Retrieval of precursors for white-type adipose conversion in brown adipose tissue

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A cellular compartment from brown adipose tissue (BAT) of newborn rats was isolated by Percoll-density-gradient centrifugation and was shown to proliferate and to undergo adipose conversion *in vitro* in primary culture. The features of the effector requirement for adipose conversion as well as the differentiated morphological and biochemical phenotype are almost identical with that of a compartment designated HCF, from white adipose tissue (WAT). A possible role for these precursors from BAT and WAT in the involution of BAT into WAT, on the one hand, and in the development of brown adipose cells among typical WAT deposits, on the other, is discussed.

INTRODUCTION

The exact relationship between white and brown adipocytes is not known. But clear-cut data exist regarding the involution of brown adipose tissue (BAT) into white adipose tissue (WAT) during ontogeny (Smalley, 1970) and the presence of potential dormant brown-fat cells in a tissue traditionally considered as a white-adipose deposit. The dormant state of these cells can be interrupted and the cells allowed to undergo differentiation into brown-adipose cells *in vivo* under cold-acclimation of animals (Young *et al.*, 1984). In this context it should be mentioned that the involution of BAT into WAT can be slowed if the animals are reared in the cold (Barnard & Skala, 1970).

We previously demonstrated the existence of at least two developmentally regulated rat adipose-precursor compartments in rat WAT (Swierczewski & Gaben-Cogneville, 1985). Both differentiate *in vitro* in primary culture with high plating efficiency under the influence of different sets of effectors (Swierczewski *et al.*, 1987). One precursor population, designated HCF (heavy-cell fraction), requires presensitization with 3-isobutyl-1-methylxanthine (IBMX) before it can be recruited for adipose conversion in the presence of insulin. We hypothesized that this cell population was probably arrested at an earlier stage of the cell-differentiation program than the other, designated LCF (light-cell fraction), which requires only insulin.

Given the involution of BAT into WAT during ontogeny, we expected at least early white-cell precursors to be present in BAT. In addition, the fact that brown-adipose cells are retrieved in typical white tissue under certain conditions suggested the possible existence of a common committed precursor for both types of tissues.

In order to evaluate these possibilities, we isolated and characterized precursor compartments in BAT and compared them with those from WAT. We demonstrate

that a cell precursor compartment presenting common characteristics does exist in both tissues. This compartment is almost identical, in terms of morphology, density, growth pattern, effector response and differentiated phenotype, with the previously described HCF compartment from WAT (Swierczewski *et al.*, 1987).

EXPERIMENTAL

Cell culture

Interscapular BAT and inguinal white fat (WAT) from 3-day-old rats were dissected under sterile conditions. For each batch, fat-pads from 40–50 pups were utilized. The tissues were pooled, weighed, thoroughly minced and digested with collagenase as described previously (Gaben-Cogneville *et al.*, 1983, 1984). After isolation from the whole tissue, the stromal cells were filtered through a 80 µm-mesh nylon filter, washed with Hanks solution and layered separately on top of a preformed Percoll density gradient as previously described (Gaben-Cogneville *et al.*, 1983). The putative precursor cells from BAT and the defined precursor cells from WAT were obtained from cell fractions of densities ranging from 1.049 to 1.062 g/ml, and were designated BCF (brown-cell fraction) and HCF (heavy-cell fraction) respectively.

After separation, the cells from both tissues were centrifuged, washed with Hanks solution and plated separately (6×10^5 cells/dish). They were grown to confluence in Parker medium 199 supplemented with 20% (v/v) foetal-calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). Cultures were maintained in a 5% CO₂ atmosphere at 37°C. Once the monolayer reached confluence (day 4–5 after plating), 10% foetal-calf serum was replaced with 20% foetal-calf serum and the cells were treated with the following effectors, either alone or in various combinations: insulin (3 nM), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), dexamethasone (10⁻⁸ M).

Abbreviations used: BAT, brown adipose tissue; WAT, white adipose tissue; BCF, brown-cell fraction; HCF, heavy-cell fraction; IBMX, 3-isobutyl-1-methylxanthine; G3PDH, glycerol-3-phosphate dehydrogenase; LPL, lipoprotein lipase.

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methasone (0.25 μM), haemin (60 μM) and noradrenaline (10 nM). IBMX and dexamethasone were removed 48 h after addition. The other drugs remained in the medium from confluence until the end of culture. Control cultures in standard medium (medium 199, 10% foetal-calf serum and glucose) were run in parallel. Culture media were changed every second day after plating.

Morphological studies

Cell preparation for ultrastructural study. Electron microscopy was performed on the freshly isolated brown and white fractions at day 0 (before plating) and on brown and white cells at days 1 and 7 of culture. At each stage, experiments were carried out on at least three different cultures.

At day 0, both brown- and white-cell suspensions were centrifuged at 800 *g* for 15 min. The pellets were then fixed with 1% glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, for 2 h at 4 °C, post-fixed in 2% OsO_4 for 30 min at 4 °C, dehydrated in graded alcohols and embedded in Araldite.

At days 1 and 7, fixation, dehydration and embedding were carried out in the culture dishes. Cultures were rinsed with Hanks solution. Cells were then fixed with 1% glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, for 2 h at 4 °C and washed overnight in the same buffer supplemented with 8% glucose. After washing in the same buffer, cells were post-fixed in 2% OsO_4 for 30 min at 4 °C, dehydrated and embedded *in situ* in Epon (Brinkley *et al.*, 1967). When embedded, the cultures can be examined by phase-contrast microscopy and retain the aspect of a living monolayer. The cells were located with a light microscope and marked with a diamond scorer. Selected zones were cut with a warm punch and mounted on Araldite blocks, as described by Picart & Tixier-Vidal (1974). Ultrathin sections of both pellets and monolayer cultures were cut with a diamond knife on a LKB Ultratome, stained with uranyl acetate and lead citrate, and examined in a Philips EM 201 electron microscope.

Cell extracts for enzyme assays

Cell extracts were prepared as described by Gaben-Cogneville *et al.* (1983). Glycerol-3-phosphate dehydrogenase (G3PDH) was assayed by the method of Wise & Green (1979). phosphoenolpyruvate carboxykinase by the procedure of Ballard & Hanson (1967) and lipoprotein lipase (LPL) by that of Nilsson-Ehle & Schotz (1976). In the LPL assay, the cells were transferred into 0.5 ml of 50 mM- $\text{NH}_4\text{Cl}/\text{NH}_3$ buffer containing heparin (4 i.u./ml). The cell suspension was sonicated twice for 20 s, and portions of the sonicated suspension were directly used for the enzyme assay. Cytochrome oxidase was measured on the mitochondrial fraction isolated from a homogenate of brown preadipocytes as described by Skala *et al.* (1969). Enzyme activity was measured as described by Smith (1955). Unless otherwise stated, results are expressed as nmol of substrate/min per mg of protein at 37 °C. All enzyme assays were performed after pooling the cells from at least two culture dishes for each independent experiment (1×10^6 – 2×10^6 cells), except for cytochrome oxidase, where 10–20 culture dishes were pooled for each experiment (1×10^7 – 2×10^7 cells).

Materials

Pig monocomponent insulin was obtained from Novo Research Institute; IBMX, dexamethasone, dihydroxyacetone phosphate, isoprenaline (isoproterenol), noradrenaline and coenzymes were from Sigma. Parker medium 199 with Hanks salts and 20 mM-Hepes was from Flow Laboratories; foetal-calf serum and collagenase were from Boehringer. Recrystallized haemin was a laboratory preparation.

RESULTS

Distribution of potential precursor compartment differs in magnitude between BAT and WAT

In our standardized density gradient, the bottom cell fraction (below 1.075 g/ml), mostly composed of red cells, represented approx. 35% of the whole stromal vascular fraction for WAT and 50% for BAT. Within the remaining cell populations, we observed a higher percentage of cells in BCF (50%) than in HCF (< 28%).

Cells from BCF and HCF exhibit similar growth pattern in primary culture

Under the experimental conditions described above, plating efficiency of cells from BAT (BCF) was about 40%. This value was lower than that observed (about 70%) for precursors from WAT (HCF), but markedly higher than in a previous report concerning cells derived from BAT of adult animals (Nechad *et al.*, 1983; Nechad, 1983). However, the growth curves presented the same pattern for BCF and HCF cell fractions, with a doubling time of about 48 h. In addition, both cell fractions entered the resting state 6 days after confluence.

The two cell types acquire identical ultrastructural features in the post-confluent stage

Before plating, both compartments were found to consist of a homogeneous population of cells with a similar morphological appearance under electron microscopy. Our preparations were free of red blood cells and endothelial cells, and thus differ from data reported previously (Nechad *et al.*, 1983; Nechad, 1983). Most of the cells from both sources presented the same general morphological appearance. They were devoid of lipid, round in appearance and abundant in rough endoplasmic reticulum. Each cell type presented a large and irregular electron-lucent nucleus with a dense peripheral layer of chromatin (results not shown). The essential difference between the two types was that in BCF the mitochondria were larger with typically dense and parallel cristae (Fig. 1*a*), whereas in HCF (Fig. 1*b*) mitochondria were sparse, with less regularly arranged cristae. These features were still observed 24 h after plating in adhered cells, but a gradual decrease in the number of mitochondria and in the content of inner membrane was noticed all through the pre-confluent period, the changes being more marked for BCF (Fig. 1*c*) than for HCF (Fig. 1*d*). In post-confluent cells at day 7 of culture, the subtle differences observed until then between the two types disappeared, and in terms of ultrastructural features they became identical (Figs. 1*e* and 1*f*), even in the presence of some effectors (i.e. noradrenaline, dexamethasone, insulin). Typically, the mitochondria were oblong and large, which are the characteristics of white-type adipose cells (Cinti *et al.*, 1985).

Fig. 1. Comparison between the mitochondria of brown- and white-adipose cells at days 0, 1 and 7 of culture: electron micrographs

At early stages (days 0 and 1), mitochondria in brown cells (*a, c*) (magnification $\times 6000$) appear distinct from those in white cells (*b, d*) (magnification $\times 12000$) and present the characteristic tightly packed cristae. At day 7, mitochondria are morphologically identical in both brown (*e*) and white (*f*) cells (magnification $\times 6000$).

Table 1. Effect of different agents on the expression of cytochrome oxidase in rat precursor cells (BCF) isolated from BAT and grown in culture

Effectors were added at confluence. Insulin (3 nM) and noradrenaline (10 nM) were added to the medium at each refeeding until the end of culture. Dexamethasone (0.25 μ M) and IBMX (0.5 mM) were maintained in the medium only for 48 h after confluence. At day 0 (cells collected from the gradient before plating) and at days 1, 4 and 8 of culture, cell extracts were prepared and the activity of the enzyme was measured as described in the Experimental section. Control cultures containing standard medium were run in parallel. Each value represents the mean $\pm$ S.E.M. obtained from at least four independent cultures (except for experiments marked*, where only one culture).

Effectors	Period of culture (days)	Cytochrome oxidase (μ mol/min per mg of protein)
None (control medium)	0	1.200 $\pm$ 0.25
	1	0.251 $\pm$ 0.04
	4	0.164 $\pm$ 0.008
	8	0.264 $\pm$ 0.05
Insulin	8	0.136 $\pm$ 0.02
Dexamethasone	8	0.108*
Noradrenaline	8	0.164*
Insulin and dexamethasone	8	0.342 $\pm$ 0.03
Insulin and IBMX	8	0.292 $\pm$ 0.02

Putative preadipocytes in BCF lose two enzyme markers during growth in culture

Along with the above-mentioned changes in the mitochondrial ultrastructural features, a significant fall in the activity of the mitochondrial enzyme cytochrome *c* oxidase was also observed in BCF (Table 1). Within 24 h after plating, the decrease was 5-fold, reaching the activity (0.25 $\pm$ 0.04 μ mol/min per mg of protein) generally observed in the mitochondria of WAT of newborn animals (Casteilla *et al.*, 1987). In parallel with the decrease in this mitochondrial enzyme, the total disappearance of cytosolic phosphoenolpyruvate carboxykinase activity in culture was also noticed during the

same period. Interestingly, such a decrease in the cytosolic enzyme activity was a feature common to both types of cells (13 $\pm$ 2.3 nmol/min per mg for HCF and 18.07 $\pm$ 5.6 for BCF before plating, and undetectable in both cases 24 h after plating). None of the effectors listed above (including noradrenaline and/or isoprenaline), either alone or in different combinations, were able to maintain these two enzyme activities in both types of cell during growth and differentiation in culture.

BCF can undergo adipose conversion in primary culture

Potential for adipose conversion was initially monitored both by phase-contrast-microscopic examination and by measuring the activity of G3PDH. Cells were exposed at confluence to insulin (3 nM), dexamethasone (0.25 μ M) and IBMX (0.5 mM), either alone or in various combinations. The results obtained are shown in Table 2, and can be summarized as follows: (i) none of the effectors mentioned above can individually favour adipose conversion of these cells; (ii) the simultaneous presence of insulin, IBMX and dexamethasone induces adipose conversion, as reflected by the marked increase in G3PDH (about 10-fold above control values at day 11), as well by the morphological appearance of cells stained with Oil Red O (results not shown); (iii) the increase in G3PDH did not occur until after IBMX and dexamethasone had been removed from the cultures (day 6 after plating). The continuous presence of these drugs is not required for efficient adipose conversion; they tend to have an inhibitory effect if present throughout the culture. All features of adipose conversion, including the effector requirement described here, are identical with those observed with cells from the HCF compartment of WAT (Swierczewski *et al.*, 1987).

Precursors of BCF and HCF follow a similar pattern of expression of lipoprotein lipase activity

LPL activity is known to be present even in the preconfluent stage of cells from the LCF compartment of WAT (Gaben-Cogneville *et al.*, 1984). We observed that both BCF and HCF in primary culture show an early presence of LPL before confluence, which increases to a maximum at day 8 (4 days after confluence), even in the absence of adipose differentiation (Table 1). When adipose conversion was favoured by the combined

Table 2. Effect of different effectors on the expression of G3PDH activity in rat precursor cells (BCF) isolated from BAT and grown in culture

Effectors were added at confluence, either alone or in combination, under the conditions described in the legend to Table 1. At days 8, 11 and 15 of culture, cell extracts were prepared and G3PDH activity was measured as described in the Experimental section. G3PDH activity during the growing phase (undifferentiated cells) did not exceed 36 $\pm$ 4 nmol/min per mg of protein. Data are means $\pm$ S.E.M. of duplicates from at least five independent cultures.

Effectors	G3PDH (nmol/min per mg of protein)		
	Day 8	Day 11	Day 15
None (control medium)	101 $\pm$ 20	92 $\pm$ 15	56 $\pm$ 7
Insulin	185 $\pm$ 4	308 $\pm$ 65	168 $\pm$ 25
Dexamethasone	94 $\pm$ 12	336 $\pm$ 46	152 $\pm$ 17
IBMX	245 $\pm$ 15	324 $\pm$ 81	167 $\pm$ 16
Insulin and dexamethasone	186 $\pm$ 2	546 $\pm$ 9	337 $\pm$ 5
Insulin and IBMX	452 $\pm$ 108	585 $\pm$ 92	389 $\pm$ 35
Insulin, IBMX and dexamethasone	743 $\pm$ 143	1034 $\pm$ 194	613 $\pm$ 120

Table 3. Effect of treatment with different effectors on the expression of lipoprotein lipase (LPL) in rat precursor cells from WAT (HCF fraction) and BAT (BCF) in culture

Effectors were added at confluence as described in the legends to Tables 1 and 2. Haemin (H; 60 μ M), when present, was added at confluence and maintained in the medium until the end of culture. Abbreviation: IDX, insulin + dexamethasone + IBMX. At each time point, cell extracts were prepared and LPL activity was determined as described in the Experimental section. LPL activity is expressed in nmol/h per mg of protein. Results are means $\pm$ S.E.M. of duplicates from at least three independent cultures.

Time after plating (days)	Cells...	LPL activity	
		HCF	BCF
1 $\rightarrow$ 2		159 $\pm$ 28	54 $\pm$ 12
4		568 $\pm$ 44	181 $\pm$ 59
8	Control	683 $\pm$ 123	225 $\pm$ 29
	IDX	1393 $\pm$ 360	479 $\pm$ 02
	IDX + H	2310 $\pm$ 362	1330 $\pm$ 235

presence of insulin, dexamethasone and IBMX, we observed a 2-fold increase in the peak activity of the enzyme in post-confluent cells of both types. However, the values obtained were consistently lower (3-fold) for BCF than for HCF. Since we have shown previously (Swierczewski *et al.*, 1987) that haemin can potentiate the effect of insulin, dexamethasone and IBMX on G3PDH activity in HCF cells, we examined the effect of haemin on LPL activity in both cell types (HCF and BCF). We found that haemin potentiates LPL activity in a similar manner (Table 3).

DISCUSSION

We have demonstrated the existence of a cellular compartment in BAT (BCF) of newborn rats which comprises cells capable of undergoing morphological and biochemical adipose conversion *in vitro* in primary culture. The resulting differentiated cells acquire white-adipose-type features in culture: few mitochondria, with a low content of inner membrane, low cytochrome *c* oxidase specific activity, increase in the specific activities of LPL, and G3PDH, elevated triacylglycerol content, all equivalent to that generally observed in typical white cells. Furthermore, the ontogenic stage of these cells (BCF) appears to be identical with that of HCF cells from white adipose tissue, as judged by the similarity in their response to a particular combination of effectors. Thus we speculate that BCF cells participate in the involution of BAT into WAT *in vivo* during ontogeny. Previously, attempts have been made to grow and differentiate in primary culture the stromal vascular fraction of human (Cigolini *et al.*, 1985) and rat (Nechad *et al.*, 1983; Nechad, 1983) BAT. A cell line (BCF-1) derived from mouse BAT has also been established (Forest *et al.*, 1987a,b). Results of these studies can be essentially summarized as follows: (i) post-confluent mitochondrial regression (as also observed in our study)

has been interpreted as a lack of specific factors in the culture medium necessary for full mitochondrial development; (ii) in contrast with mature brown adipocytes, neither the cell line nor preadipocytes from mouse BAT in primary culture express the brown-tissue-specific 32 kDa protein thermogenin (Forest *et al.*, 1987a,b); (iii) Forest *et al.* (1987a,b) found, as we did, that adipose conversion does occur, as demonstrated by the emergence of several enzyme markers and triacylglycerol accumulation, the general pattern of which largely resembles that of white-adipose conversion.

The novel aspect of our work consists of the fact that we have studied a density-defined homogeneous population of cells from BAT and that these cells are almost identical in several respects with a cell population isolated by density-gradient centrifugation from WAT. Our data suggest the possibility that these precursors from BAT are responsible for involution of BAT into WAT during ontogeny. We do not know at present whether these precursors can undergo brown-type adipose conversion. Until now no group, to our knowledge, has succeeded in establishing culture conditions which direct precursors from BAT towards the brown phenotype in culture.

It is noteworthy that Young *et al.* (1984) were able to induce *in vivo*, by cold-acclimation, brown-adipocyte regions in an area of typical WAT. In this context it is relevant to question whether these cells, which are smaller than average, correspond to the precursor fraction, namely HCF of WAT. Since we have shown in the present study that BCF and HCF behave identically in culture, they might very well represent a common precursor compartment responsible for the involution of BAT into WAT during ontogeny, as well as the emergence of brown-adipose cells in WAT during cold-acclimation. However, failure to differentiate *in vitro* the putative brown precursors into brown-type cells prevents us, at present, to speculate on the existence of a common precursor for brown/white type conversion.

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