Galactosialidosis:
Studies on Residual Enzymes in Early and Late Onset Clinical Phenotypes

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(Received March 31, 1987)

Summary Enzymatic studies were performed on fibroblasts from patients with different clinical phenotypes of galactosialidosis. Residual activities of β-galactosidase and neuraminidase were relatively low in infantile patients as compared with those in adults, and an asymptomatic adult showed higher residual activities of these enzymes than did adult patients with neurosomatic manifestations. The ability of thiol protease inhibitors to activate β-galactosidase in fibroblasts was similar in infant and adult cases. Both β-galactosidase and neuraminidase were activated slightly more by addition of the “protective protein” preparation to the culture medium of fibroblasts in the asymptomatic adult than in infants and symptomatic adults. Sucrose density gradient centrifugation revealed three peaks of β-galactosidase activity in crude homogenates of fibroblasts: very high, high, and low molecular weight fractions. The peak of the high molecular weight fraction was predominant in control fibroblasts, while it was extremely low in galactosialidosis patients, especially in the infantile cases. It was suggested that the severity of clinical manifestations was inversely correlated with amounts of residual enzyme activities and “protective protein” among these different phenotypes.

Key Words: galactosialidosis, β-galactosidase, neuraminidase, protective protein, leupeptin

Galactosialidosis is a hereditary metabolic disease occurring mainly in young adults, with clinical manifestations of progressive neurosomatic signs and symptoms; progressive ataxia, action myoclonus, cherry-red spots, corneal opacity, and slight-to-mild dysmorphism. It is transmitted as an autosomal recessive trait...
Cases with onset in infancy have recently been reported [3–5]. They presented in the neonatal period with hepatomegaly, dysmorphic somatic changes, and psychomotor retardation, and died in infancy.

Both forms of the disorder are characterized biochemically by deficiency in somatic cells of two lysosomal enzymes, \( \beta \)-galactosidase and neuraminidase, although plasma \( \beta \)-galactosidase is apparently normal [6–8]. \( \beta \)-Galactosidase activity was partially restored in fibroblasts by protease inhibitors, and both enzyme activities were almost completely restored by cell extracts containing a specific 32 kDa (kilodalton) protein ("protective protein"), which was deficient in fibroblasts from patients with this disease. This protein is necessary both for aggregation and stabilization of monomeric \( \beta \)-galactosidase molecules and for catalytic activity of neuraminidase molecules [2, 9–11].

In this report galactosialidosis patients with various clinical manifestations were compared biochemically on the basis of residual enzyme activities and the molecular size of \( \beta \)-galactosidase in cultured fibroblasts.

**MATERIALS AND METHODS**

Human skin fibroblasts were obtained from patients and control subjects, and cultured in Ham's F-10 medium supplemented with 10% fetal calf serum and antibiotics. The diagnosis of galactosialidosis was established by demonstration of \( \beta \)-galactosidase and neuraminidase deficiencies in leukocytes and fibroblasts, associated with a normal activity of \( \beta \)-galactosidase in plasma. Clinical manifestations were variable in these patients. In most of them, clinical symptoms, appearing at 10–20 years of age, were loss of vision, gait disturbance, action myoclonus, cerebellar ataxia, vertebral deformities, angio-keratoma, cherry-red spots, and corneal opacity [12]. One adult patient was referred to us because cherry-red spots were detected on examination for loss of vision ("asymptomatic" case, 22-year-old female). A newborn infant presented with somatic dysmorphism and visceromegaly associated with severe neurological signs, and died at 6 months of age [3]. An affected fetus was reported elsewhere [13].

Fibroblasts were harvested by scraping, washed with isotonic saline, suspended in water, disrupted by vigorous mixing (for neuraminidase) or by sonication (for \( \beta \)-galactosidase), and used for enzyme assays. The assay conditions were described previously [11]. \( \beta \)-Galactosidase and neuraminidase were assayed with the fluorogenic substrates 4-methylumbelliferyl \( \beta \)-D-galactopyranoside (Nakarai Chemicals, Kyoto) and 4-methylumbelliferyl N-acetyl-\( \alpha \)-neuraminitate ammonium salt (supplied by Dr. H. Kushida, Zeria Pharm. Co., Tokyo). Protein was determined by the method of Lowry et al. [14]. Leupeptin was purchased from Peptide Research Foundation (Osaka).

The "protective protein" fraction was prepared from control or \( G_m \)-gangliosidosis fibroblasts by the procedure reported by Hoogeveen et al. [10]. Confluent fibroblasts in dishes of 6 cm in diameter were cultured with 4 ml of F-10 medium.
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containing 10 mM ammonium chloride for 2 days. The medium was dialyzed against phosphate-buffered saline and concentrated finally to 50 µl with a PM-10 Amicon membrane filter. The combined concentrate (200 µl) and 4 ml of the culture medium were added to each 6 cm diameter culture dish, and the cells were cultured for 3 days prior to enzyme assays.

Sucrose density gradient centrifugation was performed according to the method described by Hoogeveen et al. [15] for the separation of β-galactosidase with different molecular sizes in fibroblasts. A linear gradient of 20–40% (w/v) sucrose in 20 mM phosphate buffer, pH 6.0, containing 100 mM NaCl and 1% (w/v) Zwittergent detergent 3-12 (Calbiochem-Behring, San Diego) (buffer A) was prepared in a total volume of 5 ml. The cell pellet was lysed by mixing in 100 µl of buffer A. This sample was centrifuged at 8,000 × g for 10 min at 4°C. The supernatant was layered on the top of the gradient and centrifugation was carried out at 150,000 × g for 15 h at 4°C. Subsequently each 200 µl fraction was collected from bottom to top of the centrifuge tube, and β-galactosidase activities were assayed. Glutamate dehydrogenase, aldolase, and malate dehydrogenase (Boehringer-Mannheim-Yamanouchi, Tokyo) were added to the loading sample as molecular weight markers [16].

RESULTS

β-Galactosidase and neuraminidase activities

Residual activities of β-galactosidase and neuraminidase in galactosialidosis patients with different clinical manifestations are shown in Table 1. Residual activities of both enzymes were lower in early onset cases than in late onset cases (p < 0.001), and an asymptomatic adult patient showed a higher residual enzyme activity than did symptomatic adults (p < 0.001).

Restoration of enzyme activities by thiol protease inhibitors and “protective protein”

In all cases of galactosialidosis β-galactosidase was remarkably elevated by leupeptin added to the culture medium of the fibroblasts, but no effect was observed on neuraminidase activity (Table 2). On the other hand, addition of the

<table>
<thead>
<tr>
<th>Galactosialidosis</th>
<th>β-Galactosidase</th>
<th>Neuraminidase</th>
</tr>
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<tbody>
<tr>
<td>Early onset</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetus (1)</td>
<td>8.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Infant (1)</td>
<td>11.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Late onset</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic (9)</td>
<td>19.5 ± 3.4</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>Asymptomatic (1)</td>
<td>35.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Controls (7)</td>
<td>285 ± 40</td>
<td>22.9 ± 3.8</td>
</tr>
</tbody>
</table>

Values are expressed as nmol of 4-methylumbelliferone released/mg protein/h (mean ± SD). Each value is the mean of 3–5 separate experiments. Numbers of patients in parentheses.

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"protective protein" induced more prominent restoration of both enzymes (Table 3). The extent of restoration was slightly higher in the asymptomatic adult case as compared with that in others, but in symptomatic adult cases there was a wide variation in the results of this addition. The values in early onset cases also fell within this range (95% confidence limit).

**Fractionation of β-galactosidase activity**

Three peaks of β-galactosidase activity were demonstrated in control fibroblasts by sucrose density gradient centrifugation: very high molecular weight (peak I), high molecular weight (peak II), and low molecular weight (peak III) fractions (Fig. 1). Peak I was small, and the other two peaks were the major fractions separated by this procedure. Molecular weights of peaks II and III were estimated as approximately 400–500 and 100 kDa, respectively (Fig. 1, inset). In galactosialidosis fibroblasts these three peaks were detected at the same fraction numbers. There was, however, a marked decrease in the activity present in peaks II and III (Table 4). Among our patients, the early onset cases showed a more drastic decrease in peak II (Fig. 2, Table 4).

Addition of leupeptin to the culture medium of fibroblasts from galactosialidosis patients resulted in the restoration of peak III, but not of peak II.
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Fig. 1. Sucrose density gradient centrifugation of β-galactosidase activity in crude homogenates of fibroblasts. Fractionation and assay of the enzyme were performed as described in Materials and Methods. a, The pattern in control fibroblasts; b, the pattern in galactosialidosis fibroblasts (a symptomatic adult). GDH, Glutamate dehydrogenase; ALD, aldolase; MDH, malate dehydrogenase.

Fig. 2. Patterns of density gradient centrifugation of β-galactosidase activity in galactosialidosis. a, Early onset patients; b, late onset symptomatic patients; c, a late onset asymptomatic patient.

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Table 4. Distribution of β-galactosidase activity fractionated by sucrose gradient centrifugation.

<table>
<thead>
<tr>
<th>Galactosialidosis</th>
<th>Peak I</th>
<th>Peak II</th>
<th>Peak III</th>
<th>II/III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early onset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetus (1)</td>
<td>0.7</td>
<td>1.0</td>
<td>7.3</td>
<td>0.13</td>
</tr>
<tr>
<td>Infant (1)</td>
<td>1.6</td>
<td>2.2</td>
<td>6.3</td>
<td>0.35</td>
</tr>
<tr>
<td>Late onset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic (9)</td>
<td>3.2±1.1</td>
<td>8.1±1.9</td>
<td>8.3±1.6</td>
<td>1.03±0.33</td>
</tr>
<tr>
<td>Asymptomatic (1)</td>
<td>2.4</td>
<td>11.0</td>
<td>14.7</td>
<td>0.75</td>
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<tr>
<td>Controls (6)</td>
<td>7.9±1.4</td>
<td>226±35.3</td>
<td>64±8.4</td>
<td>3.53±0.37</td>
</tr>
</tbody>
</table>

Values are expressed as nmol of 4-methylumbelliferone released/mg protein/h (the total cumulative activity of each peak; mean±SD). Peak I, Very high molecular weight fraction; peak II, high molecular weight fraction; peak III, low molecular weight fraction; II/III, ratio of peak II to peak III.

Fig. 3. Effects of “protective protein” and leupeptin on the pattern of density gradient centrifugation of β-galactosidase activity in galactosialidosis (a symptomatic adult patient). a, Patterns with (●—●) and without (○---○) “protective protein”; b, patterns with (●—●) and without (○---○) leupeptin.

the "protective protein" caused an almost complete normalization of the fractionation pattern in these cells (Fig. 3). The extent of these changes in the patterns of fractionation was almost identical in various types of galactosialidosis patients; no differences were observed among early and late onset cases.

**DISCUSSION**

Galactosialidosis occurs mainly in young adults, but cases have been reported with severe neurosomatic signs and symptoms in early infancy [3–5, 13]. Furthermore, we found two adults without any significant clinical manifestations except for cherry-red spots and a slight loss of vision. One of these ("asymptomatic") patients was evaluated biochemically in this report in comparison with other adults with typical neurological abnormalities and somatic dysmorphism.

All these patients were biochemically well characterized by deficiency of β-galactosidase and neuraminidase, and by restoration of their activities by thiol protease inhibitors and a protein fraction containing "protective protein" [2, 9–11]. Palmeri et al. [17] recently reported molecular heterogeneity in galactosialidosis. They classified their patients, on clinical criteria, into three different phenotypes: early infantile, late infantile, and juvenile-adult forms. Their infantile form corresponds to our early onset cases; in fact the fibroblasts from an affected fetus in the present study were obtained from one of the authors cited above [17]. All other cases in our report were classified into the juvenile-adult form except one case which was without any definite clinical manifestations other than cherry-red spots. We described this female case as "asymptomatic," but it is still possible that she will develop other clinical signs and symptoms in the future. Therefore at this stage she may be better called "presymptomatic."

In the study of Palmeri et al. [17] the residual amounts of the 52 kDa precursor of "protective protein" were slightly different between early infantile and juvenile-adult patients, although the 32 kDa protein was not detected in either of these patients. Their late infantile type was unique since neuraminidase activity was elevated by leupeptin in the culture medium of fibroblasts. In our study no such elevation was observed in any fibroblast strains from patients with galactosialidosis.

Hoogeveen et al. [15] separated the β-galactosidase activity in fibroblasts into two peaks by sucrose density gradient centrifugation. The major, high molecular weight fraction was low in preparation from galactosialidosis patients and was restored by addition of "protective protein." In this study we separated three peaks, instead of two, with the same procedure. Our peaks II and III seem to correspond to the two fractions reported by Hoogeveen et al. [15]. Apparently they did not detect the very high molecular weight fraction. However, another molecule larger in size than the multimer of β-galactosidase was also demonstrated in a concentrated and stabilized human placental glycoprotein preparation, and it was shown to be closely associated with neuraminidase activity [18]. Peak I in our study was variable in proportion to the other peaks in normal and galacto-
sialidosis fibroblasts. It was not affected by thiol protease inhibitors or "protective protein." It is not clear whether this fraction represents a physiological molecular form or an artifactual product formed during the operational procedure.

The data from this study suggest that the residual enzyme activities were inversely correlated with the clinical severity of patients with galactosialidosis, although the number of early onset patients was too small for statistic evaluation. Furthermore, fractionation of β-galactosidase activity on the basis of molecular size demonstrated a quantitative difference in multimeric aggregate molecules between early onset and late onset cases. This result indicates a difference in the intracellular amount of a high molecular weight and stable form of this enzyme. This result can be explained by a quantitative difference in a specific protein ("protective protein") that functions to aggregate and stabilize monomeric enzyme molecules [9] in fibroblasts of these patients with different clinical manifestations. However, our study does not exclude the possibility of a genetically determined qualitative difference in this protein. Further studies are currently in progress in this direction.

The authors wish to thank Dr. A.T. Hoogeveen (Department of Cell Biology and Genetics, Erasmus University, Rotterdam) and Dr. T. Fujinaga (Department of Pediatrics, Gunma University, Maebashi) for fibroblasts from an affected fetus and an infantile patient, respectively. This work was supported by grants from the Ministry of Education, Science and Culture, and from the Ministry of Health and Welfare of Japan.

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