DEVELOPMENT OF LIPOPROTEIN LIPASE IN CULTURED 3T3-L1 CELLS

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Summary

The 3T3-L1 mouse fibroblast resembles an adipocyte after reaching a confluent stage of growth. Lipoprotein lipase activity was released with heparin and was present in acetone-ether extracts of these cells. During the early post-confluent period both activities increased rapidly. A wide variation in enzyme activities was noted in subclones suggesting that spontaneous heritable change continues to take place in these cells. Since lipoprotein lipase activity was measurable before triglyceride accumulation, it may be the earliest marker of adipocyte expression in this line. This system appears to offer a unique opportunity to study the processes of cellular differentiation and fat metabolism *in vitro*.

The 3T3-L1 cell line, derived from the established mouse line 3T3, is distinguished by the accumulation of triglyceride after reaching a confluent stage of growth, eventually resembling adipocytes in appearance (1). This conversion does not occur during exponential growth and is arrested by bromodeoxyuridine in stationary cells (2). Green and Kehinde have shown that the rates of incorporation of acetate, palmitate and glucose into triglyceride are significantly higher in these cells than in the original 3T3 cell line and that this accumulation of triglyceride is modulated by hormones and drugs known to affect adipocytes (3). More recently, Mackall *et al.* have shown that [^{14}C]acetate incorporation into triglyceride by 3T3-L1 cells is paralleled by increased activity of several enzymes of fatty acid biosynthesis (ATP-citrate lyase, acetyl coA carboxylase, fatty acid synthetase). These enzyme changes occur about eight days after the cells have reached confluence (4). A further property of adipose tissue is the production of lipoprotein lipase, an enzyme that is characterized by being activated by serum and by an alkaline pH optimum (5). Following synthesis by the adipocyte, the enzyme is apparently activated upon release from the adipocyte and is transferred to the capillary endothelium where it is membrane bound for hydrolysis of triglyceride into glycerol and free fatty

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acids (6,7). Although Van et al. have measured lipoprotein lipase activity in the stromal-vascular fraction of human adipose tissue (8), no sustaining cell culture model for the study of lipoprotein lipase is available. Because of the potential usefulness of such an in vitro model, the 3T3-L1 line was investi gated for the presence of lipoprotein lipase activity.

Materials and Methods

3T3-L1 cells were obtained from Dr. Howard Green (Massachusetts Institute of Technology) and grown in Dulbecco-Vogt modified Eagle's medium supplemented with 10% fetal calf serum (Gibco) and gentamicin (50 ug/ml, Microbiological Associates). The cells were grown at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Medium was changed every two to three days. Confluent cells in 16 oz glass culture bottles were rinsed with phosphate buffered saline and incubated with 0.25% trypsin in phosphate buffered saline. The detached cells were resuspended in medium and approximately 5 x 10^6 cells dispersed into 16 oz culture bottles. Four days later, cells for lipoprotein lipase assays were harvested. Following rinsing with phosphate buffered saline, cells were removed by scraping the cell monolayer with a rubber policeman into Krebs-Ringer phosphate buffer and collected after centrifugation for 5 min at 400 X g and 4°. The pellet was then resuspended in 5.0 ml Krebs-Ringer phosphate buffer. Additional culture bottles were rinsed with phosphate buffered saline, and cells were collected after incubation with trypsin solution and counted in a hemocytometer.

Lipoprotein lipase was measured both as the activity releasable into the medium from cells incubated with heparin and as the activity present in the ammonium chloride (NH_4Cl) extract of an acetone-ether powder of cells. For activity releasable with heparin, 1.2 ml of Krebs-Ringer phosphate buffer suspended cells were incubated with heparin (2 units/ml, Upjohn intestinal mucosa) for 45 min. The mixture was then centrifuged at 400 X g for 5 min at room temperature and 1.0 ml was taken off and incubated in a Dubnoff shaker with 0.2 ml substrate at 37° for 30-60 min. Activity in acetone-ether powders was assayed using 1.2 ml of the Krebs-Ringer phosphate buffer cellular suspension according to Nilsson-Ehle et al. (9). After drying with nitrogen, the cellular powders were homogenized in 0.4 ml of 0.05 M NH_4Cl (pH 8.2) and then incubated with 0.2 ml of substrate in a Dubnoff shaker at 37° for 30-60 min. The reaction was terminated by adding 10 ml of Dole's extraction mixture and free fatty acids were extracted as outlined by Pykäläistö et al. (10). The substrate was prepared using 100 µl of unlabeled triolein (25 mg/ml in benzene, Sigma), 0.5 ml of 1[^{14}C]triolein (2 µCi in benzene, Amersham) and 10 µl egg lecithin (12 mg/ml in chloroform-methanol, 1:1) purified by W.C. Vogel by column chromatography (11). Emulsification of the triolein and lecithin was carried out with a mixture of 10% fatty acid-poor bovine serum albumin, normal human serum, 2M Tris HCl buffer and distilled water (4:1.5:5:9.5) for 100 seconds of sonification (10 seconds on, followed by 8 seconds rest for 10 cycles) using a Branson 125 Sonifier at 4°.

Results

Enzyme activity releasable with heparin from cells and in acetone-ether powders of cells was found in 3T3-L1 cells and increased linearly with length of incubation (Fig. 1). The removal of serum from the assay decreased the

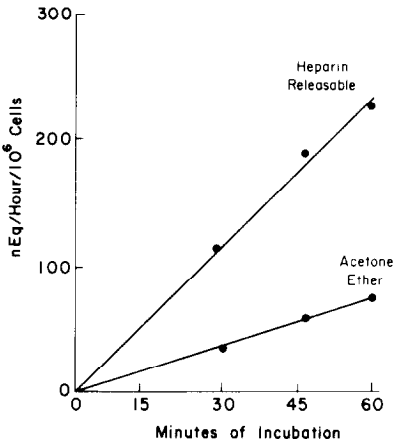


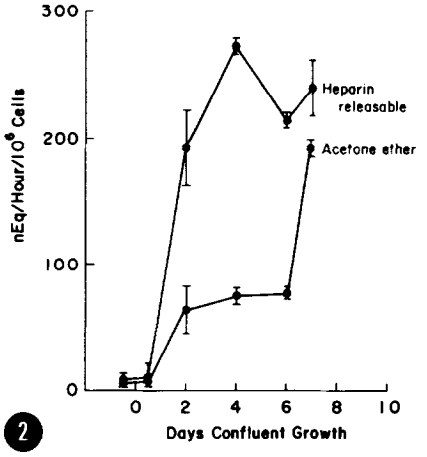
Figure 1: Heparin releasable and acetone-ether cellular powder lipoprotein lipase activities as a function of incubation time. Linearity is demonstrated up to 60 minutes.

Table I. Heparin releasable and acetone-ether cellular powder lipoprotein lipase activities in the presence and absence of serum. Control 3T3-C cells were assayed in the presence of serum only.

CELL	SERUM	Lipoprotein Lipase (nEq Free Fatty Acids/hour/10^6 cells)	
		Heparin Releasable	Acetone Ether
3T3-L1	+	50.3	12.0
		45.5	10.5
3T3-L1	-	2.3	0.9
		2.3	
3T3-C	+	3.8	2.0
		4.0	1.7

activity of the enzyme by 90% (Table I). Lipolytic activity measured in the presence of serum was very low in 3T3 control cells confluent for a similar period of time documenting that the enzyme is present only in those cells that accumulate triglyceride.

To determine if the stage of cellular growth had any effect on lipoprotein lipase activity, initial cell densities of 5×10^4, 1×10^5 and 5×10^6



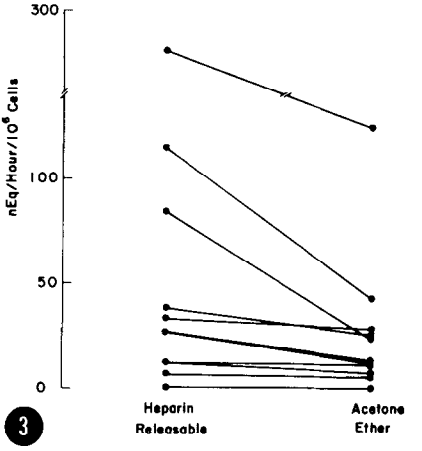


Figure 2: Heparin releasable and acetone-ether cellular powder lipoprotein lipase activities as a function of duration of confluent growth. On the abscissa, the points before 0 represent subconfluent cultures. Means ± 1 S.D. of duplicate assays are indicated.

Figure 3: Lipoprotein lipase activities in eleven subclones. Heparin releasable and acetone-ether cellular powder activities for each subclone are connected by a straight line.

cells/plate (100 mm culture dishes, Falcon Plastics) were used and cells were harvested by scraping in the presence of 0.25% trypsin at different stages of confluent growth. Cell numbers used to measure lipoprotein lipase varied from about 0.1×10^6 per assay in subconfluent cultures to 3.0×10^6 per assay in seven-day confluent cultures. Minimal activities were present in subconfluent cells (Figure 2). An increase in both the activity releasable with heparin and that present in acetone-ether powders of cells occurred between 12 and 48 hours of confluent growth.

Single cells were isolated from a single culture dish of 3T3-L1 cells and subclones were established. In these subclones, a wide variability in lipoprotein lipase activities were found (Figure 3).

Discussion

Adipose tissue contains several different lipase activities - hormone-sensitive lipase, phospholipase, mono- and diglyceride lipases and lipoprotein lipase, although only the latter appears to be important in the transfer of

triglyceride fatty acids into the adipocyte (10,13). Assays of adipose tissue enzymatic activity on triglyceride substrate potentially might reflect activities of both hormone sensitive lipase and lipoprotein lipase. By performing the assay at pH 8.4, lipoprotein lipase activity is optimized (optimum pH is 7.0 for hormone sensitive lipase)(14). Unlike the other lipases, lipoprotein lipase requires a serum lipoprotein activator, thus the loss of 90% or greater activity in the absence of serum favors lipoprotein lipase (5,15).

The discovery of lipoprotein lipase in 3T3-L1 cells provides further evidence of the adipocyte-like properties of this cell line. In fact, development of lipoprotein lipase may be the earliest manifestation of adipocyte differentiation in these cells. Morphological change (16) and induction of fatty acid biosynthetic enzymes appear to occur later (4). The amount of enzyme activity seems to be dependent on the degree of confluency present. This suggests that contact inhibition is related to adipocyte expression, perhaps the calories utilized in exponential growth are now stored as intracellular lipid. The changes in lipoprotein lipase activity with alterations in growth are not unique to this enzyme or to this cell line. For example, in chick fibroblasts, replicating cells have been shown to consume more glucose than contact inhibited cells, preferentially increasing the activity of the glycolytic pathway (17). In working with this cell line it rapidly became apparent that the accumulation of triglyceride was variable between cells in any one culture as well as between different cultures. The lipoprotein lipase activities measurable in these cells also varied between different cultures. Since Green and Kehinde had previously described similar morphological alterations in these cells (12), lipoprotein lipase activity was measured in subclones of these cells, and activities were found over a wide range. These observations suggest that the presence of this enzyme activity is neither a uniform nor a constant property of these cultures.

To further evaluate the similarity of lipoprotein lipase found in 3T3-L1 cells with that in adipocytes, characteristics of the induction and hormonal

regulation of the enzyme need to be determined. The localization of lipoprotein lipase within 3T3-L1 cells and the previous demonstration of triglyceride accumulation and induction of activity of several fatty acid biosynthetic enzymes with adipocyte expression suggest that the 3T3-L1 fibroblast offers a unique opportunity to study the processes of cell differentiation and fat metabolism *in vitro*.

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