

## **Dietary and other factors involved in the proliferation, determination and differentiation of adipocyte precursor cells**

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White adipose tissue, as a specialized connective tissue, is found in a recognizable form in a wide range of animal species (Pond, 1978, 1986; Nir *et al.* 1988) where its contribution to lipid metabolism and energy balance is well accepted (Cryer & Cryer, 1990). Initially, studies on the development of white adipose tissue concentrated on a description of the changes in mature adipocyte cell size and number that occur in a variety of species (for review, see Cryer & Cryer, 1990) during growth. The findings, arising from such studies, indicate clearly that, contrary to early propositions, the cellular development that accompanies adipose tissue growth involves both cellular hyperplasia and hypertrophy at virtually all stages of life (for example, see Faust & Miller, 1981) and that both processes can be affected by nutritional manipulation (Faust *et al.* 1978).

Although there has been a suggestion that fully differentiated unilocular adipocytes may proliferate under appropriate conditions *in vitro*, it is still the case that the overwhelming weight of evidence from studies both *in vivo* (Geloan *et al.* 1989) and *in vitro* (Ailhaud, 1982) supports the view that the major proliferative activity in adipose tissue is in the interstitial pool of cells which includes, as a major component, the so-called adipocyte precursor cell (Cryer, 1980; Hauner & Loffler, 1987*a,b*).

At the current state of development in the field, an increasing amount of information on the nature and behaviour of the adipocyte precursor *in vitro* is forthcoming (for example, see Ailhaud *et al.* 1991; Cryer & Cryer, 1991). However, because of the experimental difficulties, much of the relevance these findings may have to the behaviour of such cells *in vivo* remains to be discerned. Clearly, the precise description of the stages through which adipocyte development passes can be addressed by experimentation *in vitro*. In particular, the biochemical descriptions that may be adduced *in vitro* should, it may be speculated, provide appropriate markers which may then be applied in order to follow temporal and induced changes in the cellular development of the tissue *in vivo*.

Thus, the major work in the area has sought to study the effectors of adipocyte precursor proliferative activity and those factors that are essential or permissive with regard to the progression of the earlier and later stages of the differentiation programme which culminate together in the generation of a mature unilocular adipocyte (for review, see Cryer & Cryer, 1990). Most recently, our own work has concentrated on defining the state of development of precursor cells isolated from adipose tissue taken from a variety of depots in various animal species (Cryer & Cryer, 1989). Such animals have been at differing stages of overall development and the cells have been studied with regard to the nature of the effectors that stimulate their proliferation and differentiation *in vitro*. The comparative biochemistry of the latter now indicates the species-specific nature of the pattern of effectors that are active and this is described in more detail elsewhere (Adams

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*et al.* 1992). The present paper will concentrate on current knowledge relating to the stage of development displayed by adipocyte precursors when they are derived from tissue sources *in vivo*.

#### METHODS

Putative precursor cells with the capacity to undergo adipose conversion were isolated from the white adipose tissue of rat, chicken, ox and sheep as described by Cryer *et al.* (1984, 1987) and Cryer & Cryer (1989) and were maintained in culture as described therein. Mature ovine adipocytes were allowed to revert to a lipid-free proliferative form using the methods described in Cryer & Cryer (1990).

Antisera with specific reactivity towards white adipocyte cell surface antigens were prepared, characterized and employed as described by Thompson & Abrahams (1979), Plaas *et al.* (1981), Lee *et al.* (1986) and Cryer *et al.* (1987). In some experiments anti-adipocyte antibodies were labelled directly with acridinium ester using the technique described by Woodhead & Weeks (1985). The preparation and characterization of white adipocyte plasma membranes employed the method of Belsham *et al.* (1980) and anti-ovine lipoprotein lipase serum was prepared as described by Al-Jafari & Cryer (1986).

RNA was isolated from cultured cells by caesium chloride ultracentrifugation following extraction in guanidinium thiocyanate. The cells were removed from the growth surface mechanically, washed in sterile phosphate buffered saline (9 g sodium chloride/l) and frozen in liquid nitrogen before the extraction. The RNA samples were studied using conventional methods and found to be free of DNA and protein.

Probes for the analysis of the RNA samples were prepared and utilized using standard techniques, as described by Maniatis *et al.* (1982) and Kille *et al.* (1990). The lipoprotein lipase (*EC* 3.1.1.34; LPL) activity of cultured cells was determined as described previously (Plaas & Cryer, 1980).

#### RESULTS AND DISCUSSION

##### *Adipocyte precursor cell growth characteristics*

When adipocyte precursor cells were isolated from the white adipose tissue of a variety of species, the rate of their proliferation in the presence of serum was shown to be similar for most species (doubling time approximately 26 h) and for most depot sources (K. S. Adams, D. J. Flint, R. G. Vernon and A. Cryer, unpublished results). However, the strain of the animal from which the cell may be derived (Donnelley *et al.* 1989) and the age of the donor (Donnelley, 1990) have been shown to have an influence, with the doubling time for cells derived from young animals being shorter than that for those isolated from mature animals. Such donor age-related differences seem not to be related to the sensitivity of the cells to growth effectors present in serum, in that such differences were also discernible when a serum-free medium of defined composition was used (A. Cryer and J. Cryer, unpublished results). However, data collected to date would suggest that there are significant differences between the species with respect to specific cell receptor specificities and responsiveness (see Adams *et al.* 1992).

When putative adipocyte precursor cells have been isolated from the white adipose tissue of a variety of species they have been found, under optimal conditions of culture

Table 1. *Ratio of immunoreactivity demonstrated by cells at confluency and at the maximum post-confluency level\**

Cells	3T3-L <sub>1</sub> (mouse)	Adipocyte precursors from:		
		Rat	Sheep	Ox
Ratio (confluency:maximum)	0:1	0.26:1	0.51:1	0.2:1

\* Specific anti-adipocyte immunoreactivity was determined using either the indirect-labelled second-antibody cellular immunoassay previously described (Plaas & Cryer, 1980) or, in the case of sheep, chemiluminescently-labelled anti-adipocyte antibodies (Cryer & Cryer, 1991). Cells at confluency were compared with those expressing maximum immunoreactivity post-confluency. The antisera used were raised on a species-specific basis.

(which varies among species), to acquire the morphological characteristics of (at least) multilocular adipocytes within approximately 14–21 d of post-confluency cell culture. Such changes in morphology are accompanied not only by increases in phenotypic enzyme activities (for example, LPL) but also by increases in the reactivity that the cells exhibit when reacted with antibodies previously shown to be adipocyte-specific (for example, see Cryer & Cryer, 1991). These approaches have, however, both in our work and that of others, been confined to a comparison of post-confluency cells with those studied at the point of confluency. Growing cells have received scant attention, partly it may be suggested because of operational reasons and partly because cloned adipocyte precursor cell lines such as 3T3-L<sub>1</sub> and others exhibit no discernible adipocyte characteristics during their growth phase in culture.

#### *The demonstration of adipocyte characteristics by precursor cells at and before confluency*

1. *Adipocyte specific immunoreactivity.* When adipocyte precursor cells from a number of species were compared, in respect of their species-defined adipocyte-specific immunoreactivity at confluency, with that demonstrated by 3T3-L<sub>1</sub> cells it was found that, unlike 3T3-L<sub>1</sub> cells, the tissue-derived precursors all exhibited adipocyte-specific immunoreactivity at confluency (Table 1) which was between 20 and 55% of that exhibited maximally by cells during the post-confluency period of culture development.

These observations were extended specifically in the case of cells from sheep where the immunoreactivity of growing cells at a variety of stages was determined. This was done, first, in relation to total antibody binding and, second, the nature of binding was investigated by studying immunoprobbed blots of polyacrylamide gels loaded with plasma membrane preparations isolated from cells harvested at various stages of culture (from growing to late confluency).

When the whole cell plasma membrane immunoassay was performed it was found that, when expressed per unit cellular protein, the reactivity of growing cells, derived from five different anatomical sites of the sheep, was invariably equal to or greater than that shown by the confluent cells and was similar to that shown by cells 10 d after confluency (Table 2) when reactivity was associated with clear morphological evidence of differentiation.

The reactivity of the membrane preparations was then analysed by Western Blotting, in which samples were subjected to polyacrylamide gel electrophoresis in the presence of

Table 2. *Changes in anti-adipocyte-specific immunoreactivity during the growth and post-confluency maintenance of sheep adipocyte-precursor cells in culture. (From Cryer & Cryer, 1991)*

(Values expressed as percentage calculated from triplicate determinations in each case)

Depot source	Stage of culture		
	Growing	Confluent	10 d post-confluent
Channel	145	121	100
Mesenteric	92	82	100
Subcutaneous	79	64	100
Perirenal	69	76	100
Intramuscular	95	66	100

sodium dodecyl sulphate followed by electro-transfer to an immobilization membrane (Immobilon-P Millipore UK) and reaction with antibody, the presence of the bound antibody being visualized using a peroxidase (*EC* 1.11.1.7)-anti-peroxidase (PAP) amplification system with 4-chloro-naphthol as chromagen. The intensity of staining obtained on such blots was compatible with the pattern revealed by the immunoassay, with the pattern of revealed bands, however, being different at each time-point studied (results not shown).

These findings are compatible with the previously reported findings obtained using fluorescent-activated cell sorting (FACS) of antibody reactive bovine adipocyte precursor cells soon after isolation from the tissue (Cryer *et al.* 1984). Thus, it is clear that high levels of adipocyte specific immunoreactivity exists in cultures of rapidly dividing adipocyte precursor cells and, as such, these cells appear to exhibit some adipocyte characteristics well before the later stages of the differentiation programme are entered into following growth arrest *in vitro*. Knowing that the adipocyte specific immunoreactivity is considered an early marker of the adipocyte phenotype (Cryer & Cryer, 1991) further investigation of a second somewhat later marker of adipocyte function, *i.e.* LPL activity, was also investigated in growing cells.

2. *LPL activity, immunoreactivity and messenger RNA content.* When 3T3-L<sub>1</sub> cells have been studied, LPL activity has only been demonstrated after the cells have entered the post-confluency stage of culture development. In previous studies, with tissue-derived adipocyte precursors, the pre-confluency growth phase has been poorly studied, although with these cells also the activity at confluency has been shown to be low and to be followed by a dramatic post-confluence increase.

With sheep cells we have re-investigated the pattern of LPL expression in both growing and post-confluency cells by measuring activity, the presence of protein reactive with an anti-LPL antibody and the presence of RNA species able to hybridize with both labelled-RNA and cDNA specific for LPL-mRNA. The activity of LPL detected in cells at seeding, during growth, when freshly confluent and during post-confluency, the activity, calculated as a percentage of the maximal activity expressed post-confluency, was 0, 87 and 9 respectively. Thus, although freshly confluent cells expressed only low levels of activity (as expected), those that were actively growing and dividing expressed comparatively high levels. This pattern was qualitatively confirmed on Western Blots of

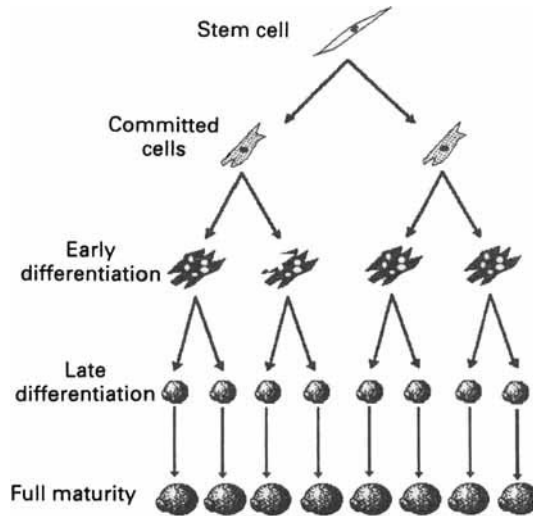


Fig. 1. Stages in adipocyte differentiation.

cellular plasma membrane preparations which were probed with anti-LPL serum followed by exposure to the PAP/4-chloro-naphthol system of visualization. This latter observation suggested that changes in the rate of synthesis of the enzyme were occurring during the maintenance of the cells in culture, rather than post-translational activity control being singularly involved. This was confirmed when the levels of LPL-mRNA were determined in the cells at the same stages of development. The determinations showed that the LPL-mRNA levels were highest in growing and post-confluent cells (where activity and immunoreactivity were also comparatively elevated) and were at their lowest following seeding and at the point of growth arrest when confluency was reached.

These findings are entirely compatible with those shown previously (p. 382) and indicate strongly that the proliferatively active precursor cell isolated from adipose tissue is highly determined during its growth phase and that it is clearly committed to the adipocyte lineage well before growth arrest, when only the later aspects of the adipocyte differentiation programme remain to be completed. A simple model of adipocyte development within adipose tissue may, therefore, be proposed (Fig 1) in which the strict division between proliferation and determination/differentiation can be eroded. However, it is clear that many problems remain to be addressed, not least of which is the definition of the earliest stages of determination and the measurement of the cell pool sizes of pluripotent stem cells, determined pre-adipocytes and adipocytes in tissue at different stages of development and a description of how such a spectrum might shift in response to nutritional change.

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