Proteoglycan Synthesis in Normal and Lowe Syndrome Fibroblasts*

Gregory S. Harper†, Vincent C. Hascall§, Masaki Yanagishita∥, and William A. Gahl‡

From the †Section on Human Biochemical Genetics, Human Genetics Branch, National Institute of Child Health and Human Development and the §Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

Lowe (oculocerebrorenal) syndrome (LS) is an X-linked disorder characterized by congenital cataracts, generalized hypotonia, mental retardation, and renal Fanconi syndrome. The basic defect remains unknown, but the possibility that fibroblasts express reduced sulfation of glycosaminoglycans has been studied in several laboratories. A mechanism involving overproduction of an enzyme (nucleotide pyrophosphatase) active against adenosine 3'-phosphate, 5'-phosphosulfate (PAPS) has been postulated. Decreased synthesis of normally sulfated glycosaminoglycans was also reported. We measured the synthesis of proteoglycans and glycosaminoglycans by incorporation of [3H]glucosamine and Na35SO4 into cultured fibroblasts from four LS patients and related it directly to the synthesis in six normal fibroblast cultures. We found that the rate of synthesis varied greatly among the normal cultures (cv, 30%), but not significantly between LS and the normal. The LS fibroblasts' ability to sulfate glycosaminoglycans was assayed as the amount of [3H]glycosaminoglycan eluting at low ionic strength on anion exchange chromatography, the amount of nonsulfated disaccharide present in chondroitinase digests of labeled proteoglycans, and the ratio of 35S to 3H incorporation into proteoglycans. Each parameter suggested that the LS cells were synthesizing normally sulfated glycosaminoglycans (e.g. % ADi-GS, 21 ± 6 in normal; 27 ± 6 in LS). The cells' ability to sulfate glycosaminoglycans was tested under conditions of markedly stimulated glycosaminoglycan synthesis, by treating the cultures with a β-D-xylotetraose, LS and normal cells responded to the treatment by elevating the rate of synthesis of normally sulfated glycosaminoglycans (3.5–5-fold in normal, 3–7-fold in LS). Nucleotide pyrophosphatase activities were found to be elevated in each of our four LS cell strains as in the previous studies, excluding genetic heterogeneity as an explanation for our findings. We conclude that LS fibroblasts do not express defects in sulfation of glycosaminoglycans or in synthesis of proteoglycans.

Lowe (oculocerebrorenal) syndrome (LS) is an X-linked disorder characterized by congenital cataracts, generalized hypotonia, mental retardation, and renal tubular Fanconi syndrome (1). A range of other histological, clinical, and biochemical findings have been reported (2-4), but no coherent explanation for all aberrant findings in LS has been constructed. The observation of urinary excretion of hyposulfated glycosaminoglycans (5, 6) has led to the hypothesis of a primary defect in proteoglycan sulfation. This hypothesis is attractive since the ubiquitous involvement of proteoglycans in basement membrane structure and function (7), tissue turgor maintenance (8), and tissue development (9) could explain the many manifestations of Lowe syndrome (3).

Glycosaminoglycan synthesis and sulfation have been studied in cultured skin fibroblasts from LS patients (4, 10, 11). Fukuda et al. (4) reported undersulfation of glycosaminoglycans produced by LS fibroblasts grown under both physiologic and reduced inorganic sulfate conditions. The undersulfation was confined to chondroitin and dermatan sulfate glycosaminoglycans, with heparan sulfates not affected. Total glycosaminoglycan synthesis was lower in LS cells than in normal (4) and depended on the concentration of inorganic sulfate in the medium. Synthesis of undersulfated glycosaminoglycans was attributed to depleted PAPS pools, reportedly resulting from elevated levels of a nucleotide pyrophosphatase active against this precursor. One study found an elevated level of this enzyme in 21 LS patients (12), with some genetic heterogeneity. The enzyme levels in cells from heterozygotes were intermediate between normal and LS cells (12).

Donnelly et al. (10) found aberrant extracellular localization of normally sulfated glycosaminoglycans in LS, as well as undersulfation, but highlighted the inconsistency of these observations with the proposed PAPS defect. They suggested a membrane defect of the Golgi complex, but found no abnormality in total glycosaminoglycan synthesis in LS cells. All the above experiments involved comparisons between cultures from a single LS patient with those from a single normal. In this paper, we assayed the effects of individual variation and genetic heterogeneity upon proteoglycan synthesis by measuring proteoglycan production and sulfation in six normal fibroblast cultures and in four LS cultures derived from three unrelated families. The studies were performed under conditions of reduced inorganic sulfate (100 μM) and under conditions which maximally stimulated chondroitin sulfate synthesis (1 mM β-D-xylotetraose) and presumably stressed the cellular capacity to synthesize sulfated proteoglycans. The results indicated substantial variation within each

5637
The proteoglycans (25) and hence potentially involved in the lies. Synthesis of intact proteoglycans, rather than isolated expression of the metabolic defect. Our preliminary experi-
and for four LS fibroblast strains from three unrelated fami-
ely, proteoglycan metabolism. For example, the degradation pathways of glycosaminoglycans have been elucidated using fibroblasts from patients with mucopolysaccharide storage disorders (24).

We compared several synthetic parameters for six normal and for four LS fibroblast strains from three unrelated families. Synthesis of intact proteoglycans, rather than isolated glycosaminoglycans (4), was studied since the presence of the core protein is essential to the localization and function of the proteoglycans (25) and hence potentially involved in the expression of the metabolic defect. Our preliminary experi-
ments involved analytical fractionation of proteoglycans la-
beled in large scale cultures under approximately physiologic sulfate conditions (1100 μM). While these experiments sug-
gested that proteoglycan synthesis and sulfation were not defective in LS, it was clear that an estimate of the normal variability of each synthetic parameter was required. Further, since Fukui et al. (4) suggested that the sulfation defect was po-
red for up to 5 days under repleted sulfate conditions, sub-
sequent experiments involved reduced sulfate media. We em-
ployed 100 μM sulfate as the reduced sulfate condition rather than 30 μM (4), because Sobue et al. (26) and Humphries et al. (27) found that very low sulfate media inhibited normal sulfation of the chondroitin glycosaminoglycans. The latter group also found that heparan sulfation was spared, which suggests that some of the characteristics previously attributed to LS cells (which included sparing of heparan sulfation) may have resulted from the stringent culture conditions employed. Small scale cultures were employed to facilitate comparison of several cell strains simultaneously. Three parameters were extensively studied: rate of glycosaminoglycan and proteoglyc-
 synthesis; localization of synthetic products into either medium or cell layer fractions; and extent of sulfation of newly synthesized proteoglycans. β-D-Xyloside was added to some cultures to study the effect of stimulated synthesis on each of these parameters.

The rates of glycosaminoglycan and proteoglycan synthesis varied considerably among normal cultures (Table III), but the range of rates encompassed those of the LS cultures. Hence, there was no significant difference between LS and normal. Compartmental localization of glycosaminoglycans was determined from synthetic rate and chondroitinase digestion data. The normal and LS cell strains secreted similar proportions of the total incorporated counts into the medium and cell layer fractions. Both distributed chondroitin/derma-
tan sulfate glycosaminoglycans into both the medium and cell layer fractions and heparan sulfate primarily into the cell layer fraction. These estimates are in accord with the findings of others (28). The extent of sulfation of the glycosaminogly-
cans synthesized by the normal and mutant cells was evalu-
ated by three methods: comparison of the rate of synthesis of species appearing in the HA fraction; comparison of the ratio of 35S to 3H incorporation into the proteoglycan species; and comparison of the proportion of ΔDi-OS disaccharide in chon-
droitinase digests of the glycosaminoglycans. Each parameter exhibited a range of values for normals, but again the LS cultures fell within this range.

β-D-Xyloside treatment of fibroblasts induces a 5-8-fold increase in 35S incorporation into medium fraction CS and DS glycosaminoglycans (29). The xyloside acts as an artificial chain initiator in the Golgi (30–33) and hence relieves the rate limitation normally exerted by core-protein supply. The xyloside-initiated glycosaminoglycans are secreted into the medium. In our synthetic rate experiments, LS cells may not have expressed defective sulfation of glycosaminoglycans be-
cause sulfate precursor pools were not sufficiently depleted. β-D-Xyloside treatment should increase the flux of sulfate through the precursor pool and supply machinery and might be expected to uncover a subtle defect in the LS cells. The rate at which LS cells incorporated [35S]sulfate into glycosaminoglycans was stimulated 2.8–7-fold by xyloside treatment (Tables III and IV), yet there was no apparent change in any of the three sulfation parameters. It seems likely that even if PAPS pools were depleted in LS cells, there remained sufficient functional capacity to supply normal amounts of sulfate for the glycosaminoglycans.

The possibility that genetic heterogeneity within the clini-
cally defined syndrome accounts for our cell strains not ex-
hibiting the sulfation defect was addressed by measuring the activity of nucleotide pyrophosphatase. This enzyme has been found to be elevated by approximately 8-fold in fibroblasts from 21 LS patients and, consistent with a gene-dosage effect, by approximately 4-fold in obligate heterozygotes (12). Nu-
cleotide pyrophosphatase was also elevated in our four LS cell strains, so genetic heterogeneity with respect to this enzyme could not explain the absence of a glycosaminoglycan sulfation defect in our studies.

Previous studies have shown the enzyme to have broad specificity, hydrolyzing UDP-Glc, ATP, and NAD, as well as PAPS (34). To exclude the possibility that UDP-hexosamine pools were significantly depleted by the over abundance of the enzyme in LS cells, the specific activity of H in the pool was estimated by measurement of the 35S/H ratio in purified disaccharides (Table V). The range of normal included the values found for the LS cells.

Donnelly et al. (10) reported a ratio of [35S]- to [14C] glucosamine incorporation into glycosaminoglycans which, at 72 h of incubation, was lower than normal in the LS intracel-
ular and pericellular fractions and higher than normal in the
medium (Fig. 3 of Ref. 10). Fukui et al. (4), using low sulfate medium for 72 h, noted only a slight decrease in the $^{35}$S/$^{3}$H ratio in chondroitin sulfate and dermatan sulfate of LS medium compared with normal. Therefore, the claim of defective sulfation in LS rested heavily upon differences in the $^{35}$S/$^{3}$H ratio measured for the cell-associated fractions, since the medium fraction did not reflect these differences (Table I of Ref. 4). The present studies suggest that fibroblasts express little specificity in localization of CS/DS proteoglycans (i.e. both medium and cell layer fractions contained proteoglycans of similar disaccharide content), and hence synthetic defects are unlikely to be limited to only one extracellular compartment. In fact, the entire difference in chondroitin sulfate sulfation noted by Donnelly et al., i.e. decreased sulfation of glycosaminoglycans in the pericellular fraction of LS cells (Table II of Ref. 10), depends upon the reproducible separation of pericellular from the cell layer fractions by trypsinization.

We conclude that previously demonstrated differences in glycosaminoglycan synthesis or sulfation between a single normal and a single LS fibroblast strain may reflect serendipitous choices of individual cultures for study. Despite elevated nucleotide pyrophosphatase activities, LS fibroblasts in culture demonstrated no obvious defects in glycosaminoglycan sulfation, displayed normal HA and proteoglycan synthesis in low sulfate medium, were stimulated in a normal way by β-D-xyloside, and synthesized normal proportions of chondroitin sulfate disaccharides.

Acknowledgments—We wish to thank Drs. Zebrower, Kieras, and Brown for supplying a preprint of a paper describing the HPLC disaccharide technique, Isa Bernardini and Diana Nomanbho for their technical assistance, and Colleen Genovese for assistance in typing this manuscript.

REFERENCES


Continued on next page.
Proteoglycan Synthesis in Lowe Syndrome

Materials

Culture designation Patient

Cell Culture

Skin fibroblasts were grown in 225 cm2 Flasks in E minimal essential medium (MEM) supplemented with 15% FCS, as a confluent monolayer. The medium was exchanged every 3rd day and the cells were split weekly when they reached 75% of the surface area.

RESULTS

Table 1. Fibroblast Cultures Used in this Study

<table>
<thead>
<tr>
<th>Culture designation</th>
<th>Patient</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>N5</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>N6</th>
<th>N7</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td></td>
<td>3549</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td></td>
<td>3540</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td></td>
<td>5010</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N4</td>
<td></td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N5</td>
<td></td>
<td>2750</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td></td>
<td>3700</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td></td>
<td>6</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td></td>
<td>1370</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each clone, the proliferative capacity (passages at confluence) was measured. The cells were labelled with [3H]-thymidine for 24 h and the medium was exchanged every 3rd day. The media were harvested at the end of the labeling period and the total radioactivity was measured.

Table 2. Inhibitory Activity of Proteoglycan Synthesis

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Cell Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS</td>
<td>N1</td>
</tr>
<tr>
<td>L2</td>
<td>L1</td>
</tr>
<tr>
<td>L3</td>
<td>N5</td>
</tr>
</tbody>
</table>

Rationale and Experimental Procedures

The experiments were performed exactly as for the small-scale, long-term experiments, except that one hour prior to labeling, proteoglycan synthesis (after confluence) was measured. The media were harvested at the end of the labeling period and the total radioactivity was measured.

Highly purified, monolayer cultures were labeled with [3H]-thymidine for 24 h and the medium was exchanged every 3rd day. The media were harvested at the end of the labeling period and the total radioactivity was measured.

The inhibitors were added to the cultures 24 h prior to labeling. The media were harvested at the end of the labeling period and the total radioactivity was measured.

Analysis of Data

The results were analyzed by Student's t-test, with the level of significance set at p < 0.05.

For each clone, the proliferative capacity (passages at confluence) was measured. The cells were labelled with [3H]-thymidine for 24 h and the media were harvested at the end of the labeling period and the total radioactivity was measured.

The inhibitory activity of proteoglycan synthesis was measured by a sensitive filter assay. The results were analyzed by Student's t-test, with the level of significance set at p < 0.05.
Proteoglycan Synthesis in Lowe Syndrome

Table III.  Glycosaminoglycans and Proteoglycans Synthesized in Exposed Surface Medium

<table>
<thead>
<tr>
<th>Cell Culture</th>
<th>Medium + Cells</th>
<th>Medium + Cells</th>
<th>Medium + Cells</th>
<th>Medium + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal - NK</td>
<td>0.9</td>
<td>1.8</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>8.4</td>
<td>5.5</td>
<td>3.6</td>
<td>4.0</td>
</tr>
<tr>
<td>100</td>
<td>10.0</td>
<td>9.5</td>
<td>9.6</td>
<td>1.0</td>
</tr>
<tr>
<td>1,000</td>
<td>13.8</td>
<td>2.4</td>
<td>1.4</td>
<td>0.12</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>8.3 ± 3.7</td>
<td>5.2 ± 3.5</td>
<td>2.2 ± 1.6</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>L5</td>
<td>8.2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>L2</td>
<td>14.8</td>
<td>4.3</td>
<td>2.5</td>
<td>1.4</td>
</tr>
<tr>
<td>L3</td>
<td>5.1</td>
<td>7.5</td>
<td>3.1</td>
<td>4.6</td>
</tr>
<tr>
<td>L4</td>
<td>8.7</td>
<td>3.6</td>
<td>2.9</td>
<td>3.3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>8.2 ± 4.2</td>
<td>4.0 ± 2.6</td>
<td>2.2 ± 1.2</td>
<td>3.7 ± 2.6</td>
</tr>
</tbody>
</table>
Proteoglycan Synthesis in Lowe Syndrome

Proteoglycan peaks observed in the synthetic rate experiments performed in low sulfate medium (Table II), were abolished by chondroitinase digestion and the digested samples were separated on thin-layer chromatography. Radioactivity migrating in each direction on the plates was expressed as a percentage of total incorporated activity. For the uptake of 50 J.C. fractions, the specific activity was reduced to 40% of the values in the corresponding series at pH 9.4, 1.00, with a single peak appearing at 4.5 kD in all the glycosaminoglycan preparations analyzed. Hypothesis was based on the observation that the glycosaminoglycan preparations contained only a single peak at 4.5 kD. The cell layer fractions from 16 normal, 41% of the chondroitin sulfate species appeared in the media, while 16% of the heparan sulfate species appeared in the cell layer fractions.

The proportion of the different glycosaminoglycans isolated into the two compartments was deduced from the synthetic rate and chondroitinase digestion data. For the normal cultures, a greater proportion of the incorporated radioactivity was recovered in the media (40 ± 5%), than was retained in the cell layer (20 ± 5%). Chondroitin sulfate to glycosaminoglycans were distributed into both the medium (30%) and the cell layer (20%) fractions. The distribution of the radioactivity in the cell layer fractions was 80% to 85% higher than in the normal culture. The distribution of the specific activity is the cell layer fraction was similar to that of the normal culture. The distribution of the sulfate species in the cell layer fractions was similar to that of the normal culture.

Analysis of Chondroitin Sulfate Synthesis in Synthetic Sulfate Media

The extent of sulfation of C6 and C8 glycosaminoglycans in the line experiment was determined by chondroitinase digestion of the mediastinum proteoglycan fraction. The chondroitinase was effectively inhibited by 10 mM L-glutamine as shown in Fig. 1. The amount of each amino residue could be quantitated and expressed as a percentage of the total. The activity in the media (100%) and the cell layer (0%) was determined. The fraction of the total sulfate present in the media was 80% to 85% higher than in the normal culture. The distribution of the sulfate species in the cell layer fractions was similar to that of the normal culture.

Fig. 1: Recovery of proteoglycan sulfate in media and cell layer fractions.

Table II: Incorporation of sulfate into proteoglycan fractions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Media</th>
<th>Cell Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>10 mM L-glutamine</td>
<td>80%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Hypothetical explanation for the increased sulfate incorporation into the media fraction was that the chondroitin sulfate in the media fraction was more heavily sulfated than in the normal culture. The increased sulfate incorporation into the media fraction was due to the increased synthesis of chondroitin sulfate in the media fraction. The increased sulfate incorporation into the media fraction was due to the increased synthesis of chondroitin sulfate in the media fraction.

Fig. 3: Elution pattern of chondroitin sulfate proteoglycan fractions from medium of synthetic sulfate medium.

Hypothetical explanation for the increased sulfate incorporation into the media fraction was that the increased synthesis of chondroitin sulfate in the media fraction was due to the increased synthesis of chondroitin sulfate in the media fraction. The increased sulfate incorporation into the media fraction was due to the increased synthesis of chondroitin sulfate in the media fraction.
Proteoglycan Synthesis in Lowe Syndrome

The specific activity was estimated from the 3H/35S ratio in the chondroitin sulfate derived from the chondroitinase-ABC treated samples, as described by Vandal et al. and Metcalfe and colleagues. Bone marrow cells were labeled with [3H]proline and [35S]sulfate, and the specific activity of the radiolabeled proteoglycans was determined. The specific activity of the proteoglycans was determined by separating the proteoglycans from the cell culture medium using a cesium chloride gradient in a Beckman ultracentrifuge. The specific activity of the proteoglycans was then measured by radioactivity analysis. The specific activity of the proteoglycans was determined by correcting the specific activity for any loss of proteoglycans due to centrifugation or other manipulations. The specific activity of the proteoglycans was then used to calculate the specific activity of the proteoglycan synthesized in the cell culture medium.

Table V. Synthesis of the Specific Activity of 3H in the Nonsense Peptide Pool

<table>
<thead>
<tr>
<th>Cell Culture</th>
<th>Pool Specific Activity (cpm/mg)</th>
<th>Nonsense Peptide Incorporation (cpm/mg)</th>
<th>Calculated Specific Activity (cpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHKt - 13</td>
<td>0.24</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>12</td>
<td>0.29</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>24</td>
<td>0.46</td>
<td>2.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

As indicated, the specific activity of 3H in the Nonsense peptide pool was determined by correcting the specific activity for the loss of proteoglycans due to centrifugation or other manipulations. The specific activity of the proteoglycans was then used to calculate the specific activity of the proteoglycan synthesized in the cell culture medium.