Uptake of a Recombinant Human \( \alpha \)-Iduronidase (laronidase) by Cultured Fibroblasts and Osteoblasts

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To examine the uptake of a recombinant human \( \alpha \)-iduronidase (laronidase) by cultured fibroblasts from a patient with mucopolysaccharidosis I (MPS I) and its effect on the cleavage of accumulated substrates, we performed enzymological, Western blotting, immunocytochemical and morphological studies. Laronidase was incorporated into the MPS I cells dose-dependently mainly via mannose 6-phosphate (M6P) receptors. Then the incorporated enzyme was transported to lysosomes and processed to the mature form, the pathological changes of the cells being improved. Furthermore, we compared the uptake of laronidase by cultured mouse osteoblasts with that by cultured mouse fibroblasts. The enzyme was incorporated into the cultured mouse osteoblasts mainly via M6P receptors, although mannose (Man) receptors were partially involved in the uptake of the enzyme, as in the cultured fibroblasts. But the uptake by the former was apparently lower than that by the latter. The administration of a high dose of the enzyme or development of a recombinant \( \alpha \)-iduronidase containing many M6P residues is required for further improvement of enzyme replacement therapy for skeletal disorders caused by MPS I.

Key words mucopolysaccharidosis I; \( \alpha \)-iduronidase; osteoblast; enzyme replacement therapy; mannose 6-phosphate receptor

\( \alpha \)-Iduronidase (IDUA; EC 3.2.1.76) is a lysosomal enzyme that catalyzes the hydrolysis of glycosaminoglycans (GAGs) heparan sulfate and dermatan sulfate. A deficiency in IDUA activity causes defective degradation of GAGs leading to their widespread accumulation, especially in skeletal, cartilage and connective tissues, and excessive excretion of GAGs into the urine. Mucopolysaccharidosis I (MPS I; McKusick 25280) resulting from a deficiency in IDUA activity exhibits a wide clinical spectrum ranging from the early-onset severe form (Hurler syndrome), with bone deformities, stiff joints, corneal clouding, mental retardation and early death, to the late-onset mild form (Scheie syndrome), with mild bone diseases, stiff joints, corneal clouding, and normal intelligence.1,2 MPS I is autosomal recessive and exhibits an incidence of approximately 1 in 100000 births. The gene encoding IDUA has been localized to 4p16.3,2) and its cDNA sequence has been isolated and expressed.3) MPS I is autosomal recessive and exhibits a wide clinical spectrum ranging from the early-onset severe form (Hurler syndrome), with bone deformities, stiff joints, corneal clouding, mental retardation and early death, to the late-onset mild form (Scheie syndrome), with mild bone diseases, stiff joints, corneal clouding, and normal intelligence.1,2) MPS I is autosomal recessive and exhibits an incidence of approximately 1 in 100000 births. The gene encoding IDUA has been localized to 4p16.3,2) and its cDNA sequence has been isolated and expressed.3) MPS I, and examined its morphological effect. Furthermore, we compared the uptake of laronidase by cultured mouse osteoblasts with that by cultured mouse fibroblasts.

MATERIALS AND METHODS

Compounds Laronidase was purchased from Genzyme Corporation (Tokyo, Japan). 4-Methylumbelliferyl-\( \alpha \)-L-iduronide was purchased from Toronto Chemicals (North York, Canada).

Cell Culture Cultured skin fibroblasts from a patient with MPS I (F17), a healthy subject (F592), and a wild-type mouse (F665) were established and cultured in Ham's F-10 medium (Invitrogen, Carlsbad, CA, U.S.A.) containing 10% fetal calf serum for mouse fibroblasts, at 37 °C under 5% CO2. The ethical committee of our institute approved the study involving the human cells (No. 1908) and informed consent of the patient and the control subject was obtained. Mouse osteoblasts (MC3T3-E1; ATCC, Manassas, VA, U.S.A.) were cultured in \( \alpha \)-MEM (Nacalai Tesque, Kyoto, Japan) containing 10% fetal calf serum, at 37 °C under 5% CO2.

Assaying of IDUA Activity and Protein Determination The IDUA activity of laronidase was measured fluorometrically, essentially according to the method of Hopwood.7) Briefly, the assay for IDUA was performed in 50 mmol/l formate buffer, pH 3.5, containing 1.0 mmol/l 4-methylumbelliferyl-\( \alpha \)-L-iduronide at 37 °C for 10 min (total volume: 100 \( \mu l \)). The reaction was stopped by adding 900 \( \mu l \) of 0.2 mol/l glycine buffer, pH 10.7. Then, the released 4-methylumbelliferone was measured with a Wallac 1420
ARVO MX Multilabel Counter (Perkin Elmer, Waltham, MA, U.S.A.), at excitation and emission wavelengths of 355 and 460 nm, respectively.

Protein concentrations were determined using a Micro BCA Protein Assay Reagent kit (PIERC, Rockfold, IL, U.S.A.) and bovine serum albumin as a standard.

A kinetic experiment was performed with 2.2 ng of laronidase and various concentrations (0.01, 0.02, 0.10, 0.20, 1.0 mmol/l) of 4-methylumbelliferyl-α-L-iduronide. Lineweaver–Burk plot was used to determine the Michaelis constant \( K_m \) value for laronidase.

For determination of intracellular IDUA activity, cell homogenates were allowed to react with 0.8 mmol/l 4-methylumbelliferyl-α-L-iduronide in 80 mmol/l formate buffer, pH 3.5, containing 9.6 mmol/l D-saccharic acid 1,4-lactone, at 37 °C for 30 min.

**Examination of the Effect of Laronidase on Cultured Human MPS I Cells**

To examine the uptake of laronidase by human fibroblasts, laronidase was added to the culture medium of fibroblasts from a patient with MPS I (F17) and a healthy subject (F592) to give concentrations of 0, 0.10, 1.0, and 5.0 µg/ml. For examination of the inhibitory effect of mannose 6-phosphate (M6P) on the cellular uptake of laronidase, fibroblasts were cultured in medium containing 5.0 µg/ml laronidase in the presence of 5.0 mmol/l M6P. After 2 d culture, the cells were harvested mechanically, washed three times with phosphate-buffered saline (PBS), pH 7.4, and then collected as a pellet by centrifugation. An appropriate amount of water was then added to the pellet and the cells were sonicated, the resulting homogenate being used for the IDUA assay and protein determination.

Processing of the incorporated laronidase was examined by means of Western blotting. F17 and F592 cells were cultured in medium containing 5.0 µg/ml laronidase in the presence or absence of 5.0 mmol/l M6P for 2 d. Then, cell homogenates (each 20 µg) were prepared and solubilized in sodium dodecylsulfate (SDS) sample buffer (30 mmol/l Tris–HCl, pH 6.8, 2% SDS, 15% glycerol and 2.5% β-mercaptoethanol), separated on 4—20% Tris–glycine polyacrylamide gels (PAG mini; Daiichi Pure Chemical Co., Tokyo, Japan), and then electrotransferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore Co., Bedford, MA, U.S.A.). The blots were blocked in Tris–buffered saline (TBS: 50 mmol/l Tris–HCl, pH 7.4, 100 mmol/l NaCl) containing 5% skim milk for 30 min at room temperature. Then, the blots were reacted with 1 : 500 diluted rabbit anti-laronidase antibodies against laronidase, washed in TBS three times, and reacted with peroxidase-conjugated donkey anti-rabbit secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL, U.S.A.) for 1 h at room temperature. Finally, the blots were washed in TBS and developed with ECL (Amersham Pharmacia Biotech) as a chemiluminescent substrate on Hyperfilm™ ECL (Amersham Pharmacia Biotech). A preliminary study had revealed that the anti-laronidase antibodies could detect 4 ng or more of laronidase (data not shown).

To investigate the localization of laronidase incorporated into cells, human fibroblasts (F17 and F592) were cultured on a Lab-Tek chamber slide (Nunc, Naperville, IL, U.S.A.) in medium containing 5.0 µg/ml laronidase for 2 d. Then, the cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 5 min, followed by blocking with 4% Block Ace (Dainippon Sumitomo Pharma, Tokyo, Japan) in PBS for 1 h. The cells were then reacted with the anti-laronidase antibodies and mouse monoclonal anti-lyosomal-associated membrane protein-1 (LAMP-1) antibodies (1:100 diluted; IgG isotype, Southern Biotechnology, Birmingham, AL, U.S.A.) for 1 h. After washing, they were reacted for 1 h with Alexa Fluor 594-conjugated goat anti-rabbit IgG antibodies (1:500 diluted; Molecular Probes, Eugene, OR, U.S.A.) and Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies (1:500 diluted; Molecular Probes). The stained cells were observed under a microscope (Axiovert 100 m; Carl Zeiss, Oberkochen, Germany) equipped with a confocal laser scanning imaging system (LSM510; Carl Zeiss).

A morphological study was performed to examine the effect of laronidase on the degradation of GAGs accumulated in cultured fibroblasts from a patient with MPS I (F17). The cells were cultured on a chamber slide (Miles Laboratories, Naperville, IL, U.S.A.) in medium containing 5.0 µg/ml laronidase for 2 d. The cells were then trypsinized, fixed in PBS containing 2.5% glutaraldehyde and 2% paraformaldehyde for 30 min, postfixed in 2% osmium tetroxide, dehydrated through a graded ethanol series, and then embedded in Epon 812. Ultrathin sections were mounted on copper grids, contrasted with uranyl acetate and lead citrate, and finally examined under an electron microscope (Hitachi H-7100; Hitachi, Tokyo, Japan).

**Comparison of Uptake of Laronidase by Cultured Mouse Osteoblasts and Fibroblasts**

To compare the uptake of laronidase by cultured mouse osteoblasts and fibroblasts, MC3T3-E1 cells as representatives of the bone system and F665 cells as ones of the connective tissue system were cultured in medium containing laronidase at the concentration of 0, 0.10, 1.0, or 5.0 µg/ml. Furthermore, the cells were cultured in medium containing 5.0 µg/ml laronidase in the presence of 5.0 mmol/l M6P or 5.0 mmol/l mannose (Man). After 2 d culture, the cells were harvested, and then the IDUA assays and protein determination were performed. Furthermore, Western blotting was performed using cells which had been cultured in medium containing 5.0 µg/ml laronidase in the presence of 5.0 mmol/l M6P or 5.0 mmol/l mannose (Man) for 2 d as samples, according to the method described above.

**Statistical Analysis**

To compare the uptake of laronidase by MC3T3-E1 cells and F665 cells, statistical comparisons were performed by means of Student’s t-test under the conditions of a two-tailed distribution and two-sample equal variance.

**RESULTS**

**Biochemical Characterization of Laronidase**

The enzymological analysis revealed that the specific activity of laronidase was 7.4 mmol/h/mg. The results of Lineweaver–Burk plot for laronidase are shown in Fig. 1. The \( K_m \) value was 0.22 mmol/l.

**Uptake of Laronidase by Cultured Human Fibroblasts**

Human MPS I (F17) and wild-type (F592) fibroblasts were cultured in medium containing the indicated concentrations of laronidase for 2 d. Then, the cells were harvested and the intracellular IDUA activity was determined. As shown in
The uptake of laronidase by these cells was strongly inhibited in the presence of 5.0 mmol/l M6P in the culture medium. The results also revealed that M6P strongly inhibited the incorporation of laronidase into the cells and that the increase in the enzyme activity was always lower in MC3T3-E1 cells than in F665 ones when laronidase was added to the medium at a low concentration, 0.10 µg/ml, there was no difference in the uptake between them. However, the increase in the enzyme activity was always lower in MC3T3-E1 cells than in F665 ones when laronidase was added to the medium at the concentration of 1.0 µg/ml or more. The statistical examination revealed that there was a significant difference in the uptake of laronidase between MC3T3-E1 cells and F665 cells (p<0.01). The uptake of the enzyme by MC3T3-E1 cells was strongly inhibited by the addition of 5.0 mmol/l M6P to the culture medium, but moderately inhibited by the addition of 5.0 mmol/l Man, as was the case in F665 cells.

Immunoblotting analysis to examine the processing of laronidase after incorporation into cells revealed that the 83 kDa precursor enzyme was processed into the mature components in both cultured mouse osteoblasts and fibroblasts, although the 69 kDa component was the major one in MC3T3-E1 cells. The results showed that M6P strongly inhibited the incorporation of laronidase into the cells and that the apparent decrease in the number of cytoplasmic inclusion bodies was observed after 2 d culture of the cells in medium containing 5.0 µg/ml laronidase (Fig. 4b).

Table 1. IDUA Activity in Cultured Human Fibroblasts after the Addition of Various Doses of Laronidase to the Culture Medium

<table>
<thead>
<tr>
<th>Laronidase (µg/ml)</th>
<th>0.10</th>
<th>1.0</th>
<th>5.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6P (mmol/l)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>F17</td>
<td>0.0±0.0*</td>
<td>1.0×10³±0.1×10³</td>
<td>3.8×10³±0.2×10³</td>
<td>4.1×10³±0.0×10³</td>
</tr>
<tr>
<td>F592</td>
<td>98.6</td>
<td>1.0×10³±0.0×10³</td>
<td>3.8×10³±0.1×10³</td>
<td>4.3×10³±0.3×10³</td>
</tr>
</tbody>
</table>

Intracellular IDUA activity is expressed as nmol methylumbellifereone released/h/mg protein. Values are expressed as means±S.D., n=3. *p<0.01. Human fibroblasts from a patient with MPS I (F17) and a healthy control (F592) were cultured in medium containing various doses of laronidase as described under “Materials and Methods”. F17 and F592 cells were also cultured in medium containing 5.0 µg/ml laronidase in the presence of 5.0 mmol/l M6P. After 2 d culture, the cells were harvested and intracellular IDUA activity was measured.
Fig. 3. Immunocytochemical Analysis of Laronidase Incorporated into Cultured Human Fibroblasts

Human fibroblasts (F17 and F592) were cultured in medium containing 5.0 μg/ml laronidase (+) for 2 d, and then double staining of the cells was carried out using polyclonal antibodies for laronidase (IDUA, red) and monoclonal antibodies for LAMP-1 (LAMP-1, green). F17 and F592 cells which were cultured in the medium not containing laronidase (−) were used as controls. Images obtained with these two fluorescent probes, phase-contrast and immunofluorescence ones, respectively, overlapped (yellow; Merge/Phase contrast). Bars = 50 μm.

Fig. 4. Morphological Effect of Laronidase on F17 Cells

(a) MPS I cells (F17). Scale bar = 2 μm. (b) F17 cells cultured in medium containing 5.0 μg/ml laronidase for 2 d. Scale bar = 2 μm.

Table 2. IDUA Activity in Cultured Mouse Osteoblasts and Fibroblasts after the Addition of Various Doses of Laronidase to the Culture Medium

<table>
<thead>
<tr>
<th>Laronidase (μg/ml)</th>
<th>M6P or Man (mmol/l)</th>
<th>MC3T3-E1</th>
<th>F665</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>12 ± 3</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>0.10</td>
<td>—</td>
<td>1.10 ± 0.0 ± 10^3</td>
<td>1.10 ± 0.1 ± 10^3</td>
</tr>
<tr>
<td>1.0</td>
<td>—</td>
<td>2.60 ± 0.1 ± 10^3*</td>
<td>4.80 ± 0.1 ± 10^3</td>
</tr>
<tr>
<td>5.0</td>
<td>—</td>
<td>2.90 ± 0.1 ± 10^3*</td>
<td>5.70 ± 0.1 ± 10^3</td>
</tr>
<tr>
<td>5.0 M6P 5.0</td>
<td>—</td>
<td>240 ± 10</td>
<td>460 ± 10</td>
</tr>
<tr>
<td>5.0 Man 5.0</td>
<td>—</td>
<td>2.3 ± 0.2 ± 10^3*</td>
<td>3.9 ± 0.3 ± 10^3</td>
</tr>
</tbody>
</table>

Intracellular IDUA activity is expressed as nmol methylumbelliferone released/h/mg protein. Values are expressed as mean±S.D., n=3. ∗p<0.01. Mouse osteoblasts (MC3T3-E1) and fibroblasts (F665) were cultured in medium containing various doses of laronidase as described under “Materials and Method”. MC3T3-E1 and F665 cells were also cultured in medium containing 5.0 μg/ml laronidase in the presence of 5.0 mmol/l M6P or 5.0 mmol/l Man. After 2 d culture, the cells were harvested and IDUA activity was measured.
DISCUSSION

Only a few reports concerning the effect of recombinant human IDUA on the degradation of accumulated substrates in cultured cells have been published. Unger et al. produced a recombinant IDUA in Chinese hamster ovary cells, and they showed that the enzyme could be endocytosed by cultured MPS I fibroblasts via M6P receptors, the accumulated sulphated GAGs being degraded, by means of $^{35}$SO$_4$- and $^3$H-galactose labelling.6)

In this study, we examined the effect of laronidase, which is now available for therapy for MPS I, on cultured MPS I fibroblasts biochemically and morphologically.

The results of the present analyses showed that the precursor form of the enzyme could be incorporated into MPS I fibroblasts dose-dependently mainly via M6P receptors. The incorporated enzyme was processed into the mature components, which were transported to lysosomes, the pathological findings being improved. These results suggest that enzyme replacement therapy should be effective for improving various manifestations due to disorders of connective tissue disorders.

However, previous studies on enzyme replacement therapy involving a canine model of MPS I revealed that a recombinant IDUA could decrease the storage of GAGs in the liver, kidneys, spleen, lymph nodes, adrenals, and lungs, but not in cartilage.9,10) As far as we know, the clinical improvement of disorders of skeletal and cartilage tissues on enzyme replacement therapy with the presently available recombinant enzymes is unknown, but is under investigation.11) Our experimental data for cultured mouse cells revealed that laronidase was incorporated into osteoblasts as well as into fibroblasts mainly via M6P receptors and partially via Man ones, which play an important role in the uptake of lysosomal enzymes by macrophages. But the uptake of the enzyme by osteoblasts was apparently lower than that by fibroblasts when the cells were cultured in medium containing 1.0 µg/ml laronidase or more. This must be one of the reasons why laronidase is not so effective for skeletal disorders in MPS I. Also, the anatomical characteristics of osteoblasts surrounded by the matrix in skeletal and cartilage tissue must cause the low uptake of the enzyme in vivo.

In conclusion, we examined the uptake of laronidase by cultured human MPS I fibroblasts and its effect on the cleavage of accumulated substrates morphologically. Furthermore, we investigated the uptake of laronidase by cultured mouse osteoblasts in comparison with that by cultured mouse fibroblasts, and revealed that laronidase could be incorporated into both the osteoblasts and fibroblasts mainly via M6P receptors, but that uptake of the enzyme by the former was lower than that by the latter.

To improve enzyme replacement therapy for skeletal disorders in MPS I, the administration of a high dose of the enzyme or the development of a recombinant IDUA containing many M6P residues is required.

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