Deficiency of density-dependent regulation of cell growth in the culture of skin fibroblasts from patients with mucolipidosis III

ATSUHIKO OOHIRA1•, FUMIKO MATSUI1, TAKASHI OKI2 and HIROSHI NOGAMI2

1Department of Embryology, Institute for Developmental Research and 2Department of Orthopaedic Surgery, Central Hospital, Kasugai, Aichi 480-03, Japan

• Author for correspondence

Summary

Cultured skin fibroblast cells were prepared from two patients with mucolipidosis III (ML III), which is a genetic disorder characterized by low activities of multiple lysosomal enzymes in fibroblasts. Genetic complementation analysis of fused fibroblast hybrids revealed that the patients were classified in different complementation groups. Growth curves of fibroblasts of ML III patients in culture were compared with those of fibroblasts of Sanfilippo's syndrome patients as well as of the normal fibroblasts. Normal and Sanfilippo fibroblasts gave essentially the same sigmoid curve of cell growth. However, although both ML III cell lines grew at the normal rate in the initial logarithmic phase, they continued to proliferate actively even after the cultures reached confluency. This is the first report to demonstrate the deficiency of density-dependent regulation of cell growth in the culture of non-transformed cell types. Therefore, the culture of skin fibroblasts of ML III patients may serve as a useful experimental model for investigating the regulation of cell proliferation in vitro.

Key words: fibroblasts, cell growth, mucolipidosis III, glycosaminoglycans.

Introduction

Mucolipidosis III (ML III, or pseudo-Hurler polydystrophy) is an autosomal recessive lysosomal storage disorder characterized by short stature, skeletal dysplasia and mild mental retardation (Kelly et al. 1975). Although patients have many clinical and roentgenological features similar to those of patients with mucopolysaccharidoses, none of them excretes excessive glycosaminoglycans in the urine (Melhem et al. 1973; Spranger et al. 1974; Kelly et al. 1975). The primary defect responsible for ML III has been shown to be a deficiency of UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine 1-phosphotransferase activity (Reitman et al. 1981; Waheed et al. 1982). This enzyme is involved in the sequential reactions to generate mannose 6-phosphate residues of the lysosomal enzymes, which are required for recognition of the enzymes by a specific transport receptor for targeting to the lysosome (Reitman & Kornfeld, 1981). The deficiency of this enzyme activity results in mislocalization of multiple lysosomal enzymes so that they are secreted from cells into body fluid, or into the medium in culture, rather than transported into lysosomes (Thomas et al. 1973; Kelly et al. 1975). Because of the low activities of multiple lysosomal enzymes in cells, there exist numerous lysosomal storage vesicles in cytoplasm of ML III cells (Kelly et al. 1975). ML III has been shown by a complementation analysis of cultured fibroblasts to be genetically heterogeneous (Honey et al. 1982; Little et al. 1986). Three complementation groups have been characterized, indicating that mutations in three different genes are implicated in the ML III phenotype.

Recently we prepared two fibroblast strains from skin of patients with ML III. In this paper we demonstrate that, although they are in different complementation groups, both cell lines give identical growth curves in culture that are distinct from that of normal fibroblasts. It is well known that transformation of cells results in loss of density-dependent regulation of cell growth in culture. Since abnormal composition and/or structure of cell surface macromolecules such as glycosaminoglycans (GAGs) and...
fibronectin are usually observed in cultures of transformed cells (Vaheri & Mosher, 1978; Mikuni-Takagaki & Toole, 1979; Keller et al. 1982), it is believed that these macromolecules are involved in density-dependent control of cell growth. The fibroblasts of ML III patients should not be considered as being transformed cells. Although the exact mechanism operating in overgrowth of the cultured fibroblasts of ML III patients is not known at present, studies using this culture system should produce useful information about growth control mechanism of cells.

Materials and methods

Clinical materials
Two boys (case-1 aged 3 years and case-2 aged 12 years) were seen, because of clinical features, such as mild skeletal dysplasia and short stature, similar to those of mucopolysaccharidoses. However, neither patients excreted excessive glycosaminoglycans in the urine (26 mg/l for case-1, 33 mg/l for case-2, and 18-32 mg/l for normal). A small piece of skin was excised from each case by biopsy to prepare the culture of fibroblasts. Fibroblasts were also prepared from the skin of a patient with Sanfilippo’s syndrome (9-year-old male) and two normal individuals (3-year-old male and 25-year-old female).

Culture of skin fibroblasts
After mincing with a no. 11 surgical blade, the pieces of skin were cultured under a coverglass in Dulbecco's modified Eagle's medium (Nissui Seiyaku, Tokyo) supplemented with 100 IU ml⁻¹ of penicillin, 100 μg ml⁻¹ of streptomycin, 2 μg ml⁻¹ of Fungizone and 10 % (v/v) foetal bovine serum (FBS from M. A. Bioproducts, Walkersville). This medium without FBS is hereinafter designated as DMEM. Fibroblasts were prepared from the skin of a patient with Sanfilippo's syndrome (9-year-old male) and two normal individuals (3-year-old male and 25-year-old female).

Lysosomal glycosidase assays
After washing with phosphate-buffered saline (PBS), cultured cells were scraped into PBS containing EDTA (2 ml per dish) at 0°C. The cell pellet, collected by centrifugation at 3000 revs/min for 10 min, was washed twice with 0.9 % NaCl (1 ml per dish). The final cell pellet was homogenized in 0.9 % NaCl (0.5 ml per dish) with a Dounce homogenizer with a Teflon pestle. The homogenate was subjected to four cycles of freezing and thawing. Using samples of the homogenate, protein was determined by the method of Lowry et al. (1951). Enzyme assays for β-N-acetylhexosaminidase (EC 3.2.1.30) and β-galactosidase (EC 3.2.1.23) were done on cell homogenates and culture media by measuring spectrophotometrically the free p-nitrophenol released from the appropriate glycosidic derivatives (Thomas et al. 1973). Assays for α-fucosidase (EC 3.2.1.51) and α-mannosidase (EC 3.2.1.24) were determined with appropriate fluorogenic 4-methylumbelliferyl derivatives (Kress & Miller, 1979). These substrates were purchased from Nakarai Chemicals, Kyoto.

[^35]Sulphated GAGs
Highly confluent cultures of fibroblasts were labelled with 15 μCi ml⁻¹ of Na₂[^35]SO₄ (carrier-free, Japan Radiisotope Association, Tokyo) in DMEM containing 10 % FBS for 48 h. GAGs were isolated from the cell layer of cultured fibroblasts by digestion with Pronase (nuclease-free, Calbiochem-Behring, San Diego) as described previously (Oohira et al. 1982). The amount of hexuronate in the GAG preparation was determined by the method of Bitter & Muir (1962). The GAG preparation was separated by two-dimensional electrophoresis on cellulose acetate film (Sepaphore III from Gelman Sciences, Ann Arbor) and each GAG component was identified enzymically as described (Oohira et al. 1986a). DNA content of the culture was determined using deoxyadenosine 5’-monophosphate (dAMP) as a standard, by the method of Burton (1956), using samples of the Pronase digest of the cell layer.

Light and electron microscopy
Cultured skin fibroblasts were fixed in culture dishes with 3 % glutaraldehyde buffered to pH 7.4 with 0.1 M-sodium cacodylate at 4°C for 2 h. Postfixation was carried out in cacodylate-buffered 2 % osmium tetroxide at 4°C for 3 h. The samples were dehydrated in a series of solutions of ethanol and acetone, and embedded in Epon 812. Embedded
samples were removed from the culture dish. Preliminary sections, parallel to the surface of the dish, were cut at 0.5 μm and stained with Toluidine Blue for the light-microscopic observation. Thin sections were stained with uranyl acetate and lead citrate. Observations were performed under a JEOL JEM-100B electron microscope.

Results

Light and electron microscopy
Cultured skin fibroblasts from both cases had numerous cytoplasmic inclusion bodies (see Fig. 1A, for case-1). The pathological cultures seemed to have a high cell density (Fig. 1A) compared to the control culture (Fig. 1B). Since there was no significant differences in the cell viability or in the initial cell density among these cultures, this observation suggests that the fibroblasts from the patients grow more actively in culture than those from the normal individuals. Ultrastructural examination revealed accumulation of lamellar figure material in these cytoplasmic vacuoles of the pathological fibroblasts (see Fig. 2A, for case-1).

Lysosomal glycosidase activities
The activities of the lysosomal β-hexosaminidase, β-galactosidase, α-fucosidase and α-mannosidase were assayed in the fibroblast homogenates of case-1 and case-2 (Table 1). In both cases, all the glycosidase activities were significantly low (at most 25% of the normal values). Of the two cases, case-2 generally had the lower glycosidase activities, especially for α-fucosidase and α-mannosidase.

The acid glycosidase activity secreted into the culture medium by fibroblast monolayers (confluent culture, 1×10⁶ cells at the time of harvest) for 3 days was also measured by the same method as that described above. The culture medium of the skin fibroblasts from either case contained the enzyme
activity almost equivalent to, or even higher than, that detected in the media of normal fibroblast lines (data not shown). From these enzymic studies, in addition to the clinical and morphological data shown in Figs 1 and 2, both cases could be diagnosed to be ML III.

Complementation analysis
The lysosomal glycosidase activities were assayed in the homogenates of multinucleated cells formed by treatment with PEG of the parental cells as well as of the fibroblast mixture of case-1 and case-2 (Table 1). When the monolayer of the normal cells was treated with PEG, the lysosomal glycosidase activities decreased slightly (by about 20% of the non-treated sample). Fusion of each cell line of ML III (hokaryon formation) did not change the levels of these intracellular enzymic activities significantly. In addition, cocultivation of the fibroblasts of case-1 and case-2 did not increase these lysosomal enzyme activities. On the contrary, heterokaryon formation for case-1 and case-2 resulted in a marked increase in the activities of these glycosidases (Table 1). These findings indicate that case-1 and case-2 are classified in different complementation groups of ML III.

Fibroblast growth curve
Fibroblasts of passage 4 ($4 \times 10^5$) were seeded in a 60-mm culture dish (Corning no. 25010) and cultured in 5 ml of DMEM containing 10% FBS. The culture medium was changed every other day. Normal fibroblast cells (from 3-year-old male) proliferated actively after a lag period of 2 days, and reached confluency (about $2 \times 10^6$/dish) on day 4 of cultivation (Fig. 3). The growth curve of another normal cell line (from 25-year-old female) coincided with that from the 3-year-old male within experimental error (see Table 2).

The growth curve of the cultured fibroblasts of ML III patients was clearly abnormal. Although the initial lag and logarithmic phases were exactly the same as those of normal cell lines, the ML III cells did not cease growth after reaching confluency (Fig. 3). Consequently, the cell density of the ML III fibroblast culture increased by about 50% of the normal in 10 days of cultivation, and almost twofold in 2 weeks (Fig. 3). It is noteworthy that, although these two cases were in the different complementation groups of ML III as described above, the growth curves of both fibroblasts in culture were exactly the same.

Sanfilippo's syndrome has some clinical and metabolic features similar to those of ML III. However, skin fibroblasts from Sanfilippo's syndrome patients did not show such an aberrant overgrowth in culture. The growth curve was almost identical to those of the normal fibroblasts (Fig. 3).

The aberrant cell density of ML III fibroblast culture on day 10 was confirmed by measuring the amount of DNA in the cultures (Table 2). The amount of DNA from a 60-mm dish of confluent culture did not vary, regardless of the age and sex of
Table 1. Correction of intracellular lysosomal glycosidase activity by heterokaryon formation using polyethylene glycol

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>β-Hexosaminidase (nmol mg⁻¹ protein h⁻¹)</th>
<th>β-Galactosidase (nmol mg⁻¹ protein h⁻¹)</th>
<th>α-Fucosidase (nmol mg⁻¹ protein h⁻¹)</th>
<th>α-Mannosidase (nmol mg⁻¹ protein h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (range, n = 4)</td>
<td>None</td>
<td>3575 (100)</td>
<td>592 (100)</td>
<td>8.34 (100)</td>
<td>5.04 (100)</td>
</tr>
<tr>
<td></td>
<td>Fused</td>
<td>2800–4310</td>
<td>333–705</td>
<td>5.58–9.56</td>
<td>4.23–5.48</td>
</tr>
<tr>
<td>Case 1</td>
<td>None</td>
<td>787 (22)</td>
<td>112 (21)</td>
<td>0.84 (10)</td>
<td>1.21 (24)</td>
</tr>
<tr>
<td></td>
<td>Fused</td>
<td>470 (79)</td>
<td>108 (15)</td>
<td>0.12 (2)</td>
<td>0.29 (6)</td>
</tr>
<tr>
<td>Case 2</td>
<td>None</td>
<td>643 (18)</td>
<td>126 (21)</td>
<td>0.19 (2)</td>
<td>0.35 (7)</td>
</tr>
<tr>
<td></td>
<td>Fused</td>
<td>772 (22)</td>
<td>111 (19)</td>
<td>0.12 (2)</td>
<td>0.29 (6)</td>
</tr>
<tr>
<td>Case-1 × case-2</td>
<td>None</td>
<td>2717 (76)</td>
<td>318 (54)</td>
<td>0.68 (8)</td>
<td>0.51 (10)</td>
</tr>
<tr>
<td></td>
<td>Fused</td>
<td>315 (76)</td>
<td>138 (23)</td>
<td>0.68 (8)</td>
<td>0.51 (10)</td>
</tr>
</tbody>
</table>

Lysosomal glycosidase activity was measured for parental cell lines, the cocultivated mixture of case-1 and case-2, and fusion cultures of these cells. The data represent the means of triplicate samples of two independent experiments. The values in parenthesis are expressed as the percentage of the non-treated normal sample.

Table 2. Amount of DNA in confluent cultures of human skin fibroblasts of various diseases

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (years)</th>
<th>Sex</th>
<th>DNA (nmol dAMP per dish) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>Male</td>
<td>13.5 ±1.3</td>
</tr>
<tr>
<td>Normal</td>
<td>25</td>
<td>Female</td>
<td>14.3 ±0.3</td>
</tr>
<tr>
<td>Sanfilippo</td>
<td>9</td>
<td>Male</td>
<td>13.5 ±0.8</td>
</tr>
<tr>
<td>Case-1</td>
<td>3</td>
<td>Male</td>
<td>21.3 ±0.6</td>
</tr>
<tr>
<td>Case-2</td>
<td>12</td>
<td>Male</td>
<td>20.7 ±1.1</td>
</tr>
</tbody>
</table>

Human skin fibroblasts of passage 4 were cultured in a 60 mm dish at an initial cell density of 4.0×10⁵ per dish. On day 10 of cultivation, cells were scraped and digested with nuclease-free Pronase for the determination of DNA (Burton, 1956). The data represent the means ± s.d. of two separate experiments. Three independent dishes were used for each experiment.

150% than those of the normal (Table 2). These findings are consistent with the results shown in Fig. 3.

[35S]sulphated GAGs in cell layer

Highly confluent cultures (usually day 7 or 8 of cultivation) were labelled with [35S]sulphate for 48 h, and labelled GAG was isolated and analysed by two-dimensional electrophoresis on a cellulose acetate film. Fig. 4A shows an Alcian Blue-stained electrophoretogram of the GAG preparation (containing 20 nmol hexuronate) from the normal fibroblast culture of 3-year-old male. Three major spots and two faint spots were detectable. These major spots were identified enzymically to be dermatan sulphate (DS), heparan sulphate (HS) and hyaluronate (HA) as described (Oohira et al. 1986a). One of the faint spots was also identified to be chondroitin sulphate (CS). Another faint spot (arrowhead in Fig. 4A) has not been identified. This material was resistant to digestion by all

Fig. 3. Growth curves of human skin fibroblasts in culture. Cells of passage 4 were seeded in a 60 mm dish at an initial cell density of 4.0×10⁵, and cultured, with feeding every other day. Cells were counted with a double Neubauer haemocytometer after dissociation of the cell layer with trypsin. The data represent the means of eight determinations of two independent experiments. Vertical bars represent the standard deviations of the data. (▲) normal fibroblasts from 3-year-old male; (△) fibroblasts from case-1; (●) fibroblasts from case-2.

Fibroblast growth of mucolipidosis III 253
GAG lyases used in this work. The autoradiogram of the electrophoretogram is shown in Fig. 4B. There were three radioactive spots representing CS, DS, and HS. Similar electrophoretograms and autoradiograms were obtained from all the other samples, but the GAG composition seemed to be different depending on the type of disease. For example, these four GAGs and the unidentified, faint spot were detected in the samples of ML III cells (Fig. 4C). Three of these GAGs were labelled with $[^{35}S]sulphate$ (Fig. 4D).

Judging from the density of these spots, the relative amount of DS in the GAG preparation of ML III cells seemed to be significantly large compared to the normal samples.

The radioactive spots were cut out to measure the $^{35}S$ radioactivities. HS was the major sulphated GAG isolated from the cell layers of the normal as well as of Sanfilippo samples (Fig. 5). The cell layer of ML III cultures contained significantly large amounts of labelled GAG (more than twofold of the normal).

Fig. 4. Two-dimensional electrophoresis of GAG preparations on cellulose acetate film. The electrophoretograms shown are from the cell layer of normal skin fibroblast culture (3-year-old male) labelled with $[^{35}S]sulphate$ for 48 h (A) and the corresponding autoradiogram (B); the preparation from the cell layer of ML III (case-2) fibroblast culture (C) and the corresponding autoradiogram (D). These Alcian Blue-stained GAG spots were identified as chondroitin sulphate (CS), dermatan sulphate (DS), heparan sulphate (HS) and hyaluronate (HA), by the enzymic method described (Oohira et al. 1986a). In addition, there was a very faint spot (arrowhead) that was shown to be resistant to digestion by all GAG lyases used in this work. Migrating positions of the reference GAGs are shown in the margins of the electrophoretogram (A, C).
Interestingly, DS was the major sulphated GAG in both cases of ML III (Fig. 5).

Discussion

Although strictly controlled growth takes place in the development and maintenance of a normal animal, the control mechanism of animal cell proliferation still remains poorly understood. In this paper, we have demonstrated that the skin fibroblasts of patients with ML III grow to a cell density greater than that of the normal human skin fibroblasts (Fig. 3). Cell behaviour in culture, such as proliferation, may vary with age of donors, passage number of cells, concentration of ingredients in the medium, and so on. As described in detail in Materials and methods, we used cells prepared from age-matched normal individuals at the same culture age for the control experiments. In addition, a control experiment using at least one of the normal cell lines was always carried out in parallel with the experiments with pathological cell lines at the same time. Therefore, we consider that the difference in the growth curve between normal and ML III fibroblasts in culture is significant.

Loss of density-dependent regulation of cell growth in culture is one of the common features of transformed cells (Holley, 1975; Vaheri & Mosher, 1978; Mikuni-Takagaki & Toole, 1979). Transformed cells grow to a cell density higher than that of non-transformed counterparts. In some cell types, transformation results in shortening the doubling time (Mikuni-Takagaki & Toole, 1979). The ML III fibroblasts grew to a cell density higher than that of the normal cell lines in prolonged culture, but they grew at the normal rate during the initial logarithmic phase (Fig. 3), showing that their doubling time is not shortened.

ML III is one of the lysosomal storage diseases and shows some clinical, morphological and biochemical features similar to those of other lysosomal storage diseases (see Melhem et al. 1973; Spranger et al. 1974; Kelly et al. 1975; Oohira et al. 1986b). However, the aberrant overgrowth of the cultured skin fibroblasts does not seem to be common in the family of lysosomal storage diseases. In addition to the Sanfilippo fibroblasts shown in Fig. 3, our preliminary experiments revealed that fibroblasts of Hunter's syndrome, another lysosomal storage disease, also gave a normal sigmoid growth curve in culture. It is of interest that both cell lines of ML III showed identical, abnormal growth curves (Fig. 3) in spite of being in different
genetic complementation groups (Table 1). The aberrant overgrowth of ML III fibroblasts in culture might be a phenomenon resulting from low activities of multiple intracellular lysosomal enzymes.

Besides many growth factors, some cell surface molecules including GAGs have been shown to be involved in control of cell proliferation in vitro either positively or negatively (Holley, 1975; Vaheri & Mosher, 1978; Hynes, 1981; Toole, 1981; Hsu & Wang, 1986). These cell surface molecules must interact with cells via specific receptors on the plasma membrane. Cells have to modulate their microenvironment by changing the content and composition of receptors as well as pericellular molecules, not only through synthesis but also by degradation of these molecules. Therefore, it is presumed that there are quantitative and qualitative abnormalities of the cell surface molecules in the culture of ML III fibroblasts, because of the low activities found for multiple lysosomal enzymes. These abnormalities may result in the abnormal overgrowth shown in Fig. 3. In the present study, we have demonstrated abnormalities in the content and the composition of GAGs in the cell layer of ML III fibroblasts (Fig. 5). The finding that DS is the major sulphated GAG is interesting, because DS and HA have been shown to stimulate proliferation of mouse dorsal fibroblasts in confluent culture under certain conditions (Yoneda et al. 1985). However, it should be mentioned that some of the excessive GAGs no doubt originate from the intracellular storage vesicles of ML III fibroblasts.

We thank Mrs Meiko Aoki for her excellent technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research (59570651 and 61106001) from the Ministry of Education, Science and Culture of Japan and Research Grant no. 59-296 from the Ishida Foundation to A.O., and by Research Grant no. 60-314 from the Ishida Foundation to H.N.

References


(Received 1 September 1986 – Accepted 24 October 1986)