Biochem. J. (1980) **186**, 971–975
Printed in Great Britain

Effect of Tunicamycin and Cycloheximide on the Secretion of Acid Hydrolases from I-Cell Cultured Fibroblasts

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(*Received 10 September 1979*)

I-cell cultured fibroblasts secrete excessive amounts of *N*-acetyl-β-D-hexosaminidase and α-L-fucosidase into the culture media as compared with normal fibroblasts. Addition of tunicamycin or cycloheximide at doses that inhibit the incorporation of [^{3}H]mannose (60–80%) and [^{14}C]leucine (40–50%) into trichloroacetic acid-precipitable material decreased the secretion of these I-cell hydrolases to normal values within 24h, but had no effect on the secretion of acid hydrolases from normal fibroblasts. These results indicate that I-cell cultured fibroblasts secrete at least two types of acid hydrolases: one is tunicamycin- and cycloheximide-sensitive and constitutes the greater proportion of the secreted hydrolases, and a smaller proportion is insensitive to tunicamycin and cycloheximide, similar to the acid hydrolases secreted by normal cultured fibroblasts.

I-cell disease is a lysosomal storage disorder characterized in part by decreased activities of multiple acid hydrolases in cultured fibroblasts, with concomitant increases of many of these enzyme activities in the fibroblast media (Leroy *et al.*, 1972; Hickman & Neufeld, 1972; Weismann & Herschkowitz, 1974). This apparent mislocalization of the acid hydrolases and the autosomal recessive mode of inheritance suggest a single gene defect in I-cell disease that is common to the final expression of acid hydrolases. The molecular basis for this disorder is not known, but evidence from several laboratories indicates that the genetic defect results in an altered oligosaccharide side chain on the I-cell acid hydrolases (Hickman *et al.*, 1974; Kress & Miller, 1979; Kress *et al.*, 1979). This altered carbohydrate may interfere with the ability of the hydrolases to be efficiently localized within the lysosome, and as a result the I-cell hydrolases are secreted to the extracellular media.

To investigate the role of hydrolase-bound carbohydrate in the localization of these enzymes within the lysosome, cultured fibroblasts from patients with I-cell disease, pseudo-Hurler polydystrophy (a variant of I-cell disease) and GM_1-gangliosidosis and normal control cultured fibroblasts were incubated with tunicamycin, an antibiotic which inhibits the formation of a dolichol–oligosaccharide intermediate involved in the glycosylation of asparagine-linked glycoproteins (Takatsuki & Tamura, 1971; Takatsuki *et al.*, 1976). Our results indicate that within 24h tunicamycin was able to decrease the rate of secretion of I-cell β-D-hexosaminidase and α-L-fucosidase to normal values, but had little or no effect on the secretion of these enzymes from normal cultured fibroblasts. Similar results were obtained by using the protein-synthesis inhibitor cycloheximide.

Experimental

Culture conditions

D-[2-^{3}H]Mannose (12Ci/mmol) and L-[U-^{14}C]leucine (354mCi/mmol) were obtained from Amersham/Searle Co. (Arlington Heights, IL, U.S.A.). Cycloheximide was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and tunicamycin was the generous gift of Professor G. Tamura, Department of Agricultural Chemistry, University of Tokyo.

Human skin fibroblasts were grown in 250ml plastic tissue-culture flasks with 20ml of Ham's F-10 medium containing 15% (v/v) heat-inactivated foetal bovine serum as previously described (DeMars & Leroy, 1967). At 5–7 days before each experiment, 35mm plastic Petri dishes were separately seeded with 2×10^5 cells from I-cell-disease, pseudo-Hurler-polydystrophy and GM_1-gangliosidosis patients and normal controls in 1ml of Ham's F-10 medium and grown just to confluency (2.0×10^6–2.5×10^6 cells/dish). Cell numbers were determined with a Coulter counter.

Incorporation of [^{3}H]mannose and [^{14}C]leucine

The medium was removed and the cells were washed with 1ml of phosphate-buffered saline [15mM-sodium phosphate (pH7.4)/0.85% NaCl]. Fresh medium (1ml) was added to the cells, with

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of without tunicamycin or cycloheximide. The cells were preincubated at 37°C for 4h, after which 10μCi of [^{3}H]mannose and 0.5μCi of [^{14}C]leucine were added to each dish. At specified time intervals the medium was removed, the cells were washed twice with 0.5ml of 0.85% (w/v) NaCl and incubated at room temperature for 30min with 1ml of 0.5% (v/v) Nonidet P-40 containing 1mg of non-radioactive leucine/ml. The lysed cells were harvested with a rubber policeman and the residual cells were harvested with an additional 0.5ml of 0.5% Nonidet P-40 solution. The cell lysates were combined and centrifuged in a Beckman TJ-6 centrifuge at 850g for 10min at room temperature. The supernatant was removed, and the cell pellet was resuspended in 0.5ml of 0.5% Nonidet P-40 and re-centrifuged as described above. The two supernatants were pooled, human serum albumin was added as a carrier (final concn. 1mg/ml) and the proteins were precipitated with an equal volume of cold 15% (w/v) trichloroacetic acid. This mixture was vortex-mixed and centrifuged in a Sorvall RC-5B centrifuge at 12500rev./min for 15min at 0–4°C in a SM-24 rotor. The pellet was resuspended in 1ml of cold 7.5% trichloroacetic acid, vortex-mixed and re-centrifuged as described above. The resultant precipitate was redissolved in 1ml of 2% (w/v) Na_2CO_3/0.4% (w/v) NaOH/0.02% (w/v) sodium tartarate/0.01% (w/v) $CuSO_4$. Concentrated HCl (0.1ml) and 8ml of Biofluor (New England Nuclear) were added to 0.5ml portions of the redissolved precipitate and the mixture was counted for radioactivity in a Beckman LS8100 scintillation counter. Protein determination on the cells was by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Determination of enzyme activity

The cultured fibroblasts were incubated as described above, without [^{3}H]mannose and [^{14}C]leucine. The intracellular and extracellular lysosomal hydrolase activities were determined in the presence and absence of tunicamycin or cycloheximide. At the specified times the medium was removed and used directly for enzyme assays. The intracellular enzymic activities were determined after washing the cells once with 0.5ml of 0.85% NaCl, harvesting with 3ml of 0.85% NaCl, and centrifuging the cells at 850g in the Beckman centrifuge (as above) for 5min. The resulting cell pellet was resuspended in 0.1ml of glass-distilled water and the suspension sonicated twice (Kontes micro-ultrasonic cell disrupter, setting 5) for 10s with a 10s interval between pulses. β-D-Hexosaminidase and α-L-fucosidase activities were assayed fluorimetrically as previously described (Leroy *et al.*, 1972; Alhadeff *et al.*, 1974).

The tunicamycin- and cycloheximide-treated cultured fibroblasts were found to be 95–100% viable, as determined by staining with a 0.5% (w/v) solution of Trypan Blue (Phillips, 1973).

Results

Fig. 1 depicts the effects of tunicamycin and cycloheximide on the incorporation of [^{3}H]mannose

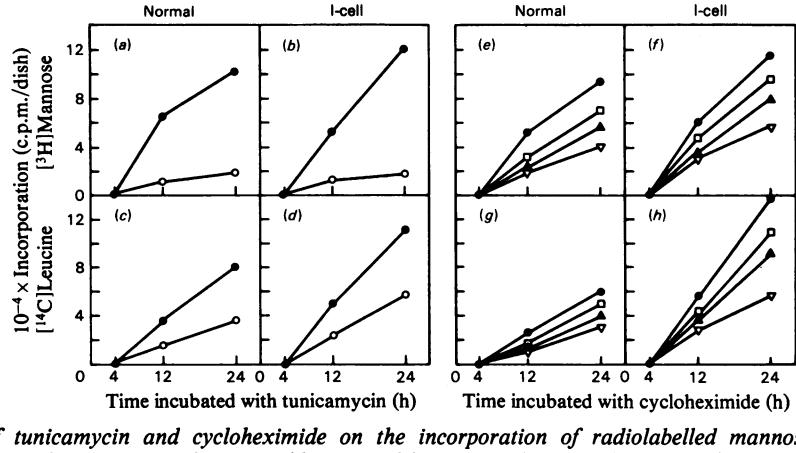

Fig. 1. *Effect of tunicamycin and cycloheximide on the incorporation of radiolabelled mannose and leucine into trichloroacetic acid-precipitable material from normal and I-cell cultured fibroblasts*

Dishes containing 2.0 × 10^6–2.5 × 10^6 cells were incubated with or without tunicamycin or cycloheximide and radiolabelled mannose and leucine for 4, 12 and 24h. The cells were then harvested, precipitated with trichloroacetic acid and counted for radioactivity (see the text for details). Values plotted are the means of at least two experiments using duplicate determinations. Variation was within ±10% of the mean. (*a*)–(*d*): ●, control; ○, tunicamycin (1.0μg/ml). (*e*)–(*h*): ●, control; □, cycloheximide (0.025μg/ml); ▲, cycloheximide (0.050μg/ml); ▽, cycloheximide (0.100μg/ml).

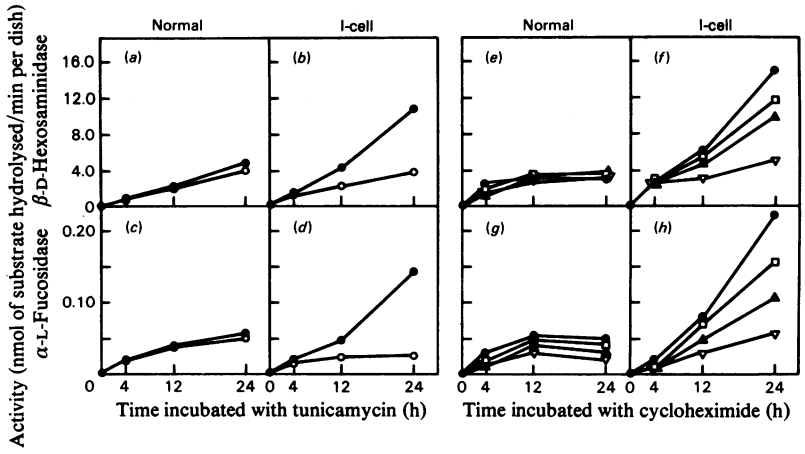

Fig. 2. *Effect of tunicamycin and cycloheximide on total extracellular activities of β-D-hexosaminidase and α-L-fucosidase from normal and I-cell cultured fibroblasts*

Dishes containing 2.0×10^6–2.5×10^6 cells were incubated wtih or without tunicamycin or cycloheximide for 4, 12 and 24h. The media were then removed and assayed fluorimetrically for β-D-hexosaminidase and α-L-fucosidase activities (see the text for details). Values plotted are the means of at least two experiments using duplicate determinations. Variation was within ±20% of the mean. (*a*)–(*d*): ●, control; ○, tunicamycin (1.0μg/ml). (*e*)–(*h*): ●, control; □, cycloheximide (0.025μg/ml); ▲, cycloheximide (0.050μg/ml); ▽, cycloheximide (0.100μg/ml).

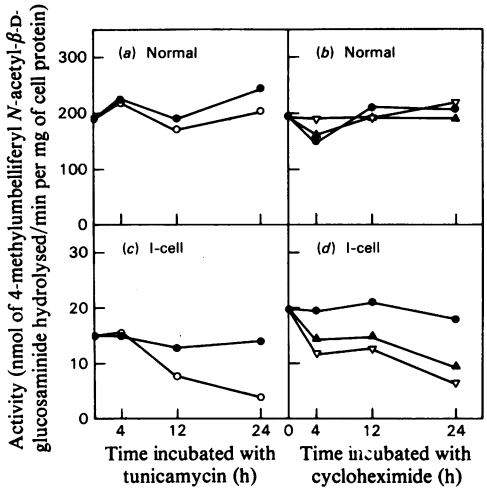

Fig. 3. *Effect of tunicamycin and cycloheximide on intracellular activities of β-D-hexosaminidase from normal and I-cell cultured fibroblasts*

Dishes containing 2.0×10^6–2.5×10^6 cells were incubated with or without tunicamycin or cycloheximide for 4, 12 and 24h. The cells were then harvested and assayed fluorimetrically for β-D-hexosaminidase activity (see the text for details). Values plotted are the means of at least two experiments. Variation was within ±20% of the mean. (*a*), (*c*): ●, control; ○, tunicamycin (1.0μg/ml). (*b*), (*d*): ●, control; ▲, cycloheximide (0.05μg/ml); ▽, cycloheximide (0.10μg/ml).

and [^{14}C]leucine into trichloroacetic acid-precipitable material from Nonidet P-40 cell lysates of normal and I-cell cultured fibroblasts. In a series of experiments, tunicamycin inhibited the incorporation of [^{3}H]mannose into macromolecular material by 60–80% (Figs. 1*a* and 1*b*), while inhibiting the incorporation of [^{14}C]leucine by 40–50% for both normal and I-cell fibroblasts (Figs. 1*c* and 1*d*). Similar results were obtained by using ^{14}C-labelled mixed amino acids (A. L. Miller, B. C. Kress, L. Lewis, R. Stein & C. Kinnon, unpublished work).

The enhanced secretion rate of β-D-hexosaminidase and α-L-fucosidase activities from I-cell cultured fibroblasts was decreased to normal values within 24h in the presence of 1.0μg of tunicamycin/ml (Figs. 2*b* and 2*d*). However, secretion of these enzymes from normal cells was not significantly affected by the same concentration of tunicamycin (Figs. 2*a* and 2*c*). When higher concentrations of tunicamycin were used (2–3μg/ml), no further decrease in the rate of secretion was seen for either the I-cell or normal fibroblasts (A. L. Miller, B. C. Kress, L. Lewis, R. Stein & C. Kinnon, unpublished work).

Because of the relatively high degrees of inhibition of protein synthesis by tunicamycin, studies were carried out to investigate whether the effects observed on I-cell enzyme secretion could be mimicked by using the protein-synthesis inhibitor cycloheximide. Cells were treated with concen-

trations of cycloheximide which caused the inhibition of [^{14}C]leucine incorporation into trichloroacetic acid-precipitable material to approximate to those observed in the presence of tunicamycin. Results show that cycloheximide gave a dose-dependent inhibition of both glycosylation (Figs. 1*e* and 1*f*) and I-cell acid hydrolase secretion (Figs. 2*f* and 2*h*). Most notable was the similarity of the effect of cycloheximide to that of tunicamycin in decreasing the rate of secretion of I-cell β-D-hexosaminidase and α-L-fucosidase to normal values while not significantly affecting the secretion of these enzymes from normal cells within 24h (Figs. 2*e*, 2*f*, 2*g* and 2*h*).

Tunicamycin and cycloheximide treatment caused a decrease in the intracellular specific activity of β-D-hexosaminidase in I-cell cultured fibroblasts (Figs. 3*c* and 3*d*), but there was no significant effect on the specific activity of this enzyme in normal fibroblasts (Figs. 3*a* and 3*b*).

Similar studies with tunicamycin and cycloheximide were carried out on skin fibroblasts from patients with pseudo-Hurler polydystrophy and GM_1-gangliosidosis. The results were similar to those for I-cell and normal fibroblasts respectively (results not shown).

Discussion

Cultured fibroblasts from patients with I-cell disease demonstrate increased acid hydrolase activities in the culture media as compared with normal fibroblasts (Hickman & Neufeld, 1972; Hickman *et al.*, 1974; Weismann & Herschkowitz, 1974). The present studies demonstrate that this increase in hydrolase activities in the culture media is due to an enhanced rate of enzyme secretion from the I-cell fibroblasts. Furthermore, our results suggest that the acid hydrolase activities secreted from I-cell fibroblasts consist of at least two types of enzyme: (1) the greater proportion of the acid hydrolase activity is tunicamycin- and cycloheximide-sensitive and is, in this regard, unlike the acid hydrolases secreted from normal fibroblasts; and (2) a smaller proportion of the secreted hydrolases from I-cell fibroblasts is relatively insensitive to the effects of tunicamycin and cycloheximide, similar to the hydrolases secreted from normal fibroblasts. This differential effect of tunicamycin and cycloheximide on hydrolase secretion from I-cell and normal fibroblasts suggests that the increased rate of secretion of the I-cell hydrolases may be via an alternative pathway to the predominant one in normal fibroblasts. It is likely that less of the total I-cell enzyme synthesized at any one time is localized within the lysosome compared with normal controls. This may be due to an altered or absent carbohydrate recognition marker on the I-cell hydrolases necessary for their localization within the lysosome (Hickman *et al.*, 1974). As a result of their inability to be localized within lysosomes, the I-cell hydrolases are secreted to the extracellular milieu, possibly via the Golgi apparatus in a manner similar to other secreted glycoproteins (Zagury *et al.*, 1970). The basal rate of secretion observed for normal hydrolases, most of which are presumably localized within the lysosome, may result from lysosomal fusion with the plasma membrane (de Duve & Wattiaux, 1966; Lloyd, 1977; Von Figura & Weber, 1978). This interpretation implies the existence of separate secretion pathways of the I-cell and normal hydrolases from cultured fibroblasts. An alternative interpretation may be that the enzymes from both cell types are secreted via similar pathways, but other regulatory mechanisms may be involved that respond differently to tunicamycin and cycloheximide in I-cell and normal fibroblasts. Our present data do not differentiate between these possibilities.

Treatment of cells with tunicamycin or cycloheximide for up to 24h resulted in a decrease in the intracellular specific activity of β-D-hexosaminidase activity for I-cell fibroblasts, whereas there was no effect on normal cells. It has been reported by others that treatment of rat embryo fibroblasts with cycloheximide (20μg/ml) for up to 4 days had no significant effect on the specific activities of intracellular acid phosphatase and β-D-glucuronidase (Amenta *et al.*, 1977, 1978). These data support the concept that in normal cells the greater proportion of these enzymes are probably packaged within the lysosome, whereas most of the intracellular I-cell acid hydrolases are probably not associated with the lysosomes. Finally, we have shown in this study that the effects of tunicamycin in cultured fibroblasts could be mimicked with cycloheximide. This should add caution to the interpretation of data obtained by using agents such as tunicamycin which have secondary effects on other cellular mechanisms.

This research was supported in part by National Institutes of Health Grant NS12138 and National Easter Seal Research Foundation Grant N-7910 to A. L. M. A. L. M. is the recipient of Research Career Development Award NS00050 from the National Institutes of Health. C. K. is the recipient of the Capstaff Scholarship.

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