitivity of the patient’s fibroblasts with respect to formation of DS-PG II argues against the involvement of a nonspecific normal galactosyltransferase. The same conclusion emerges from the results of experiments with the artificial substrate, p-nitrophenyl β-D-xylidose. Again illustrating the discrepancy between the results of in vitro and in vivo studies, the experiments showed no measurable galactosyltransferase I activity in extracts of the patient’s cells at a xylidose concentration of 5 mM, but a marked stimulation of glycosaminoglycan synthesis occurred in vivo in the presence of 1 mM xylidose. At elevated culture temperature, the decrease in glycosaminoglycan chain formation was substantially greater in the mutant cells than in normal cells, supporting the view that the mutant enzyme and not a nonspecific galactosyltransferase was the target of the thermal effect.

Interestingly, a deficiency in galactosyltransferase II, which catalyzes the third glycosyl transfer step in polysaccharide chain assembly, was also observed. Although an explanation of this finding is not yet apparent, the possibility may be suggested that the two galactosyltransferases share a common subunit, which is the target of the mutation. It is also possible that galactosyltransferase II participates in the formation of a multienzyme complex containing all glycosyltransferases required for synthesis of the glycosaminoglycan-protein linkage region and that the instability of galactosyltransferase I may render other components of the complex more labile. The known ability of galactosyltransferase I to form complexes with xylosyltransferase in vitro (23, 24) is consistent with this notion. On the other hand, no deficiency in xylosyltransferase was observed in the patient’s cells, and the finding that xylosyltransferase and galactosyltransferase I are not coordinately synthesized and degraded (22) leaves doubts as to the physiological importance of the complex formation observed in vitro.

The different chondroitin sulfate/dermatan sulfate and heparan sulfate proteoglycans contain the same glycosaminoglycan-protein linkage region (13). Studies with Chinese hamster ovary cell mutants suggested that a single xylosyltransferase (16) and a single galactosyltransferase I (25) are involved in the biosynthesis of these proteoglycans. It is not
known whether human fibroblasts express one or several proteins with galactosyltransferase I activity. If human fibroblasts behave like Chinese hamster ovary cells, defects in the biosynthesis of the large dermatan sulfate proteoglycan and of heparan sulfate proteoglycans would be expected in the patient. However, normal urinary excretion of heparan sulfate and normal [35S]sulfate incorporation into fibroblast proteoglycans other than DS-PG II have been observed previously (6). In light of the fact that the mature DS-PG II from the patient contains abnormally long dermatan sulfate chains, [35S]sulfate incorporation studies do not exclude the possibility that the core proteins of the large dermatan sulfate proteoglycan and/or of heparan sulfate proteoglycans are substituted with a reduced number of glycosaminoglycan chains. Further studies are required to exclude with certainty a defect in the biosynthesis of these proteoglycans.

In spite of the many unanswered questions outlined above, it appears certain that we have observed a human defect in the biosynthesis of the carbohydrate backbone of a connective tissue polysaccharide, and the diploid human cell line carrying the defect should be a valuable tool in future studies of various cell/matrix interactions.

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