Defective catabolism of low-density lipoprotein by fibroblasts from patients with I-cell disease

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Skin fibroblast cultures from patients with I-cell disease (mucolipidosis II), an autosomal inherited fatal childhood disorder, are characterized by a range of deficiencies in the activities of multiple enzymes (Leroy et al., 1972; Leroy & O’Brien, 1976; Wenger et al., 1976; Poiter et al., 1977). Culture medium from I-cell skin fibroblasts and extracellular fluids from I-cell-disease patients show elevated activities of several lysosomal hydrolase activities (Wiesmann et al., 1971; Hickman & Neufeld, 1972; Kress & Miller, 1979).

Biochemical evidence suggests that I-cell disease is the result of an alteration in a post-translational process involving glycosylation, which is common to the final expression of the lysosomal enzymes (Kress et al., 1980; Hasilik & Neufeld, 1980a,b; Tabas & Kornfeld, 1980; Miller et al., 1981). Results further indicate an absence of the phosphomannosyl recognition marker on lysosomal enzymes in I-cell disease (Bach et al., 1979; Hasilik & Neufeld, 1980b; Tabas & Kornfeld, 1980). Recent studies suggest that the absence of the phosphomannosyl marker is due to the deficiency of a UDP-N-acetylgalcosamine lyosomal enzyme, N-acetylgalcosamine 1-phosphatetransferase (Hasilik et al., 1981; Reitman et al., 1981).

The work of Goldstein & Brown (1977) has delineated the pathway for binding, uptake and catabolism of LD lipoprotein by normal fibroblasts and the regulation of HMG-CoA reductase that follows on the uptake of LD lipoprotein. They have shown that in fibroblasts from patients with Wolman’s disease and with cholesteryl ester storage disease the uptake and degradation of LD-lipoprotein protein occurs normally but the cholesteryl esters are hydrolysed poorly and thus cholesterol synthesis is poorly regulated. Studies in our laboratory (Pittman et al., 1979) have demonstrated a partial reduction of acid cholesteryl ester hydrolase activity in skin fibroblasts and post-mortem liver specimens from several I-cell-disease patients. In the present studies we have examined the binding, internalization and degradation of 125I-labelled LD lipoprotein by I-cell fibroblasts, their content of acid proteinase activity and also the regulation of HMG-CoA reductase by LD lipoprotein in these cells.

Materials and methods

Materials

Tissue culture media, foetal calf serum, Dulbecco’s phosphate-buffered saline and trypsin were...
obtained from Grand Island Biological Laboratories (Grand Island, NY, U.S.A.); [1-14C]acetate (sodium salt) and carrier-free 125I were from Amer-sham–Searle Corp. (Arlington Heights, IL, U.S.A.); dithiothreitol and o-phthalaldehyde were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 7-oxo-cholesterol was from Steroids (Wilton, NH, U.S.A.); and cholesterol oxidase and cholesteryl ester hydrolase were obtained from Boehringer-Mannheim (Germany). Serum samples from patients with I-cell disease were shipped to us either fresh (on ice) or frozen.

Fibroblast cultures

Human skin fibroblasts from a preputial biopsy of a normal infant and fibroblasts from a patient with homozygous familial hypercholesterolaemia were grown in monolayer culture. Metabolism of lipoproteins by these cell lines has been described previously (Stein et al., 1976; Miller et al., 1977). The cells were maintained in DME medium containing 10% (v/v) foetal calf serum (Stein et al., 1976). Skin fibroblasts from two patients with I-cell disease (lines K.Z. and L.T.) were maintained in Ham's F-12 medium containing 15% (v/v) foetal calf serum. Analysis of these patients' fibroblasts confirmed that at least eight lysosomal hydrolase activities were deficient compared with the enzyme activities of the normal fibroblast control cultures. Cells were all transferred to DME medium 24h before study and used at or near confluency.

Lipoprotein preparation

LD lipoprotein was isolated from fresh human serum by preparative ultracentrifugation and purity was demonstrated as described previously (Miller et al., 1977). Lipoprotein-deficient foetal calf serum was prepared as described previously (Weinstein et al., 1976). 125I-labelled LD lipoprotein was prepared by a modification of the iodine monochloride method of McFarlane (1958) as described previously (Weinstein et al., 1976). Less than 2% of the radioactivity in the final preparations was trichloroacetic acid-soluble or extractable into chloroform/methanol (2:1, v/v). All lipoprotein preparations were sterilized by passage through 0.22 μm Millipore filters (Millipore Corp., Bedford, MA, U.S.A.) and stored at 4°C. Protein content was determined by the method of Lowry et al. (1951) after extracting the coloured reaction product with one-half the volume of chloroform to eliminate lipid opalescence.

Lipoprotein catabolism

The growth medium was removed from the culture dishes 18–24h before the beginning of each experiment and was replaced with 2ml of fresh DME medium containing 5% (v/v) lipoprotein-deficient serum. For lipoprotein-binding experiments at 4°C, the cells were placed on crushed ice for 10min before the medium containing 125I-labelled LD lipoprotein was added. The cells were kept on ice in a 4°C cold box for 60min and then the medium was removed and a portion was assayed for total radioactivity. The cell monolayers were washed three times with 2ml of Dulbecco's phosphate-buffered saline at 4°C, followed by two washes with cold Dulbecco's phosphate-buffered saline containing 2mg of bovine serum albumin/ml and finally with the buffered saline alone. The cell monolayers were removed from the dishes by treatment with 1ml of 0.2m-KOH for 1h at room temperature. A portion of the cell digest was removed for protein analysis (Lowry et al., 1951) and for measurement of total radioactivity, which is a measure of the cell-surface-bound lipoprotein.

Binding, internalization and degradation of 125I-labelled LD lipoprotein was measured after pre-incubation of the cells in DME medium containing 5% (w/v) lipoprotein-deficient serum for 24h and in some experiments for 48h. After incubation with 125I-labelled LD lipoprotein at 37°C the medium was removed and lipoprotein degradation was measured as trichloroacetic acid-soluble 125I radioactivity in the medium after removal of [125I]iodide as described previously (Weinstein et al., 1976). Pre-incubation of I-cell fibroblasts in lipoprotein-deficient medium for 36–48h increased the degradation rate of LD lipoprotein by only 10–15% compared with the 24h period. All the data in this paper are based on the 24h induction period. Net degradation was calculated as the difference between values obtained from identical incubations in the presence and absence of cells.

Since lysosomal enzymes may leak into the culture medium and contribute to the apparent rate of degradation of 125I-labelled LD lipoprotein, control incubations were performed using 'conditioned medium', i.e. medium that had been exposed to I-cell fibroblasts for 24h. Proteolysis of 125I-labelled LD lipoprotein by 'conditioned' culture medium was negligible at all LD-lipoprotein concentrations tested.

After removal of the medium, the cells were washed as described above for binding studies at 4°C and were harvested by trypsin treatment as described previously (Stein et al., 1976). The radioactivity released by trypsin treatment was considered to represent lipoprotein bound to the cell surface. The cell pellet was dissolved in 1ml of 0.2m-KOH and a portion was removed for measurement of lipid-soluble radioactivity [extractable into chloroform/methanol (2:1, v/v)]. The non-lipid trichloroacetic acid-precipitable radioactivity remaining associated with the cells after trypsin
Low-density-lipoprotein catabolism in I-cell fibroblasts

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Low-density-lipoprotein catabolism was protein by reductase, the rate-limiting enzyme in cholesterol synthesis, was measured as described by Goldstein & Brown (1974).

Cell cholesterol synthesis

The effects of LD lipoprotein on sterol and fatty acid synthesis were measured by using the [1-14C]acetate incorporation described previously (Weinstein et al., 1976). The activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, was measured as described by Goldstein & Brown (1974).

Cholesterol content

Serum samples were extracted with 20 vol. of chloroform/methanol (1:1, v/v) at 4°C for 18 h. The extract was washed with water to remove non-lipid contaminants and portions were removed for total cholesterol analysis by the o-phthalaldehyde method of Rudel & Morris (1973). The cholesterol and cholesteryl ester content of the cells was measured by the enzymic method of Gamble et al. (1978).

Proteolytic degradation of 125I-labelled LD lipoprotein by cell-free extracts

Cells from 10 dishes (60 mm x 15 mm) were washed free of growth medium with Dulbecco’s phosphate-buffered saline and were scraped off the dish with a rubber policeman into the same buffer at 4°C. Cells were collected by centrifugation (2500 g for 5 min at 4°C) and the cell pellet was suspended in 1 ml of water and sonicated for 15 s at maximum power using a micro-ultrasonic cell disruptor (Kontes, Vineland, NJ, U.S.A.). Each assay contained 0.1 m-acetate or 0.1 m-Tris/HCl buffer at the indicated pH, 10 μg of 125I-labelled LD lipoprotein (190 c.p.m./ng)/ml, 5 mm-dithiothreitol, 0.1 mm-EDTA and 80 μg of cell-extract protein. The incubation and assay were performed as described by Goldstein et al. (1975a).

Results

Degradation of 125I-labelled LD lipoprotein was first studied over a 6-h period. As shown in Fig. 1, the degradation at all LD-lipoprotein concentrations was abnormal in the two lines studied. In one cell line (L.T.) the degradation was almost as low as it was in a receptor-negative line of cells from a patient with homozygous familial hypercholesterolemia. In the other cell line (K.Z.), degradation was reduced to less than 50% of that in the control fibroblasts. This clear deficiency in rate of LD-lipoprotein degradation is not attributable to deficient binding, as shown by the results in Fig. 2. Cell line L.T., in which degradation was almost completely absent, showed binding that was indistinguishable from the binding to normal fibroblasts at all LD lipoprotein concentrations. Cell line K.Z. showed binding that was perfectly normal at low LD
lipoprotein concentrations but slightly less at higher concentrations of LD lipoprotein both at 37°C (Fig. 2) and at 4°C (results not shown). Still the differences were much smaller than the differences in observed degradation rates (Fig. 1). As shown in Fig. 3, the amount of 12I-labelled LD lipoprotein found intracellularly in I-cell cultured fibroblasts at the end of the incubation ('internalized') was greater than that in normal control cells, indicating the accumulation of undegraded LD lipoprotein. When the amount of LD lipoprotein internalized and the amount degraded were summed, providing an index of total uptake over the 6 h, the differences between I-cell fibroblasts and control fibroblasts (Fig. 4) were much smaller than the differences in degradation (Fig. 1). Thus degradation by L.T. cells was less than 10% of control values, and total uptake was 75% of control values.

As shown in Fig. 5, neither I-cell line showed any significant inhibition of HMG-CoA reductase activity in 6 h even at concentrations of LD lipoprotein cholesterol up to 40 μg/ml, in contrast with the greater than 80% inhibition observed in normal fibroblasts.

The effects of LD lipoprotein (20 μg/ml) were also tested with regard to rates of acetate incorporation into cell sterols, as shown in Table 1. In normal cells, as expected, there was a significant inhibition, approx. 40% in the presence of 20 μg of LD lipoprotein/ml. On the other hand, there was little or no effect in the two I-cell lines. These cells lines did, however, show a marked inhibition of acetate incorporation into sterols in the presence of 7-hydroxysterol, indicating that the systems regulating endogenous cholesterol biosynthesis are responsive and that the deficiency relates specifically to the metabolism of LD lipoprotein.

As shown in Fig. 3, the pool of internalized undegraded LD lipoprotein in the I-cell fibroblasts at 6 h was increased, particularly in the L.T. cells. A
Table 1. Effects of LD lipoprotein and 7-oxocholesterol on the incorporation of \[1^4\]Cacetate into sterols and fatty acids

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No additions</th>
<th>LD lipoprotein (20 (\mu)g/ml)</th>
<th>7-Oxocholesterol (5 (\mu)g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sterols</td>
<td>Fatty acids</td>
<td>Sterols</td>
</tr>
<tr>
<td>Normal</td>
<td>20 100</td>
<td>99 700</td>
<td>12 110</td>
</tr>
<tr>
<td>I-cell (K.Z.)</td>
<td>9 140</td>
<td>44 200</td>
<td>8 110</td>
</tr>
<tr>
<td>I-cell (L.T.)</td>
<td>19 100</td>
<td>103 000</td>
<td>18 500</td>
</tr>
</tbody>
</table>

Fig. 6. Comparison of the degradation of \[125\]I-labelled LD lipoprotein in 24 h by normal fibroblasts (●), fibroblasts from a patient with homozygous familial hypercholesterolaemia (▲) and fibroblasts from two I-cell-disease patients (L.T. (●) and K.Z. (▲))

The experimental conditions were identical with those described in Fig. 1, except that degradation was measured after a 24 h incubation with \[125\]I-labelled LD lipoprotein (sp. radioactivity 180 c.p.m./ng). Results are means of duplicate determinations.

progressive increase in the concentration of LD lipoprotein available inside the cell might enhance the rate of its degradation and so in longer incubations the rate in I-cells might more nearly approach control values. For this reason we re-studied the process of LD lipoprotein uptake and degradation in 24-h incubations. As shown in Fig. 6, the LD lipoprotein degradation by the I-cell lines in 24 h was still less than that in the control fibroblasts but the difference was less striking than in the 6-h experiments (Fig. 1).

This might reflect a progressive increase in substrate concentration in the lysosome with consequent increases in the net rate of LD lipoprotein degradation, approaching the rate in control cells. Indeed, the total amount of \[125\]I accumulating in the I-cells over a 24 h interval was 8 to 12 times that accumulating in the normal cells. This contrasts with the intracellular accumulation at 6 h, which was 1.6 to 3 times that in control cells.

Fig. 7 shows the time course for \[125\]I-labelled LD lipoprotein degradation in normal cells, the I-cell line K.Z. and the familial-hypercholesterolaemia line. There was a longer initial lag period in the case of the K.Z. line compared with the control cells. Between 12 h and 24 h the degradation rate in the K.Z. line was only about 30% lower than that in the control cells. The results are compatible with the conclusion that at steady state the difference in LD lipoprotein degradation between the I-cells and normal fibroblasts may be small or may not even be observed, probably due to the progressive accumulation of LD lipoprotein in the lysosomes of I-cell fibroblasts thus increasing the available concentration of protein substrate in these lysosomes.

The effects of a 24 h incubation with LD lipoprotein on HMG-CoA reductase activity were examined. As shown in Fig. 8, reductase activity in the control cells was inhibited by more than 80% and there was little if any effect in the familial-hypercholesterolaemia cells. The I-cell line L.T. now showed some inhibition, about 37%, but the inhibition was much less than that seen in the normal cells.

Cell cholesterol content was determined after a 24 h incubation in the presence of lipoprotein-deficient serum with or without 100 \(\mu\)g of LD lipoprotein/ml. As shown in Table 2, the two I-cell lines contained more cholesterol, both unesterified and in the ester form, than control cells whether incubated overnight in lipoprotein-deficient serum or with LD lipoprotein. The ester cholesterol content of I-cells...
incubated in lipoprotein-deficient serum was three times that of the control cells. After incubation with LD lipoprotein all cell lines showed an increase in cholesterol content but the increment was smaller in the I-cell lines.

Homogenates of fibroblasts were prepared by

Table 2. Increase in cell cholesterol content after 24-h incubation with lipoprotein-deficient serum or with LD lipoprotein (100 µg/ml)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Addition to lipoprotein-deficient medium</th>
<th>Unesterified</th>
<th>Ester</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>58</td>
<td>7</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>LD lipoprotein</td>
<td>78</td>
<td>23</td>
<td>101</td>
</tr>
<tr>
<td>I-cell line K.Z.</td>
<td>None</td>
<td>77</td>
<td>21</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>LD lipoprotein</td>
<td>85</td>
<td>32</td>
<td>117</td>
</tr>
<tr>
<td>I-cell line L.T.</td>
<td>None</td>
<td>82</td>
<td>26</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>LD lipoprotein</td>
<td>88</td>
<td>42</td>
<td>130</td>
</tr>
</tbody>
</table>
sonication and assayed for their ability to degrade 125I-labelled LD lipoprotein as a function of pH. As shown in Table 3, the rates of degradation in normal cells and cells from a patient with homozygous familial hypercholesterolaemia were approximately the same, and both showed optimum activity at pH 4. Degradation by homogenates from the I-cell lines was also optimal at pH 4 but was only 20–30% of the control values. Thus the deficiency in degradation of LD lipoprotein may be attributable to a deficiency of lysosomal cathepsin activity.

### Discussion

The deficiency of multiple lysosomal glycohydrolases, acid lipase and aryl sulphatase in fibroblasts from patients with I-cell disease is well recognized (Leroy et al., 1972; Leroy & O'Brien, 1976; Wenger et al., 1976; Pittman et al., 1979). The present studies are the first to directly demonstrate an acid proteinase deficiency in I-cell fibroblasts. These latter results are consonant with the findings of Hasilik & Neufeld (1980b) that the mannose moiety of cathepsin D is not phosphorylated in I-cells and with the evidence for a reduced degradation by intact I-cells of albumin–mannose 6-phosphate conjugates (Karson et al., 1980). In one of the I-cell lines studied, degradation at 6 h was less than 10% of that in control cells and in the other line about one-third that in control cells. In contrast, binding of LD lipoprotein to the I-cells was essentially normal and the overall uptake into them was within 25% of normal values. Thus the demonstrated defect in degradation is attributable primarily to a defect in proteolytic degradation.

This conclusion is supported by the greater accumulation of intracellular LD lipoprotein in I-cells than in control cells. This was evident at 6 h but more striking at 24 h, when the increase was 8–12 times that seen in control cells. The lesser defect in degradation rate seen at 24 h probably reflects this accumulation and a consequent increase in the availability of LD lipoprotein substrate in the lysosomes.

The consequences of the demonstrated defect in cell culture with regard to LD lipoprotein metabolism in vivo are difficult to predict. In vivo it is possible that the intracellular pools might go on increasing until the absolute degradation rate matched the rates of LD lipoprotein internalization. In that case, one might not see abnormalities in the rate of degradation of plasma LD lipoprotein at steady state. On the other hand, if there were defective degradation, it might be reflected in an increased steady-state concentration of plasma LD lipoprotein. Data on LD lipoprotein concentrations in I-cell disease are not available. However, we have been able to obtain serum samples for total cholesterol determination in seven patients. Total serum cholesterol exceeded the 95th percentile for age-matched controls in four of these cases (Lipid Research Clinics Population Studies Data Book, 1980). In two of them, the value was more than 50% above the 95th percentile value and in two others just above the 95th percentile value. The significance of this limited and uncontrolled sampling is difficult to assess. It may indicate a primary defect in LD lipoprotein removal in vivo or it may reflect an indirect end result of other metabolic consequences of the disease.

In normal fibroblasts, incubation with LD lipoprotein decreases HMG-CoA reductase activity and this is believed to be dependent on prior hydrolysis of the cholesterol ester moiety (Goldstein & Brown, 1977). The failure of LD lipoprotein to affect reductase activity in I-cells could reflect the proteinase deficiency, the previously demonstrated deficiency in acid cholesterol esterase (Pittman et al., 1979) or both. The acid cholesterol esterase activity in the L.T. line studied here was about 15% of normal values (Pittman et al., 1979), which is less of a deficit than that found in the cells of patients with

**Table 3. Degradation of 125I-labelled LD lipoprotein by cell-free homogenates**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>pH 3.0</th>
<th>pH 4.0</th>
<th>pH 5.0</th>
<th>pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>78.2</td>
<td>68.1</td>
<td>170.6</td>
<td>14.3</td>
</tr>
<tr>
<td>Homozygous familial</td>
<td>86.1</td>
<td>70.1</td>
<td>180.3</td>
<td>21.7</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-cell (L.T.)</td>
<td>16.0</td>
<td>133.8</td>
<td>34.2</td>
<td>3.1</td>
</tr>
<tr>
<td>I-cell (K.Z.)</td>
<td>22.7</td>
<td>189.1</td>
<td>46.6</td>
<td>3.6</td>
</tr>
</tbody>
</table>
cholestereryl ester storage disease (Goldstein et al., 1975b). Yet reductase activity in the L.T. line showed only slight inhibition even after 24h of incubation with 100μg of LD lipoprotein/ml, whereas Goldstein et al. (1975a,b) found that under the same conditions reductase activity in cholestereryl ester storage disease cells was almost completely inhibited. This difference suggests that the proteinase deficiency in I-cells plays a role in their lack of response to LD lipoprotein.

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