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Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O

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Summary. Cultured 3T3-F442A cells differentiate into adipocytes and accumulate lipid droplets in the cytoplasm. When fat cells are stained with Oil red O, the degree of staining seems to be proportional to the extent of cell differentiation. We report here a fast and simple method to quantitate the extent of adipose conversion by staining the accumulated lipid with Oil red O and determining the amount of extracted dye at 510 nm. The results show that Oil red O specifically stains triglycerides and cholesteryl oleate but no other lipids. This technique is a valuable tool for processing large numbers of cell cultures or samples in which adipose differentiation and/or accumulated triglycerides is to be quantitated.

Introduction

Under appropriate culture conditions, 3T3-F442A cells undergo adipose differentiation in vitro, changing their fibroblastic shape to rounded cells with intracytoplasmic fat droplets (Green and Meuth 1974). The cells acquire a new set of metabolic pathways, increasing several-fold the activities of some lipogenic enzymes (for reviews see Green 1979; Ailhaud 1982). Since the activity of glycerophosphate dehydrogenase increases up to 500-fold in terminally differentiated cells (Kuri-Harcuch et al. 1978; Wise and Green 1979), the assay of this lipogenic enzyme has been widely used as a sensitive marker to determine the extent of adipose conversion (Pairault and Green 1979).

Intracytoplasmic lipid accumulation seems to be directly proportional to the extent of differentiation (Kuri-Harcuch and Green 1978). This relationship has been commonly used as a qualitative marker of adipose conversion after staining with Oil red O (see Fig. 1 in Kuri-Harcuch and Green 1978), a dye that is soluble in lipids and suitable for the histochemical staining of neutral fats and cholesteryl esters (Pearse 1968; Fowler and Greenspan 1985; Kruth 1984; Kasturi and Joshi 1982). A fast a simple method was developed to quantitate the extent of adipose conversion by determining the amount of the intracytoplasmically accumulated lipid in Oil red O-stained 3T3 adipose cells.

Materials and methods

Reagents

Insulin, Trizma base, nicotinamide adenine dinucleotide (NAD), dihydroxyacetone phosphate, d-biotin, transferrin, cholesterol, cholesteryl esters, lecithin, linoleic acid, oleic acid and Oil red O were purchased from Sigma Chemicals (St. Louis, Mo., USA). Triolein was purchased from Calbiochem (La Jolla, Calif.). The recombinant form of epidermal growth factor was a generous gift from Dr. George Donascimento (Chiron Corp.). Fetal bovine serum was obtained from M.A. Bioproducts (Walkersville, Md., USA), calf serum from Colorado Serum Co. (Denver, Colo.), and cat serum was obtained by bleeding adult domestic cats. All other reagents used were analytical grade.

Cell culture

The 3T3-F442A cells (Green and Kehinde 1976) were inoculated into 35-mm Falcon tissue culture dishes at a density of 6×10^3 cells per dish in 2 ml of Dulbecco and Vögt-modified Eagle's medium (DMEM) supplemented with 7% calf serum. For some experiments, growing cells were fed every other day with medium supplemented with 10% fetal bovine serum, 5 μg/ml insulin and 10^{-6} *M* d-biotin (Kuri-Harcuch and Green 1978). In biotin defficient conditions, cultures were fed with medium supplemented with fetal bovine serum depleted of biotin by exhaustive dialysis (Kuri-Harcuch et al. 1978). In some other experiments, growing cells were fed 3 days after seeding with medium supplemented with 4% cat serum (nonadipogenic medium; Kuri-Harcuch and Green 1978), containing insulin and biotin as above; the medium was changed every other day. To promote adipose conversion, 2 days after confluence

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cultures were changed to medium supplemented with 2% cat serum, 0.25% calf serum, 5 μg/ml insulin, 10^{-6} *M* d-biotin, 40 μ*M* 2-mercaptoethanol, 5 μg/ml transferrin, 0.01 ng/ml epidermal growth factor and the indicated concentrations of fetal bovine serum (adipogenic medium; Nixon and Green 1984). These cultures were maintained without further change of medium.

Assay of adipose conversion

Cultures were fixed for at least 1 h with 10% formalin in isotonic phosphate buffer, then washed with water, stained for 2 h by complete immersion in a working solution of Oil red O and exhaustively rinsed with water. Excess water was evaporated by placing the stained cultures at a temperature of about 32° C. In order to determine the extent of adipose conversion, 1 ml of isopropyl alcohol was added to the stained culture dish, the extracted dye was immediately removed by gentle pipetting and its absorbance monitored spectrophotometrically at 510 nm. Adipose conversion was also quantitated by glycerophosphate dehydrogenase (EC 1.1.1.8) activity as described previously (Kozak and Jensen 1974; Kuri-Harcuch et al. 1978).

The working solution of Oil red O was prepared as described (Humason 1972), by dissolving 4.2 g of Oil red O in 1200 ml absolute isopropanol and leaving overnight without stirring at room temperature. The solution was filtered through analytical filter paper 589-WH (Schleicher and Schuell); then, 900 ml of bidistilled water was added and the solution was left overnight at 4° C without stirring and filtered twice. This working solution could be stored at room temperature for 6–8 months. Protein was determined by the method of Bradford (1976).

Triglyceride enzymatic assay

Intracytoplasmic triglyceride accumulation was determined on total cell extracts using a clinical enzymatic assay kit (no. 470694, Boehringer Mannheim Diagnostica). Briefly, cell cultures were rinsed three times with phosphate-buffered saline (PBS). The cells from each 35 mm dish were scraped, suspended in 0.2 ml of 0.05 *M Tris*-HCl, 1 m*M* EDTA, 1 m*M* β-mercaptoethanol, pH 7.4, at 5° C, and disrupted by sonication for 10 s at 40 W. Total cell extracts were vigorously stirred, and 0.02 ml aliquots were added to 1 ml of assay mixture containing 73.6 m*M Tris*/citrate buffer pH 8.2, 30.6 m*M* $MgCl_2$, 7.8 m*M* sodium cholate, 0.19% isotridecanol polyglycol ether, 0.049 m*M* ATP, 0.29 m*M* phosphoenol pyruvate, 0.24 m*M* NADH, 3 U/ml esterase, 4.0 U/ml pyruvate kinase, 0.5 U/ml lactate dehydrogenase (LDH) and 1.3 U/ml glycerol kinase. After mixing, samples were incubated at 25° C for 10 min and absorbance at 340 nm was measured to calculate triglyceride concentration (see Wahlefeld 1974; Nägele et al. 1985).

Triolein calibration curves

Triolein was dissolved in hexane at a concentration of 25 mg/ml and stored at −20° C. Aliquots of the triolein solution were placed in polystyrene tubes and the solvent evaporated with N_2; the tubes were stained with Oil red O and rinsed with water as described above for assay of adipose conversion. The Oil red O was extracted with 1 ml of isopropanol and its absorbance determined at 510 nm. As indicated, aliquots of other lipid solutions were subjected to the same procedure as triolein.

Results

Cultures of mature 3T3-F442A adipocytes were stained with Oil red O for various periods of time; the dye was extracted and its optical density was determined at the maximum absorbance peak, 510 nm (Fig. 1, inset). Figure 1 shows that the highest extent of staining was reached after 2 h incubation with Oil red O. Therefore in all subsequent experiments, intracellular lipids were stained for 2 h in fixed cultures.

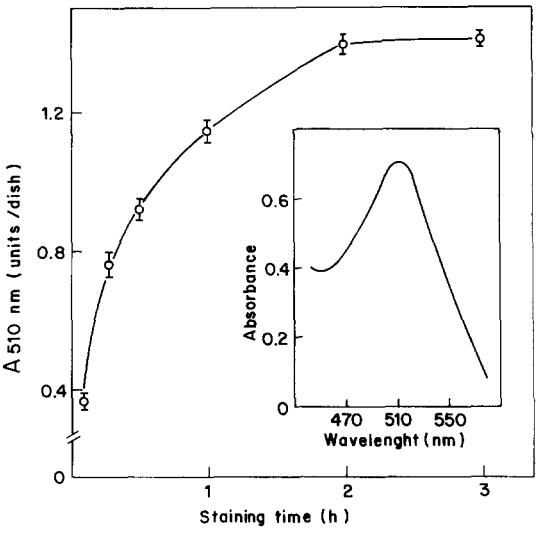

Fig. 1. Time course of staining of intracytoplasmic lipids in 3T3 adipocytes. The cells were grown in medium supplemented with 10% fetal bovine serum and were refed every 3 days. Fifteen days after seeding, cultures were fixed and stained at the indicated times. Absorbance was monitored at 510 nm (o—o), the wavelength at which Oil red O has its maximum absorbance peak (*inset*). Values are the average of three independent experiments; *bars* indicate standard deviation

The extent of 3T3-F442A adipose conversion was quantitated by staining the intracytoplasmic lipids and compared with glycerophosphate dehydrogenase activity. The 3T3-F442A cells were stimulated to differentiate into adipocytes with various concentrations of fetal bovine serum. Figures 2 and 3 show that the extent of adipose conversion, determined by the extraction of Oil red O from stained cultures or by glycerophosphate dehydrogenase activity in cell extracts, was similar at any concentration of fetal bovine serum tested. However, at different culture times the increase in this lipogenic enzyme activity appeared to occur earlier than lipid accumulation (Fig. 3).

Under biotin defficient conditions, the 3T3 preadipocytes differentiate to mature adipose cells without triglyceride accumulation (Kuri-Harcuch et al. 1978). However, after adding biotin to the culture medium, intracytoplasmic lipid accumulation is proportional to the concentration of biotin (Kuri-Harcuch et al. 1978), although the activity of glycerophosphate dehydrogenase remains at the same high levels independently of biotin addition (Kuri-Harcuch et al. 1978). Therefore, the extent of the accumulated lipids was quantitated in terminally differentiated 3T3-F442A cells cultured under various concentrations of biotin. The data of Fig. 4 show that the amount of Oil red O extracted from stained cultures correlated with the triglyceride content of 3T3 adipocytes, as determined with a triglyceride enzymatic assay.

Due to the close relationship between Oil red O extraction and triglyceride content of fat cells (Fig. 4), we attempted to show whether Oil red O specifically stains

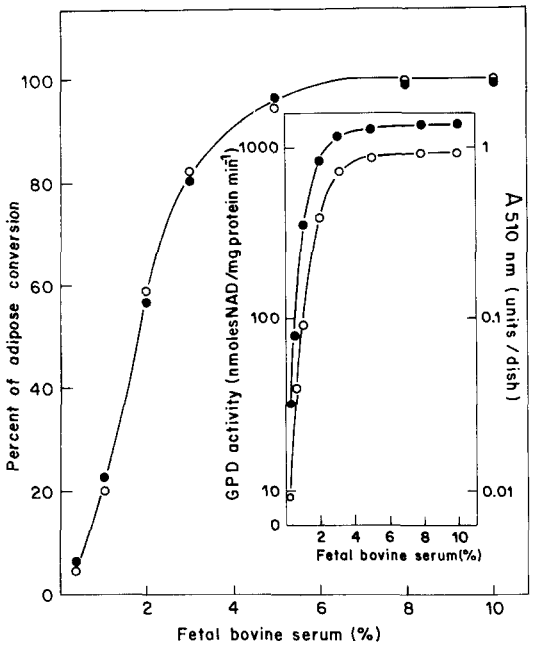

Fig. 2. Comparison of the extent of adipose conversion, determined by lipid accumulation and by glycerophosphate dehydrogenase activity. Cells were grown under nonadipogenic conditions (see the Materials and methods). At confluence, cells were stimulated to differentiate into adipocytes by adding fetal bovine serum. Eight days later, the extent of adipose conversion was assessed by extracting the dye from stained cultures (○—○) or by glycerophosphate dehydrogenase activity (●—●), and corresponds to the per cent of maximum value obtained by each procedure. Values are the average from three independent experiments

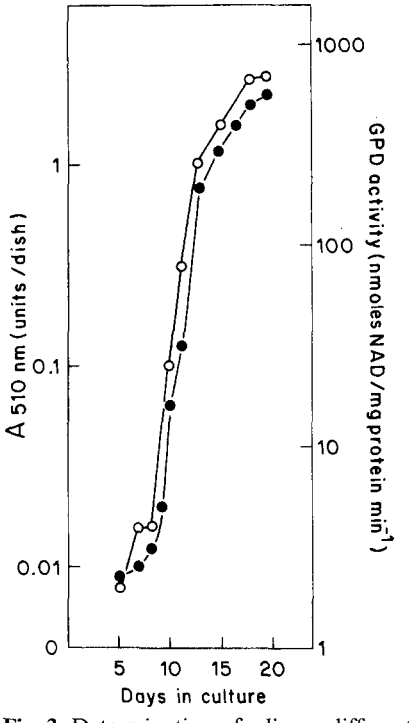

Fig. 3. Determination of adipose differentiation of 3T3 adipocytes during time in culture. Cells were grown under the same conditions described in Fig. 2; adipose conversion was stimulated with 5% fetal bovine serum. At the indicated culture times, the extent of adipose differentiation was assesed in duplicated dishes by staining with Oil red O (●—●) or by glycerophosphate dehydrogenase activity (GPD; ○—○)

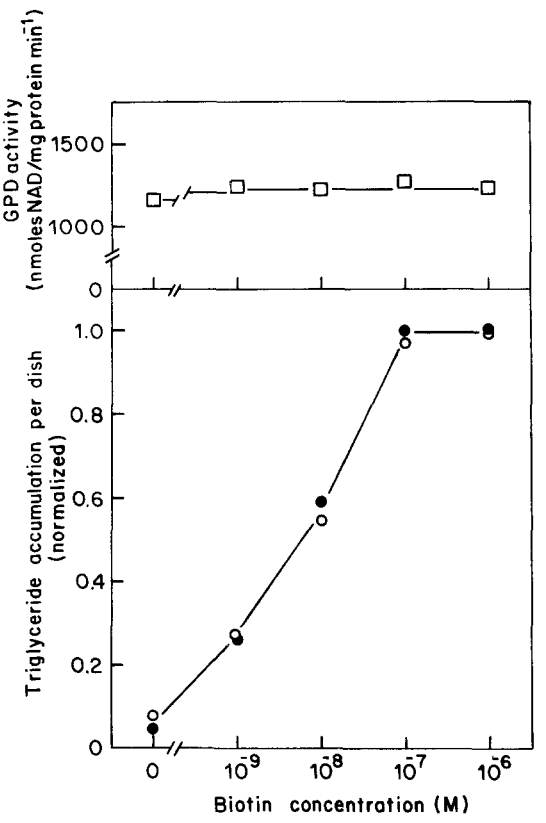

Fig. 4. Assessment of adipose differentiation by staining depends on the rate of lipid accumulation. 3T3-F442A cells were cultured in biotin defficient conditions. Twelve days after seeding, cultures were refed with medium containing increased concentrations of biotin. After 2 days, lipid accumulation was determined by the enzymatic assay (○—○) or by Oil red O extraction (●—●); values of absorbance units were normalized to the maximum value (1.0) of each procedure. Glycerophosphate dehydrogenase activity was also determined in parallel cultures (□—□). For each experiment duplicated dishes were used. Values are the average from four independent experiments and variation between duplicates was not higher than 6%

triglycerides. Various types of lipids were smeared on polystyrene tubes and after drying, lipids were stained with Oil red O for 2 h; the dye was extracted with isopropanol and its absorbance determined at 510 nm. Table 1 shows that triolein and cholesteryl oleate, among all the lipids tested, were the only lipids that stained significantly with Oil red O. A standard calibration curve was thus made using different concentrations of triolein (Fig. 5). The calibration curve was linear over a range from 0 to 200 μg of triolein with a correlation coefficient of 0.999. From the slope of the curve, it was calculated that each milligram of triolein shows 2.06 absorbance units at 510 nm.

Since triglyceride and cholesteryl oleate can be quantitated by the extraction of Oil red O from stained samples, we attempted to assess the triglyceride content of 3T3 adipocytes by this method in order to obtain a comparison with that obtained by the enzymatic assay of triglycerides. The 3T3-F442A cells were stimulated to adipose conversion with different concentrations of fetal bovine serum. When adipose conversion was reached, cultures were fixed and stained with Oil red O; parallel

Table 1. Oil red O extraction from several lipid samples

Lipid amount (μg)	Absorbance at 510 nm							
	Triolein	Tripalmitin	Cholesteryl oleate	Cholesteryl palmitate	Cholesterol	Lecithin	Linoleic acid	Oleic acid
25	0.045±0.016	0.000±0.002	0.145±0.018	0.0	0.0	0.0	0.0	0.0
50	0.170±0.004	0.000±0.007	0.293±0.026	0.0	0.0	0.0	0.0	0.0
100	0.388±0.008	0.035±0.009	0.481±0.032	0.0	0.0	0.0	0.0	0.0
200	0.663±0.035	0.068±0.001	0.690±0.067	0.0	0.0	0.0	0.0	0.0

Values are the average from three different experiments (±SD)

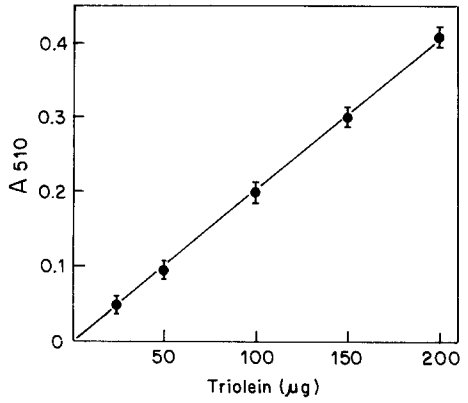

Fig. 5. Standard triolein calibration curve, prepared as described in the Materials and methods. *Bars* indicate standard deviation. Variation between duplicates was not higher than 6%. The data represent average values from five experiments

Table 2. Determination of intracytoplasmic triglyceride content of 3T3-F442A adipocytes

Fetal bovine serum added (%)	Triglyceride content (μg/dish)	
	Oil red O extraction	Enzymatic assay
0	68.9±4	73.9±8
0.5	160.0±2.4	185.5±11
1.0	334.0±8.7	346.0±8.5
2.5	686.0±4.3	699.0±7.7
5.0	838.0±5.6	840.0±13.1

The 3T3-F442A cells were grown in 35-mm culture dishes as described in the Materials and methods. Two days after confluence, adipose conversion was stimulated with fetal bovine serun. Duplicate cultures were fixed and stained with Oil red O, or used for enzymatic assay. Values are the average from four experiments (±SD)

cultures were subjected to triglyceride determination by the enzymatic assay. The number of absorbance units at 510 nm obtained from the extraction of Oil red O was used to calculate the triglyceride content according to the triolein calibration curve (Fig. 5). Table 2 shows that no significant differences were found in the triglyceride content as determined by either procedure. It can be concluded that Oil red O stains triglycerides and cholesteryl oleate. Therefore, this histological dye can be used to quantitate the triglyceride content from stained cultures of 3T3-F442A adipocytes, since these cells seem to accumulate triglycerides (Green and Kehinde 1974) but not significant amounts of cholesteryl oleate.

Discussion

The staining of fat cells with Oil red O has been commonly used macroscopically to visualize adipose cell colonies from cultures of preadipose cell lines. There is a close relationship between the extent of adipose conversion and the degree of staining of 3T3 adipose cells with Oil red O (Kuri-Harcuch and Green 1978). Oil red O can be easily extracted from stained fat cell cultures and its optical density measured at 510 nm (Fig. 1). Thus the method described is adequate to assess adipocyte differentiation and is as accurate and sensitive as the procedures based on glycerophosphate dehydrogenase activity (Figs. 2 and 3).

Dye extraction from stained cell cultures constitutes an inexpensive, fast and simple method for processing cell cultures in which adipose conversion must be quantitated. Recently some of the adipogenic factors have been isolated and characterized from fetal bovine serum; these experiments required a simpler and faster technique than those already available, for measurement of cell differentiation in a large number of cell cultures. In this case, Oil red O staining and extraction was a better quantitative method than the assay of enzyme activity in the cell extracts (Ramírez-Zacarías et al. manuscript in preparation).

Moreover, we found that triolein and cholesteryl oleate were the only type of lipids among those tested that strongly stained with Oil red O. However, in order to rule out the possibility that lipids other than triolein and cholesteryl oleate were not stained because they would not adsorb onto polystyrene tubes, all the tested lipid samples were also added to Whatman paper filters which subsequently were stained with the dye. The red spot appeared only on filters containing triglycerides (triolein or tripalmitin); cholesteryl oleate was not tested in the filter paper. We can conclude that Oil red O specifically stains triglycerides and cholesteryl oleate. In addition, by constructing a calibration curve we found that the amount of Oil red O extracted from stained lipid is directly proportional to the triolein content. The standard calibration curve was linear in the range of 0–200 μg triolein with a slope of 2.06 A_{510} units/mg of

triolein. The method described is accurate and sensitive to determine intracytoplasmic triglyceride content and showed a very close correlation ($r=0.995$, $P<0.001$) with enzymatic assays of triglycerides in 3T3-F442A adipocytes.

The results reported here indicate that the triglyceride colorimetric assay is equally reproducible as the enzymatic determination. In our hands, variation between duplicate dishes and experiments was not higher than 3–7% on those samples processed by either the dye staining or the enzyme assay. A possible source of variation in the Oil red O method could be related to losses of intracytoplasmic lipids during fixation of cell cultures. Some authors have described lipid and protein losses of 0.5% and 4.5%, respectively, during formalin and glutaraldehyde fixation of samples for histological and electron microscopy studies (Morgan and Huber 1967; Mays et al. 1984; Leist et al. 1986). However, fixation times up to 72 h did not significantly modify the results obtained from the colorimetric determinations (data not shown). It must be noted that, in order to obtain fast results, the fixation step was, in most cases, not longer than 1 h.

The determination of lipids with Oil red O can be used for any in vitro cultured cells that may accumulate triglycerides or cholesteryl esters, whether they are adipocytes or nonadipocytes. In the case of 3T3 adipocytes, lipid accumulation is mainly due to the increase of triglyceride synthesis (Green and Kehinde 1974, 1975; Spooner et al. 1979), and these neutral lipids constitute 90% of the total extractable cellular lipids; cholesterol or its derivatives did not seem to accumulate during adipocyte differentiation (Green and Kehinde 1974). The colorimetric assay has also been used on other cell culture systems; we have determined the extent of fat accumulation, mainly triglyceride, in primary cultures of hepatocytes to evaluate steatogenic capacity of several drugs (Mendoza-Figueroa et al. 1988). However, it is important to determine first by other procedures the intracytoplasmic proportion of triglycerides and cholesteryl oleate in cells that may accumulate both types of lipids. On the other hand, since slight changes in absorbance seem to occur with different lots of Oil red-O (results not shown), calibration curves for triolein or cholesteryl oleate must be made for every new lot of Oil red O used.

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