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COMMENTARY

Proadipocyte cell lines: models of cellular proliferation and differentiation

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INTRODUCTION

The relationship between growth arrest and differentiation is complex and not well defined. Frequently, cells that are competent to differentiate remain undifferentiated while exposed to proliferative stimuli and undergo differentiation only upon removal of such stimuli (Zentella and Massague, 1992). In some cell types, suppression of proliferation is itself sufficient to induce differentiation (Nadal-Ginard, 1978; Munroe et al., 1984; Bianchi-Scarra et al., 1986), while inhibition of growth appears to be controlled separately from differentiation in other cells (Sorrentino et al., 1990). In the case of adipocyte differentiation, it is known that reversible growth arrest must occur before most proadipocytes can commit to the differentiation program (Scott et al., 1982a,b; Spiegelman and Farmer, 1982; Sparks et al., 1990). Growth factors and other mitogens, therefore, may inhibit adipogenesis indirectly by preventing arrest in the appropriate stage of the cell cycle (Hayashi et al., 1981; Negrel et al., 1981; Ignotz and Massague, 1985; Navre and Ringold, 1989). Growth arrest itself, however, does not induce adipocyte differentiation (Krawisz and Scott, 1982; Wille and Scott, 1982; Umek et al., 1991). As the differentiation program proceeds, changes in proliferative capacity occur and irreversible loss of proliferative potential is generally a characteristic of terminal differentiation. The potential to dissociate the molecular events involved specifically in the loss of mitogenic responsiveness from those involved in adipogenesis per se, indicates the value of adipocyte cell lines in deciphering the relationship between cellular proliferation and differentiation.

A number of proadipocyte cell lines have been isolated, including the following: 3T3-L1 (Green and Kehinde, 1974), 3T3-F442A (Green and Kehinde, 1976), TA1 (Chapman et al., 1984), Ob17 (Negrel et al., 1978), 1246 (Darmon et al., 1981), ST 13 (Hiragun et al., 1980) and PFC6 (Ailhaud, 1982). Our studies have focused on A31T6 proadipocytes, also referred to as Balb/c-3T3 T and A31T proadipocytes, which were cloned from the fibroblast-like cell line Balb/c-3T3 clone A31 (Diamond et al., 1977). During exponential growth, all of these cell lines are morphologically indistinguishable from murine fibroblasts. Following growth arrest, they differentiate into adipocytes under appropriate culture conditions. Extensive studies in proadipocyte cell lines have established morphologic, enzymatic and biochemical aspects of adipocyte differentiation (reviewed by Ailhaud, 1982; Spiegelman, 1988; Spiegelman et al., 1993). Proadipocytes have been particularly valuable in elucidating mechanisms that control differentiation of normal and neoplastically transformed cells, with particular emphasis on the uncoupling of proliferation and differentiation during carcinogenesis (Filipak et al., 1989 and references therein; Grimaldi et al., 1984; Freytag, 1988; Serrero, 1985; Cherington et al., 1988).

Adipocyte models have proved valuable in studies of the mechanisms regulating glucose uptake and metabolism, gene expression in proadipocytes and adipocytes, and in studies investigating controls on proliferation and differentiation. A number of genes have been shown to be differentially expressed during adipocyte differentiation. Some, such as those involved in lipid synthesis and storage, are related specifically to expression of the adipogenic phenotype (Bernlohr et al., 1984; Chapman et al., 1984; Amri et al., 1986; Ntambi et al., 1988; Spiegelman, 1988; James et al., 1989; Kaestner et al., 1989; Moustaid et al., 1990; Zechner et al., 1991; Enerback et al., 1992). Others, such as novel transcription factors that are induced during differentiation, could be related either to processes involved in irreversible loss of proliferative potential or to expression of the acquired specialized function (Cao et al., 1991; Samuelsson et al., 1991; Umek et al., 1991; Lin and Lane, 1992). In addition, a recent report identifies a preadipocyte gene product whose expression is abundant in preadipocytes and abolished during adipocyte differentiation (Smas and Sul, 1993).

Commitment to the differentiation program typically occurs in the presence of serum or plasma, though the exact plasma-derived factors that are responsible for the induction of differentiation have yet to be identified. The nature of the active components that are responsible for adipogenic conversion has, however, been partially elucidated in studies focusing on the mouse clonal cell lines 3T3-L1, 3T3-F442A, Ob17 and TA1, in addition to primary cells

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isolated from adipose tissue derived from a wide range of mammalian species (reviewed by Li et al., 1989). Studies in 3T3-F442A cells, for example, have shown that growth hormone is involved (Zezulak and Green, 1986). In addition to those agents involved in commitment to the adipogenic program, a number of positive and negative regulators of adipocyte differentiation have been identified. Positive regulators include the following agents, either alone or in various combinations: dexamethasone, indomethacin, insulin, methylisobutylxanthine, the antidiabetic agent AD4743 and clofenapate (Verrando et al., 1981; Yun and Scott, 1983; Freytag, 1988; Sparks et al., 1991; Smyth and Wharton, 1992a, 1993). Negative regulators of adipogenesis include transforming growth factor β (TGF-β) (Ignotz and Massague, 1985; Sparks and Scott, 1986; Gimble et al., 1989; Torti et al., 1989), tumor necrosis factor (TNF) (Torti et al., 1985; Filipak et al., 1988; Gimble et al., 1989), preadipocyte factor 1 (pref-1) (Smas and Sul, 1993), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Diamond et al., 1977; Shimizu et al., 1983; Yun and Scott, 1983; Muller et al., 1984), fibroblast growth factor (FGF) (Hayashi et al., 1981; Muller et al., 1984; Navre and Ringold, 1988), platelet-derived growth factor (PDGF) (Hayashi et al., 1981; Navre and Ringold, 1988), retinoic acid (Murray and Russell, 1980; Kuri-Harcuch, 1982) and interleukin-1α (IL-1) (Gimble et al., 1989). A number of these are landmark studies on the inhibition of differentiation in that, for example, the initial determination of the inhibitory effects of TPA and TNF on differentiation were carried out in adipocyte models. As indicated above, a number of the negative regulators of adipocyte differentiation are growth factors. In some cases, their effects are indirect and result from stimulation of cell proliferation (Sparks and Scott, 1986; Sparks et al., 1986), whereas in other cases the effects have been shown to be independent of effects on cell proliferation. The inhibitory effects of FGF and TPA in TA1 cells and of TNF and TGF-β in A31T6 cells, for example, have been shown to be independent of effects on cell growth (Navre and Ringold, 1988; Sparks and Scott, 1986; Filipak et al., 1988). In contrast, it has been suggested that if pref-1 functions as a growth factor, its down-regulation may be involved in the cessation of growth required for adipocyte differentiation in 3T3-L1 cells (Smas and Sul, 1993). In addition, dexamethasone has been found to be capable of stimulating growth or inducing differentiation in A31T6 cells, by unrelated actions (M. J. Smyth and W. Wharton, unpublished observations). The ability of growth factors to inhibit the differentiation of determined stem cells therefore provides a useful experimental paradigm in which proliferation-independent actions of growth factors can be investigated.

In this commentary, we will focus on: (1) differential expression of genes involved in both adipogenesis and the loss of mitogenic responsiveness during the program of adipocyte differentiation; (2) the value of specific preadipocytes in identifying genes of critical importance in negative growth control, by facilitating the functional separation of loss of mitogenic responsiveness from expression of genes involved in the specialized adipogenic functions; and (3) flow cytometry, which has added a new dimension to investigations of adipogenesis by providing a unique and quantitative method for evaluating progress of the adipogenic program at both the phenotypic and biochemical level.

CHANGES IN GENE EXPRESSION DURING ADIPOCYTE DIFFERENTIATION

Studies involving F442A, 3T3-L1, A31T6 and TA1 cells have been particularly fruitful in investigating the fundamental mechanisms underlying changes in gene expression occurring during adipocyte differentiation. As preadipocytes differentiate into adipocytes, many new proteins and enzymes are expressed (reviewed by Spiegelman, 1988; Watt, 1991; Spiegelman et al., 1993), while growth-regulated genes decrease in expression (Spiegelman and Farmer, 1982; Sparks et al., 1993; Smas and Sul, 1993). A number of genes encoding differentiation-specific proteins have been cloned (Spiegelman et al., 1983; Chapman et al., 1984; Bernlohr et al., 1984; Bernlohr et al., 1985; Smith et al., 1988; Dani et al., 1989; Jiang and Serrero, 1992). Many of these gene products are involved in fatty acid and triglyceride synthesis, but other new classes of proteins, such as transcription factors that are potential regulators of adipocyte differentiation, are also induced (Birkenmeier et al., 1989; Friedman et al., 1989). Cloned genes whose expression increases during adipogenesis include insulin-regulated glucose transporter (IRGT, also called Glut4), lipoprotein lipase (LPL), glycerol-3-phosphate dehydrogenase (GPD), fatty acid synthetase, malic enzyme, aP2 and adipsin. Expression of some of these genes begins to increase prior to morphologic differentiation, while others increase concurrent with morphologic differentiation (Chapman et al., 1984, 1985; Christy et al., 1989; Torti et al., 1985, 1989; Kaestner et al., 1990).

The regulation of these genes has been investigated at the molecular level. Common regulatory sequences are present in the 5′ flanking regions of some adipocyte-specific genes (Graves et al., 1985; Christy et al., 1989; Cao et al., 1991; Lin and Lane, 1992) suggesting that they might be induced by a common regulatory pathway. A 5′ flanking sequence of the aP2 gene, for example, appears to contain an adipocyte-specific enhancer sequence and is regulated by a factor in the NF-1 family (Graves et al., 1985). This is the first evidence for a functional enhancer with specificity for adipose cells. In contrast, the Fos-Jun complex is known to negatively regulate transcription of the differentiation-specific gene aP2, via a regulatory region in its promoter called fat specific element 2 (FSE2) (reviewed by Spiegelman et al., 1988). This type of mechanism may also regulate other differentiation-specific genes.

Regulatory genes that control muscle differentiation have been identified, the first of which was the transcription factor *MyoD* (Davis et al., 1987; reviewed by Olson, 1990). The muscle transcription factors are the only mammalian factors identified to date that can generate a complete differentiated phenotype (reviewed by Olson et al., 1991). Several lines of evidence suggest that adipocyte differentiation may be regulated in a similar fashion. Experiments analogous to those early studies in which genomic transfection experiments demonstrated the existence of myoblast con-

version activity (Lassar et al., 1986), have demonstrated the existence of adipogenic conversion activity (Chen et al., 1989). Also, the original system used for isolation of *MyoD* from myoblast cells (i.e. 5-azacytidine treated $10T_{\frac{1}{2}}$ cells) also gives rise to proadipocytes (Davis et al., 1987). This suggests that adipocyte differentiation could be initiated/regulated by a putative '*AdipoD*' in a manner analogous to that of myogenic differentiation.

Potential candidates for regulatory genes/factors in adipocyte differentiation have been identified. These include the CCAAT enhancer binding proteins (*C/EBP*) α, β and δ (Cao et al., 1991) which are sequence-specific DNA binding proteins with preference for binding to CCAAT boxes (Johnson et al., 1987). It has been suggested that these transcription factors may play a general role in establishing and maintaining the differentiated, non-proliferative state (Friedman and McKnight, 1990). *C/EBP*α is a basic leucine zipper transcription factor whose expression increases at both the message and protein level during differentiation and which enhances or trans-activates expression of a number of adipocyte-specific genes that contain common 5′ flanking regions (Birkenmeier et al., 1989; Christy et al., 1989; Herrera et al., 1989; Kaestner et al., 1990; Cao et al., 1991; Umek et al., 1991). *C/EBP*α is virtually undetectable in proliferating proadipocytes. Lin and Lane (1992) found that antisense *C/EBP*α mRNA inhibited both expression of the differentiation-specific genes *422* (*aP2*), *SCD1*, and *Glut4* (*IRGT*) and expression of the differentiated phenotype in 3T3-L1 cells, demonstrating that *C/EBP*α is required for differentiation to occur. Premature expression of *C/EBP*α, using transfected expression vectors, impedes proliferation of 3T3-L1 cells while, in the presence of an appropriate stimulus, differentiation is enhanced (Umek et al., 1991). Premature expression of *C/EBP*α, however, is not sufficient to induce differentiation in the absence of enhancers of differentiation. This suggests that additional regulatory proteins must be involved in inducing adipocyte differentiation.

*C/EBP*β and *C/EBP*δ, recently cloned from proadipocytes based on sequence similarities to *C/EBP*α (Cao et al., 1991), exhibit the same structural characteristics as *C/EBP*α and bind to the same recognition sequence. In contrast to *C/EBP*α, however, both are expressed in growing cells, and their true function in adipoycte differentiation has yet to be elucidated (Cao et al., 1991). Transcription of the *C/EBP*β and *C/EBP*δ genes is induced directly by adipogenic hormones. A transient increase in expression of *C/EBP*β and *C/EBP*δ is seen shortly after exposure to the differentiation stimulus, as is seen for the *MyoD* family of muscle regulatory genes. It has been suggested that the profiles for the expression of *C/EBP*β and *C/EBP*δ during adipogenesis raise the possibility that these transcription factors perform important regulatory functions during the early processes of differentiation. Specifically, the possibility that *C/EBP*β and *C/EBP*δ are responsible for the induction of *C/EBP*α has been raised (Cao et al., 1991). The fact that TGF-β blocks differentiation in A31T6 cells at an early point in the adipogenic process (Sparks and Scott, 1986; Sparks et al., 1993) further supports the theory that a sequence of regulatory events controls adipocyte differentiation. It has recently been shown that expression of *C/EBP*β and *C/EBP*δ is not exclusive to adipocytes. *C/EBP*β has also been identified as *AGP/EBP* (Chang et al., 1990), *NF-IL6* (Akira et al., 1990), *CRP2* (Williams et al., 1991) and *LAP* (Ron and Habener, 1992), while *C/EBP*δ has also been identified as *CRP3* (Williams et al., 1991).

A compelling body of evidence indicates that the c-*myc* proto-oncogene is involved in control of the intimately related processes of cell proliferation and differentiation (Hoffman-Liebermann and Liebermann, 1991). Enforced expression of c-*myc* in 3T3-L1 proadipocytes, by transfection with expression vectors containing a recombinant c-*myc* gene, inhibits differentiation (Freytag, 1988). The block is not due merely to transformation and can be overcome by high expression of *myc* antisense RNA. It has been suggested that myc may act as a molecular switch that directs cells to a pathway leading to continued growth or to terminal differentiation (Freytag and Geddes, 1992). Overexpression of myc not only inhibits adipogenesis, but also prevents the normal induction of *C/EBP*α. In addition, forced expression of *C/EBP*α can overcome the *myc*-induced differentiation block. Freytag and Geddes suggested that deregulation (overexpression) of *myc* promotes proliferation (or competence to proliferate) and in turn inhibits expression of the differentiation promoter *C/EBP*α. This interpretation is in general agreement with previous reports on transformation and/or loss of differentiation control in proadipocytes (Scott and Maercklein, 1985; Cherington et al., 1988; Umek et al., 1991; Sparks et al., 1993). While it has not been shown that *myc* directly inhibits the induction of *C/EBP*α, it is known that the promoter of the *C/EBP*α gene (Christy et al., 1991) contains a *myc* binding site (Blackwell et al., 1990; Prendergast and Ziff, 1991). If adipocyte differentiation is controlled by a sequence of regulatory events, then it is also plausible that *myc* affects the cascade of events at an earlier point in the sequence than does *C/EBP*α.

VALUE OF ADIPOCYTE MODELS IN INVESTIGATION OF NEGATIVE GROWTH REGULATION

Proliferation and differentiation are usually alternative and mutually exclusive pathways for cells (Freytag, 1988; Freytag and Geddes, 1992). However, both the nature of the molecular switch that controls the graded loss of proliferative potential during the differentiation program, and the mechanisms by which this is controlled, remain obscure. Accumulating experimental evidence regarding gene expression during differentiation in eukaryotes suggests that the differentiated state is maintained by active continuous regulation, both by positive and negative regulators (Blau, 1992). Studies employing in vitro differentiation models should, therefore, lead to an understanding of the regulation of proliferative potential during the differentation program and help dissect and identify significant regulatory events in negative growth control.

An extensive body of literature is accumulating detailing various aspects of the regulation of mammalian cell proliferation (Murray and Kirschner, 1989; Pardee, 1989, and references therein). These studies have, however, focused

largely on the positive regulation of growth control through transcriptional activation of genes. While numerous such genes have been identified (Bishop, 1991; Cantley et al., 1991), various lines of evidence have recently converged on the idea that the mammalian genome also carries a subset of genes that is normally involved in negatively regulating cellular proliferation (reviewed by Marshall, 1991; Weinberg, 1991). Despite the acknowledged importance of such genes, their isolation has proved elusive. Intensive research in recent years has resulted in the isolation of only a small number of genes for which roles as negative effectors of cell growth or transformation have been demonstrated (Friend et al., 1986; Kitayama et al., 1989; Call et al., 1990; Fearon et al., 1990; Wallace et al., 1990; Levine et al., 1991; Nuell et al., 1991). There appear to be three functional categories of genes that negatively regulate cell proliferation: (1) genes that play a negative role in the cell cycle, such as *BTG1* (Rouault et al., 1992) and *GAS* genes (Schneider et al., 1988), as determined by differential expression during quiescence; (2) tumor suppressor genes such as *RB* (Dunn et al., 1989) and *p53* (Levine et al., 1991), identified on the basis of an association between neoplasia and loss of normal function; and (3) genes involved in negatively regulating cell proliferation during differentiation (*MyoD*) or senescence (*prohibitin*) (Davis et al., 1987; Nuell et al., 1991). The mechanisms by which these genes interact with cellular regulatory processes are not yet understood and the categories established here may be simplistic. For example, *RB* functions both as a tumor supressor gene and a regulator of cell cycle traverse (Goodrich et al., 1991). While these categories of genes have not been investigated in adipocyte models, the *C/EBP* family of transcription factors has been postulated to play a role in the control of growth arrest during differentiation, as described above.

The relationship between growth arrest and differentiation has received intensive investigation in the A31T6 model. In this system, differentiation can be induced either at density arrest, or when the cells are arrested at low density while maintained in medium containing heparinised human plasma (Scott et al., 1983). The manner in which the cells are arrested proves to be an important variable in defining the relationship between proliferation and differentiation. Scott and co-workers have studied this relationship in A31T6 cells, arrested at low density, in heparinised medium containing human plasma. In order for initiation of adipogenesis, growth arrest must occur at a distinct stage in the G_1 phase of the cell cycle, called G_D (Scott et al., 1982a). G_D is distinct from other G_1 growth arrest states, including those induced by density-dependent growth inhibition, growth factor deprivation and nutrient deprivation (Wille and Scott, 1982). It has subsequently been shown that other proadipocytes, including 3T3-L1 cells, also arrest in this distinct state (Freytag, 1988). Indeed, it has been suggested that the arrest of Swiss 3T3 cells in the presence of sodium butyrate is similar to arrest at G_D, in that it predisposes the cells to respond to agents that induce terminal differentiation (Toscani et al., 1990). A31T6 cells which have arrested at the predifferentiation arrest state, G_D, but which do not yet express the adipocyte phenotype, can be induced to reinitiate DNA synthesis and traverse the cell cycle by addition of a variety of mitogens (Scott et al., 1982b). As differentiation proceeds, a progressive loss of responsiveness to growth factors is seen and increasing concentrations are required to elicit an effect (Wier and Scott, 1986; Hoerl and Scott, 1989). Failure to stimulate entry into S phase by numerous known growth factors, mitogens and de-differentiation-inducing agents, eventually results (Wier and Scott, 1986). The molecular basis for this loss of mitogenic responsiveness during the program of differentiation has not been defined. A functionally distinct protein (aproliferin) that induces transition from the non-terminal to the terminal differentiation state has, however, been identified in human plasma (Wier and Scott, 1986, 1987). In addition, a decrease in the abundance of a subset of highly conserved basic nuclear proteins has been found to correlate with the loss of proliferative potential seen in association with the process of terminal differentiation in murine mesenchymal stem cells and human keratinocytes (Minoo et al., 1989).

In contrast, investigations of density-arrested A31T6 cells may add a new dimension to deciphering this relationship between proliferation and differentiation. At density arrest, these cells respond in a manner typical of Balb/c-3T3 fibroblasts, in that combinations of mitogens are required for optimal mitogenesis (Smyth et al., 1990; Smyth et al., 1992). Responsiveness to differentiation-promoting agents also develops at confluence (Smyth and Wharton, 1992a). By 48 hours following initiation of the differentiation program, in response to specific combinations of differentiation-promoting agents, these cells become refractory to subsequent growth stimulation. This irreversible growth arrest, which we have called ‘terminal growth arrest’ (Smyth and Wharton, 1992b), precedes acquisition of the differentiated phenotype in that cytoplasmic lipid droplets are not present until after day 3 (Smyth and Wharton, 1992a). The fact that terminal growth arrest occurs prior to morphological expression of the adipogenic phenotype reflects the power of this model for analysis of negative controls on cell growth, in that changes in gene expression related to the irreversible loss of mitogenic responsiveness can be investigated independently of expression of adipocyte-specific genes. This is in contrast to the situation where A31T6 cells are arrested in plasma, when irreversible loss of proliferative potential occurs considerably later, and subsequent to full expression of the adipogenic phenotype (Scott et al., 1982b). It is also of interest that, depending on the nature of the inducer, differentiation in density-arrested A31T6 cells can occur in the absence of terminal growth arrest. For example, while exposure to the combinations of either insulin and indomethacin or insulin, dexamethasone and indomethacin causes adipogenesis and terminal growth arrest, cells exposed to dexamethasone alone or to dexamethasone in combination with indomethacin, undergo adipogenesis in the absence of terminal growth arrest (Smyth and Wharton 1992a, 1993, and unpublished observations).

An understanding of the molecular events governing the irreversible loss of proliferative potential occurring during the differentiation program will contribute to an understanding of the balance between proliferation and differentiation. Uncoupling of these two normally interdependent processes is an obligatory step in the generation of the trans-

formed phenotype, and hence of cancer (Jetten, 1989). Therefore, an understanding of the molecular coordination of differentiation and proliferation is imperative. Indeed, it has been suggested that defective negative regulation is as important as autonomous mitogenic stimulation for the completion of malignant transformation (Bohmer, 1991).

We postulate that investigation of irreversible growth arrest in adipocyte models could result in the characterization of a dominant negative inhibitor of growth, as has been found in the senescence and myoblast models. As discussed above, it has been suggested that *C/EBP*α may play a general role in establishing and maintaining the differentiated, nonproliferative state (Umek et al., 1991). We suggest that there may be multiple classes of such proteins in adipocytes, based on similarities to results obtained in the myoblast system. While there is now some information regarding nuclear proteins that accompany or regulate adipocyte differentiation, the A31T6 system specifically allows a direct evaluation of the role of the *C/EBP* family of transcription factors and other factors in terminal growth arrest as distinct from adipogenesis.

FLOW CYTOMETRY: A UNIQUE QUANTITATIVE METHOD FOR MONITORING THE PROGRESS OF THE ADIPOGENIC PROGRAM

The most obvious phenotypic manifestation of the adipogenic program is the accumulation of cytoplasmic lipid droplets. Adipogenesis has traditionally been quantified by monitoring the accumulation of these droplets by phase microscopy. Focus has recently shifted to measurement of the induction and maintenance of adipocyte-specific mRNAs. It has become clear, however, that molecular criteria alone are not necessarily appropriate markers of adipocyte differentiation. This is because a number of adipocyte-specific genes can be induced in response to treatment with individual agents that are themselves incapable of inducing accumulation of cytoplasmic lipid droplets (Toscani et al., 1990). Monitoring of lipid accumulation, therefore, in addition to measurement of adipocyte-specific gene expression, is necessary for accurate quantification of adipogenesis.

As cells undergo differentiation in a parasynchronous fashion, a wide range of differentiated phenotypes is present at any given time. Analysis of such complex heterogeneous cell populations presents a number of technical challenges. When phase microscopy is employed to quantify adipogenesis, cells are scored as adipocytes if the number of detectable droplets (often stained with Oil Red O) exceeds a threshold value (Sparks et al., 1992). As proadipocytes differentiate, however, they become spherical and mask the presence of the flatter fibroblast-like non-differentiated or early-stage differentiating cells in a high-density monolayer (as shown in Smyth and Wharton, 1992a). The presence of the latter two categories of cells is, therefore, underestimated when phase microscopy is used to monitor progress of the adipogenic program. This technique is also limited by the slow rate at which cells can be examined.

Flow cytometry, which allows rapid analysis and separation of cells based on physical, biochemical and functional properties, has proved to be a valuable tool for analysing other complex heterogeneous cell populations (Lehnert and Steinkamp, 1986). In order to overcome the problems related to accuracy, precision and speed in quantifying adipogenesis, therefore, a flow cytometric assay was developed (Smyth and Wharton, 1992a). This assay employs a multilaser/multiparameter flow cytometer (Steinkamp et al., 1991) and provides unique, precise and quantitative information on the accumulation of cytoplasmic triglyceride in individual A31T6 proadipocytes undergoing differentiation into adipocytes (Smyth and Wharton, 1992a, 1993). Cells are stained with the fluorescent dye, Nile red, which emits gold fluorescence from neutral lipids (Greenspan et al., 1985). Using this assay, the accumulation of cytoplasmic lipid has been monitored by fluorescence measurements (Smyth and Wharton, 1992a, 1993) and by perpendicular light scatter values (Smyth and Wharton, unpublished observations). Fluorescence values allow determination of the relative amount of lipid in each individual cell, in addition to measurement of the rate of differentiation. The ability to measure the increase in cytoplasmic lipid in cells already committed to the differentiation program, defined as ‘maturation’ (Smyth and Wharton, 1993), is proving to be a particularly powerful aspect of this assay, allowing investigation of classes of adipogenic agents that selectively and independently affect both the rate and extent of differentiation. Perpendicular light scatter, which is proportional to the diffractive, reflective and refractive properties of both internal and external cellular components (Salzman et al., 1991), is proving to be valuable in analysing not only the presence of cytoplasmic lipid droplets but also the number, size, and distribution of droplets within the cell (Smyth and Wharton, unpublished observations). Investigations involving measurement of perpendicular light scatter values provide the additional advantage of avoiding use of stain, thereby facilitating re-plating of the cells for subsequent experiments. Flow cytometry has recently been employed to monitor adipogenesis in murine stromal cell lines, based on fluorescence and light scatter parameters (Dorheim et al. 1993).

Flow cytometry has also allowed investigation of adipogenesis on a biochemical level, by determination of the expression of various polypeptides or their receptors. This has advantages over traditional measurements of the induction of lipogenic enzymes at the RNA or protein level, where the results reflect only the characteristics of the average cell in the population and provide no indication of the degree of heterogeneity. A flow cytometric immunofluorescence procedure utilizing a specific antibody to rat adipose tissue lipoprotein lipase (LPL), for example, allows determination of the relative abundance of precursor cells in adipose tissue regions from the female rat and evaluation of the importance of ovarian factors in influencing regional differences in precursor cell development (Krakower et al., 1988). Wright (1992) established conditions for utilizing monoclonal antibodies and fluorescence-activated flow cytometry to study expression of an adipocyte-specific cell surface antigen in primary porcine stromal-vascular cells cultures under various conditions.

In addition to monitoring adipogenesis on the basis of the accumulation of lipid droplets in individual cells or by

biochemical criteria, flow cytometry also allows isolation of homogeneous populations of cells by sorting. Cells with desired properties are isolated from other cells in the population with high statistical precision, resulting in highly purified populations of cells that can then be investigated under controlled conditions. Flow sorting is, therefore, a valuable and versatile aid to studying many aspects of adipocyte differentiation.

In conclusion, we suggest that use of flow cytometry will enable significant contributions to studies of adipogenesis to be made, with the range of applications expanding as both the instrumentation and the cytochemical staining techniques are advanced. While it might be premature to suggest that this technology will prove valuable for investigations of internal antigens, it should now be possible to correlate expression of surface antigens or receptors with extent of accumulation of cytoplasmic lipid, adding a new dimension to investigations of adipogenesis. In addition, measurement of increases or decreases in the amount of lipid in a cell in response to various agents has implications for investigations of adipogenesis in vitro and also for the study of adipose tissue biology and metabolic disorders.

PERSPECTIVES

There is every reason to expect that cell culture models of adipogenesis will not only continue to provide important insights into the nature of the adipogenic process but will also add a new dimension to investigations of positive and negative regulation of cell proliferation. It is expected that investigations involving adipocyte models will lead to a fundamental understanding of the irreversible loss of proliferative potential that is an integral component of the terminally differentiated state. One of the challenges for the future is to define the molecular events involved in irreversible loss of proliferative potential during the differentiation program. By virtue of the ability to uncouple terminal growth arrest from the phenomenon of adipogenesis, adipocyte models will make significant contributions to unravelling the issue of negative control of cell proliferation. With the application of a range of recently advanced molecular and technical approaches, it is anticipated that proadipocyte cell lines will, indeed, prove to be powerful models of cellular proliferation and differentiation.

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