Enhanced Accumulation of Hyaluronate in the Culture of Skin Fibroblasts from Two Patients with Coffin-Lowry Syndrome

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Miyazaki, K., Yamanaka, T. and Oohira, A. Enhanced Accumulation of Hyaluronate in the Culture of Skin Fibroblasts from Two Patients with Coffin-Lowry Syndrome. Tohoku J. Exp. Med., 1989, 158 (4), 325-334 —— Cultured skin fibroblasts were prepared from two unrelated adult patients with full expressions of Coffin-Lowry syndrome. Glycosaminoglycans (GAGs) were isolated either from the medium or from the cell layer of cultured skin fibroblasts. Two-dimensional electrophoresis of GAG preparations on cellulose acetate film revealed that hyaluronate was the major component both in the medium and in the cell layer. Quantitative analysis of GAGs was carried out by measuring optical density at 615 nm of Alcian blue-stained GAG spots on electrophoretograms. Increase in the hyaluronate content was found both in the culture medium and in the cell layer of Coffin-Lowry fibroblasts. In addition, the incorporation of [14C]glucosamine into hyaluronate was similarly activated in skin fibroblasts from patients, suggesting the active biosynthesis and/or the suppressed degradation of hyaluronate by cultured skin fibroblasts from Coffin-Lowry syndrome. The abnormal metabolism of hyaluronate in Coffin-Lowry fibroblasts may be implicated in some of the clinical aspects of this genetic disorder. ——— Coffin-Lowry syndrome; fibroblasts; glycosaminoglycans; hyaluronic acid

Coffin-Lowry syndrome is a genetic disorder characterized by severe mental retardation, short stature, characteristic face, large and soft hands with tapered fingers, loose skin, and several skeletal abnormalities (Coffin et al. 1966; Lowry et al. 1971; Temtamy et al. 1975). This condition is not very rare, and more than 50 cases have been reported in the literature (Young 1988). Clinical expressions

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Abbreviations used are as follows: GAG, glycosaminoglycan; HA, hyaluronate; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; FBS, fetal bovine serum; dAMP, deoxyadenosine 5'-monophosphate; GlcN, glucosamine.
in females are variable and less severe than males, suggesting X-linked dominant inheritance (Temtamy et al. 1975). Recently, it is suggested that the Coffin-Lowry locus may be located on the distal part of the short arm of chromosome X by multipoint linkage analysis (Hanauer et al. 1988; Partington et al. 1988).

The primary defect of Coffin-Lowry syndrome is still unknown. Because the patients with Coffin-Lowry syndrome have abnormalities in connective tissues such as skin and bone, detailed analyses of extracellular matrix macromolecules synthesized by cultured skin fibroblasts should be useful to elucidate the pathological mechanism of this disorder. So far, however, few biochemical studies on these macromolecules have been carried out except for that of Beck et al. (1983), who showed the metabolic abnormalities of proteodermatan sulfate secreted by Coffin-Lowry fibroblasts.

In this paper, we present biochemical analyses of GAGs isolated from the culture of skin fibroblasts from two unrelated male patients with Coffin-Lowry syndrome. The aberrant composition of GAGs mainly due to the enhanced accumulation of HA is emphasized.

**Patients and Methods**

**Patients**

Two unrelated Japanese male patients (Case-1 aged 20 years and Case-2 aged 20 years) were diagnosed as Coffin-Lowry syndrome from clinical and roentgenographic findings. Both patients showed severe mental retardation, short stature, clumsy broad gait, peculiar face, large and soft hands with tapered fingers, loose skin, and kyphoscoliosis. Radiographic examinations showed thickened facial bones, prominent tufting of distal phalanges on hands, and vertebral defects with narrowed intervertebral spaces. The mother of Case-1 also showed tapered fingers and prominent tufting of distal phalanges on hand radiographs. She was mildly mentally retarded and has suffered from schizophrenia.

**Materials**

The following commercial materials were used: cellulose acetate film (10 x 10 cm) (Sepaphore III) from Gelman Sciences, Ann Arbor; Sepharose CL-4B from Pharmacia, Uppsala; cetyltrimethylammonium bromide (CTAB) from Nacalai Tesque, Kyoto; and hyaluronidase from Streptomyces hyalurolytics and the GAG marker kit composed of HA, CS, DS, and HS from Seikagaku Kogyo, Tokyo.

**Cultures of skin fibroblasts**

This study was approved by the institutional human research committee, and parental informed consent was obtained prior to this study. A small piece of skin was obtained at biopsy or at operation from two patients and four individuals for controls (Table 1). Fibroblasts were cultured in Dulbecco's modified Eagle's medium (Nissui Seiyaku, Tokyo) supplemented with 100 IU/ml penicillin, 100 μg/ml of streptomycin, 2 μg/ml of fungizone, and 10% of FBS (M.A. Bioproducts, Walkersville, MD, USA). As described previously (Oohira et al. 1987), about 1 x 10^6 cells were seeded and subcultured in a 100-mm culture dish, and cells of passage 4 were used for all experiments in this work. Thus all cultures were matched for total culture age. Fibroblasts were observed under a phase-contrast microscope on day 2 of cultivation.
Preparations of GAGs

Fibroblasts of passage 4 were fed on day 1, 3 and 5. Under the culture conditions used, both pathological and normal fibroblasts reached confluency on day 4. On day 7, the cultures were harvested to prepare GAGs both from the cell layer and from the medium by the following methods. The cell layer was washed with 10 mM phosphate buffered saline, pH 7.0, and the wash was combined with the medium. The solution was centrifuged at 2000 rpm for 10 min to remove the cellular debris. After dialysis against cold water, macromolecules in the medium were precipitated by adding 3 volumes of 95% ethanol containing 1.3% potassium acetate. The cell layer was solubilized with 2% sodium dodecyl sulfate-50 mM Tris-HCl, pH 7.5 (Oohira et al. 1983), and macromolecules were precipitated from the sodium dodecyl sulfate solution as described above. DNA content of the culture was determined using dAMP as a standard by the method of Burton (1956), using an aliquot of the cell layer thus solubilized.

GAGs were prepared by the sequential treatments with Pronase-E (Kaken Seiyaku, Tokyo), 0.4 N NaOH, 5% trichloroacetic acid, and precipitation with 75% ethanol containing 1% potassium acetate, as described previously (Oohira et al. 1986). Hexuronate content in the GAG preparations was determined by the method of Bitter and Muir (1962).

The GAG preparations (10 nmole hexuronate for the medium and 50 nmole hexuronate for the cell layer) were separated by two-dimensional electrophoresis on cellulose acetate using 0.1 M pyridine/0.47 M formic acid, pH 3, in the first dimension at a constant current of 1 mA per cm for 1 hr and 0.1 M barium acetate in the second dimension for 5 hr (Hata and Nagai 1972), and each GAG component was identified enzymatically as described previously (Oohira et al. 1986).

Preparation of [14C] GlcN-labeled GAGs

Confluent cultures of fibroblasts (day-5 of cultivation) were labeled with 1 uCi/ml of D-[14C(U)]glucosamine HCl (300 mCi/mmmole, New England Nuclear, Boston, MA, USA) in Dulbecco’s modified Eagle’s medium containing 10% FBS for 48 hr. [14C]GlcN-labeled GAGs were prepared as described above. An aliquot of the labeled GAG preparation was analyzed by gel filtration on a column (1.0 x 48 cm) of Sepharose CL-4B at room temperature in 0.2 M NaCl-50 mM Tris-HCl, pH 7.5, containing 0.2% Nonident P-40 and 1 mM NaN3.

Quantitative determination of HA

GAG preparations from the medium and the cell layer were dissolved in distilled water at the hexuronate concentration of 0.5 nmole/µl and 2 nmole/µl, respectively. Aliquots of the solution (1 to 5 nmole of hexuronate for the medium and 10 to 40 nmole of hexuronate for the cell layer) were separated by electrophoresis on cellulose acetate film using a buffer

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Diagnosis</th>
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<tbody>
<tr>
<td>Case-1</td>
<td>20</td>
<td>M</td>
<td>Coffin-Lowry syndrome</td>
</tr>
<tr>
<td>Case-2</td>
<td>20</td>
<td>M</td>
<td>Coffin-Lowry syndrome</td>
</tr>
<tr>
<td>Control-1</td>
<td>2</td>
<td>M</td>
<td>Mental retardation</td>
</tr>
<tr>
<td>Control-2</td>
<td>3</td>
<td>M</td>
<td>Normal individual</td>
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<td>25</td>
<td>F</td>
<td>Normal individual</td>
</tr>
<tr>
<td>Control-4</td>
<td>39</td>
<td>F</td>
<td>Normal individual</td>
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</table>

M, male; F, female.
system containing 0.1 M pyridine/0.47 M formic acid at a constant current of 0.5 mA per cm for 2 hr. GAG spots were stained with 0.5% Alcian Blue in 3% acetic acid for 10 min. Under these conditions, the spot of HA was clearly observed, so that the optical density at 615 nm was measured by a densitometer (dual-wave length TLC scanner CS-910; Shimadzu Kyoto).

Our preliminary experiments revealed that the absorbance at 615 nm was linear with the amount of HA at least up to 3 \( \mu \)g per spot.

**Quantitative determination of CS, DS and HS**

Aliquots (20 to 50 nmole hexuronate) of GAG preparations were separated by two-dimensional electrophoresis on cellulose acetate film. Quantitative determination of each GAG thus separated was done by the colorimetric method of Hata and Nagai (1973), with some modifications as previously described (Oohira et al. 1986).

**RESULTS**

**Cell growth and morphology**

Skin fibroblasts were cultured under the same conditions as previously reported (Oohira et al. 1987). The growth curves of Coffin-Lowry fibroblasts were similar to those of normal fibroblasts (data not shown). In fact, DNA content in the cultures of Coffin-Lowry fibroblasts on day 7 was not significantly different from that of normal fibroblasts in each experiment (Table 2).

The fibroblasts on day 2 of cultivation were observed under a phase-contrast microscope, but no morphological abnormalities were found. There was also no difference in the number of cytoplasmic inclusion bodies between normal and Coffin-Lowry fibroblasts (data not shown).

**Separation of GAGs**

For the characterization, GAG preparations were separated by two-dimensional electrophoresis on cellulose acetate. Fig. 1 shows Alcian blue-stained electrophoretograms. There appeared one major spot (identified enzymatically to be HA) and one faint spot (CS) in the sample of normal culture medium from Control-3 (Fig. 1a), and three major spots (HA, HS and CS) and one faint spot (DS) in the sample of normal cell layer (Fig. 1b). Fig. 1c and 1d show the electrophoretograms of the GAG preparations from the fibroblast culture of Case-1. All the GAG spots found in the normal samples were detected. However, the spot of HA in Coffin-Lowry sample showed relatively greater Alcian blue-staining both in the culture medium and in the cell layer, suggesting the aberrant composition of GAGs in the culture of skin fibroblasts of Case-1. An apparent increase in the relative amount of HA was also observed in Case-2.

**Quantitative determination of GAGs**

As shown in Fig. 1, HA can be separated completely from other GAGs by the electrophoresis in the buffer system used for the first run. Therefore, HA can be estimated by the densitometric method after electrophoretic separations as de-
<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA†</th>
<th>Hexuronate</th>
<th>Hyaluronate</th>
<th>Chondroitin sulfate</th>
<th>DS</th>
<th>HS</th>
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<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>Cell layer</td>
<td>Medium</td>
<td>Cell</td>
<td>Medium</td>
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<td></td>
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<tr>
<td>Case-1</td>
<td>65.2</td>
<td>3.46</td>
<td>1.51</td>
<td>1164.5 ± 25.1 (156)</td>
<td>45.5 ±</td>
<td>0.2(129)</td>
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<td>Control-3</td>
<td>71.1</td>
<td>2.71</td>
<td>1.73</td>
<td>744.7 ± 46.4 (100)</td>
<td>35.3 ±</td>
<td>2.8(100)</td>
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<td>Case-2</td>
<td>69.0</td>
<td>4.82</td>
<td>3.00</td>
<td>1805.3 ± 142.2(280)</td>
<td>225.7 ±</td>
<td>41.6(352)</td>
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<td>2.38</td>
<td>2.21</td>
<td>878.8 € (136)</td>
<td>189.9 ±</td>
<td>25.3(296)</td>
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<td>66.4</td>
<td>2.36</td>
<td>2.39</td>
<td>644.8 ± 81.3 (100)</td>
<td>64.1 ±</td>
<td>8.9(100)</td>
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<td>Experiment 3</td>
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<tr>
<td>Case-2</td>
<td>70.3</td>
<td>5.64</td>
<td>2.19</td>
<td>1072.7 ± 50.4 (144)</td>
<td>149.3 ±</td>
<td>19.4(208)</td>
</tr>
<tr>
<td>Control-2</td>
<td>60.7</td>
<td>4.21</td>
<td>2.14</td>
<td>747.4 ± 89.3 (100)</td>
<td>71.8 ±</td>
<td>3.4(100)</td>
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<tr>
<td>Control-4</td>
<td>73.1</td>
<td>3.29</td>
<td></td>
<td>739.6 ± 52.4 (99)</td>
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</table>

*Values refer to means ± s.d. of two to four separate measurements.
†Mean of two separate measurements.
‡The values in parentheses are expressed as the percentage of a normal sample.
§One measurement.
—Not determined.
scribed under Methods, and other GAGs such as CS, DS, and HS were measured by the colorimetric method after two-dimensional electrophoretic separation.

The amounts of GAGs in the culture of skin fibroblasts are summarized in Table 2. In each experiment, HA content of Coffin-Lowry syndrome, based on the amount of DNA, was about 1.3- to 3.5-fold higher, both in the culture medium and in the cell layer, than in normal adult controls. There was no orderly difference in the contents of sulfated GAGs between the cultures of Coffin-Lowry fibroblasts and those of normal fibroblasts (Table 2).

[^14C]GlcN-labeled GAGs

[^14C]GlcN-labeled GAG preparations from cultures of skin fibroblasts (equivalent to 10 nmole dAMP-DNA for the medium and 80 nmole dAMP-DNA for the cell layer) were chromatographed on Sepharose CL-4B. The sample from the normal adult cell layer (Control-3) was separated into two peaks, which were designated as I and II in the order of elution (Fig. 2b). Peak I was susceptible to digestion with hyaluronidase, indicating that it is hyaluronate (HA). In Coffin-Lowry sample, [^14C]radioactivities in peak I (HA) were significantly higher than in normal adult sample. Peak II was obtained from both normal and Coffin-Lowry samples, and showed no significant difference between them. Though the properties of peak II were not clear, this peak could not be considered as pathological.
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[14C]GlcN-labeled GAG preparation from the normal medium eluted as a very broad peak from Sepharose CL-4B (Fig. 2a). More than 95% of the radioactivity in this broad peak was removed by digestion with hyaluronidase, showing that it is HA. The radioactivity in this broad peak of Coffin-Lowry sample was higher than that of normal sample, but the elution position was almost identical to that of normal sample. From these observations, it is considered that the hydrodynamic size of HA synthesized and secreted by Coffin-Lowry fibroblasts is normal. These observations, together with the data shown in Table 2, indicate that Coffin-Lowry fibroblasts, compared with normal fibroblasts, secrete a larger amount of newly synthesized HA into the culture medium.

DISCUSSION

In this study, we have demonstrated the abnormal GAG content in the culture of skin fibroblasts from patients with Coffin-Lowry syndrome, which is a genetic disorder of unknown etiology. The synthetic activities for GAGs of cultured skin fibroblasts change in an age-related manner. Turakainen (1983) demonstrated that, by the study of incorporation of [3H]glucosamine into HA and sulfated GAGs by skin fibroblasts, the synthesis of HA and sulfated GAGs depended on the age of fibroblast donor. For example, the synthesis of HA and sulfated GAGs is the highest in the fibroblasts from the fetus and the newborn, and thereafter marked age-related decline is found during infancy. Our experi-
ment showed that HA content in the cultures of Coffin-Lowry fibroblasts was significantly higher than those of fibroblasts from the adult controls (Control-3 and -4) and was also higher than those of fibroblasts from the infant controls (Control-1 and -2). Thus, taking into consideration the age-related decline of GAG synthesis, we conclude that the increase in HA content was obvious both in the medium and in the cell layer of cultured pathological fibroblasts.

No increased number of cytoplasmic inclusion bodies was observed in Coffin-Lowry fibroblasts, nor was there any observable deficiency of a lysosomal enzyme which is involved in HA degradation. In fact, we measured the activities both of lysosomal β-hexosaminidase and of β-glucuronidase with appropriate fluorogenic 4-methylumbelliferyl derivatives (Kress and Miller 1979). The levels of β-hexosaminidase activity and of β-glucuronidase activity in Coffin-Lowry fibroblasts fell in the control ranges (2300-3000 nmole/mg protein/hr for β-hexosaminidase; 1.55-1.78 nmole/mg protein/hr for β-glucuronidase). However, there is still a possibility that HA degradation is inhibited in Coffin-Lowry fibroblasts. Alternatively or additionally, HA biosynthesis would be activated in this syndrome. It has been demonstrated that mammalian cells have a protein factor which modulates HA synthesis (Matuoka et al. 1987).

Previously, Beck et al. (1983) proposed that the abnormal proteoglycans were secreted from Coffin-Lowry fibroblasts, and these functionally altered proteoglycans accumulated extracellularly owing to the reduced capability of secreted proteoglycans for being endocytosed. If this was the case, an accumulation of sulfated GAGs in the extracellular space, or in the culture medium, should be observed in the culture of Coffin-Lowry fibroblasts. However, the amount of sulfated GAGs in the culture medium was not elevated in the present study (Table 2). In our preliminary experiments using [35S]sulfate for proteoglycan labeling, the incorporation of [35S]sulfate into the medium proteoglycans of Coffin-Lowry fibroblasts during 48 hr cultivation was about 70% of that of age-matched normal fibroblasts. Thus, we could not demonstrate the accumulation of [35S]labeled proteoglycans in the culture medium of Coffin-Lowry fibroblasts. Therefore, it is postulated that the abnormal accumulation of HA, rather than sulfated proteoglycans, is implicated in the pathological features of this disease.

There have been several reports indicating the abnormal metabolism of HA in heritable connective tissue disorders. The urinary excretion of HA and/or the synthesis of HA by fibroblasts have been found to be elevated in osteogenesis imperfect (Turakainen 1983), progeria (Brown et al. 1985), Werner syndrome (Brown et al. 1985), mucopolysaccharidosis I-S (Scheie) (Satake et al. 1983), and other genetic diseases. Therefore, one should not conclude that the enhanced accumulation of HA in the cultures of skin fibroblasts is specific for Coffin-Lowry syndrome. But it is reasonable to consider that the composition of extracellular matrix macromolecules in this disorder is abnormal, and that Coffin-Lowry syndrome is a kind of heritable connective tissue disorder. HA provides a highly
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hydrated extracellular milieu, holding much water intramolecularly (Toole 1981), and HA is distributed in almost all animal tissues. Owing to this property, the elevated accumulation of HA may well explain some clinical pictures of this disease such as loose and easily stretchable skin, large and soft hands, and marked extensibilities of joints.

References

17) Temtamy, S.A., Miller, J.D., Dorst, J.P., Hussels-Maumenee, I., Salinas, C., Lacassie,

