Down-regulation of Platelet-derived Growth Factor Receptor Expression during Terminal Differentiation of 3T3-L1 Pre-adipocyte Fibroblasts*

(Received for publication, January 22, 1996, and in revised form, March 26, 1996)

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The transcription and expression of platelet-derived growth factor (PDGF) receptors (PDGFRs) is down-regulated as a consequence of entry into the replicative cell cycle (Vaziri, C., and Faller, D. V. (1995) Mol. Cell. Biol. 15, 1244–1253). In this study, we have investigated the expression of PDGFRs during terminal differentiation, a process in which cells exit from the cell cycle. When treated with appropriate hormonal stimuli, 3T3-L1 fibroblasts initiate a differentiation program resulting in conversion to lipid-accumulating, adipocyte-like cells. Pre-adipocytes express amounts of PDGFRα and PDGFRβ mRNA and protein that are similar to levels expressed in other murine 3T fibroblasts. In contrast, the expression of both α and β receptor transcripts is greatly reduced in differentiated 3T3-L1 cells. The loss of PDGFR mRNA following induction of differentiation precedes morphological conversion as well as the induction of many adipocyte-specific genes. The amounts of cell surface PDGFR protein diminish in parallel with the mRNA levels during differentiation, as shown by Western blotting and PDGF-binding assays. The reduced expression of PDGFRs does not reflect a general down-regulation of growth factor receptors, as expression of the type 1 FGFR is unaffected by terminal differentiation. The PDGFRβ promoter drives strong expression of a luciferase reporter gene in pre-adipocytes, but not in differentiated cells, indicating that the decrease in PDGFR expression following induction of differentiation is a transcriptionally regulated event. Decreased PDGFR expression in differentiated cells is associated with impaired biological responsiveness to PDGF, as shown by reduced activation of mitogen-activated protein-kinase following PDGF stimulation, and decreased chemotactic responsiveness to PDGF. Our data suggest that PDGFR down-regulation is an important mechanism for reducing PDGF-responsiveness in terminally differentiated 3T3-L1 cells.

Platelet-derived growth factor (PDGF)* is a potent mitogen

* This work was supported in part by National Institutes of Health, NCI Grant CA50459, and by a grant from the Council for Tobacco Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a Senior Postdoctoral Fellowship from the American Cancer Society, Massachusetts Division, Inc.

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The abbreviations use are: PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; DMEM, Dulbecco's modified Eagle's medium; MAP, mitogen-activated protein; MBP, myelin basic protein; CAT, chloramphenicol acetyltransferase; C/EBPα, CCAAT/enhancer-binding protein α; PPARγ, peroxisome proliferator-activated receptor γ; RSV, Rous sarcoma virus; FGF, fibroblast growth factor; FGFR, FGF receptor; GPD, glycophosphate dehydrogenase.
cellular expression of PDGFRs. Such regulatory mechanisms are likely to be important determinants of a cell’s ability to respond to PDGF. We previously demonstrated transcriptional down-regulation of the PDGFR expression following stimulation of Balb/c-3T3 fibroblasts with specific combinations of mitogens, or as a result of transformation with viral oncopogens (11). These experiments provided a molecular basis for previous observations that proliferating cells are less responsive to the chemotactic actions of PDGF (12) and established that expression of the PDGFR is subject to transcriptional regulation in response to a variety of growth-promoting agents. Moreover, these data suggest that regulated changes in the levels of the PDGFR may provide an important mechanism for modulating cellular responsiveness to the actions of PDGF during the course of the cell cycle. In this work, we have identified another phase of the cellular life cycle, terminal differentiation, in which regulated changes in the expression of PDGFRs and concomitant modulation of PDGF sensitivity, may have important biological consequences.

The adipogenic 3T3-L1 cell line, originally isolated as a lipid-accumulating subclone of mouse 3T3 fibroblasts (13), has provided a valuable model system for the study of adipoblast differentiation. During exponential growth, 3T3-L1 cells display properties that are typical of other murine 3T3 fibroblast lines. However, upon attaining confluence, 3T3-L1 fibroblasts tend to differentiate into adipocyte-like cells. Treatment with appropriate stimuli (a combination of insulin, methylisobutylxanthine, and dexamethasone) results in dramatic potentiation and acceleration of the adipose-conversion process (14). Differentiation of 3T3-L1 fibroblasts is associated with activation of many adipose-specific genes, including those encoding adipose-specific transcription factors and lipid-metabolizing enzymes (15, 16).

Continued proliferation and terminal differentiation are considered to be mutually exclusive events in 3T3-L1 fibroblasts, as well as in other cell lines that undergo differentiation. Indeed, activation of mitogenic signaling pathways, for example by ectopic expression of oncogenes (17) or treatment with tumor promoters (18), can prevent differentiation. Conversely, terminally differentiated cells do not proliferate and are insensitive to mitogens. Therefore, it is likely that specific mechanisms exist to maintain the differentiated phenotype and restrict proliferation of terminally differentiated cells. Putative blocks to cellular proliferation could occur at several stages of the mitogenic signaling cascade ordinarily initiated by activated growth factor receptors. We hypothesized that down-regulation of growth factor receptors might be one mechanism whereby differentiating cells become desensitized to the biological action of growth factors. Accordingly, we have investigated the expression of PDGFRs during differentiation of adipogenic 3T3-L1 cells.

MATERIALS AND METHODS

Cells and Culture—3T3-L1 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown at 37 °C in Dulbecco modified Eagle’s medium (DMEM) containing 10% heat-inactivated donor calf serum supplemented with glutamine and penicillin-streptomycin. To initiate adipocyte differentiation, 2-day post-confluent cultures were fed with fresh DMEM containing 10% serum plus a combination of insulin, methylisobutylxanthine (Sigma), 1 μM dexamethasone (Sigma), and 10 μg/ml insulin (Sigma), hereafter referred to as MDI treatment. After 2 days, the MDI-containing medium was removed and replaced with fresh DMEM containing 10% serum. 5–7 days after MDI treatment, 50–80% of the cells had assumed an adipocyte-like appearance.

RNA isolation and Northern (RNA) blot analysis were carried out as described previously (11). cDNA probes for PDGFRs and the FGFR were obtained from previously described sources (11). cDNA probes for murine GPD and p27 were provided by Claire Steppan and Deb Dobson, and by Randy Poon and Tony Hunter, respectively. The p21 probe was amplified from mouse genomic DNA by polymerase chain reaction as described previously (19).

PDGF-BB-binding assays, preparation of cell membranes, and immunoblot detection of PDGFR expression were carried out as described previously (11).

Assay of Mitogen-activated Protein Kinase (MAP Kinase) Activity—Confluent monolayers of control and differentiated 3T3-L1 cells in 10-cm culture dishes were placed in 0.5% serum-containing medium. After 5 h of serum starvation, different doses of PDGF were added directly to the medium. After 5 min of PDGF treatment, the monolayers were rinsed with phosphate-buffered saline and lysed by scraping into 10 mM Tris (pH 7.5), 0.5% Triton X-100, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM benzamidine. Lysates were centrifuged at 10,000 × g to pellet nuclei. Supernatants were removed and stored at −70 °C for up to 3 days prior to assay of MAP kinase activity. To assay myelin basic protein (MBP)-kinase activity, 50 μg of cell extracts were incubated for 20 min at 37 °C in buffer containing 20 mM Hepes (pH 7.4), 10 mM MgCl2, 100 μM ATP containing 2 μCi of [γ-32P]ATP, 2 mM EGTA, 1 mM dithiorthreitol, 100 μM okadaic acid, 100 μM Na3VO4, and 1 μM of myelin-basic protein (Sigma). Reaction mixes were then spotted onto P81 filters (Whatman). Filters were washed in 50 mM phosphoric acid and dried with ethanol. Radioactively labeled MBP bound to the washed filters was determined by scintillation counting.

Chemotaxis Assay—2-day postconfluent 3T3-L1 cells were fed with fresh medium containing 10% serum with or without MDI. After 2 days, control and differentiating cultures of cells were washed with DMEM, trypsinized, and resuspended at 100,000 cells/ml in medium containing 0.5% serum. 100-μl aliquots of cell suspensions were placed in the upper wells of Transwell chambers (Costar). Each lower well contained 600 μl of medium containing 0.5% serum and varying concentrations of PDGF-BB. After 4 h at 37 °C, the medium was removed from each upper well. The upper surface of each membrane was washed firmly with a cotton wool tip, rinsed with phosphate-buffered saline, and wiped again to remove nonmigrating cells. The membranes were fixed in methanol, and cells that had migrated through the membrane were stained with 1% crystal violet. Each filter was cut out of the Transwell device and mounted on a microscope slide. Stained cells were counted under a microscope.

Transient Transfections—Monolayers of undifferentiated and MDI-treated differentiating 3T3-L1 cells were transfected by calcium phosphate co-precipitation of plasmid DNA (10 μg) and carrier salmon sperm DNA (20 μg) for 15 h. After removal of the transfection medium, cells were given fresh DMEM containing 10% serum for a further 8 h, prior to harvesting cell extracts for assay of reporter gene activities. Transfected monolayers were washed with phosphate-buffered saline, detached by scraping into 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and pelleted by centrifugation at 10,000 × g for 10 s. Cell pellets were lysed by freeze-thawing, or by detergent lysis, to obtain extracts for assay of chloramphenicol acetyltransferase (CAT) or luciferase activities, respectively. Cell lysates were normalized for protein content prior to reporter gene assays. To assay CAT activity, extracts were incubated with [3H]chloramphenicol and acetyl-CoA. Acetylated [3H]chloramphenicol reaction products were separated by thin layer chromatography on silica gel plates and detected by autoradiography according to standard procedures. Luciferase activity was assayed using a luciferase kit, as described by the manufacturer (Promega).

The following plasmid DNAs were used for transfections: pCAT, a promoterless CAT vector; RSV-CAT, a plasmid containing Rous sarcoma virus promoter elements upstream of the CAT gene; pGL2b, a promoterless luciferase vector; and pGL2KB1, pGL2b containing 1.4 kilobase pairs of PDGFR promoter sequence inserted upstream of the luciferase reporter gene.

RESULTS

Expression of PDGFR mRNA Is Specifically Reduced following Differentiation of 3T3-L1 Cells—3-day postconfluent 3T3-L1 cells were induced to differentiate by treatment with MDI. After 6 days, greater than 70% of the MDI-treated cells had assumed an adipocyte-like morphology. We performed RNA blot analysis using RNA samples from differentiated cells in parallel with RNA from control cells that were not treated with MDI. As shown in Fig. 1, the control, pre-adipocyte 3T3-L1 cells expressed levels of PDGFR mRNA (lanes 1 and 3)
that were similar to those expressed in Balb/c-3T3 fibroblasts (lane 5). However, PDGF\(\beta\)R mRNA was barely detectable in samples of total RNA from the MDI-treated cells (lanes 2 and 4). By contrast with 3T3-L1 cells, Balb/c-3T3 fibroblasts are less prone to adipose conversion upon attaining confluence. Although MDI treatment of Balb/c-3T3 cells occasionally resulted in slight decreases in PDGF\(\beta\)R mRNA (Fig. 1, lane 6), induction of differentiation in 3T3-L1 cells was always accompanied by large decreases in PDGF\(\beta\)R mRNA levels.

The same RNA samples were probed for expression of the PDGF\(\alpha\)R (Fig. 1B). As with other murine fibroblast lines, 3T3-L1 cells express only low levels of the PDGF\(\alpha\)R (accounting for only 20% of the total cell surface PDGFR population). The PDGF\(\alpha\)R cDNA probe detected low levels of PDGF\(\alpha\)R transcripts in RNA samples from Balb/c-3T3 fibroblasts (Fig. 1B, lane 5) and 3T3-L1 pre-adipocytes (Fig. 1B, lanes 1 and 3), but not in samples from the differentiated cells (Fig. 1B, lanes 2 and 4). To determine whether the reduced levels of PDGF\(\beta\)R mRNA in differentiated adipocytes reflected a general decrease in expression of growth factor receptors, we probed the RNA samples from control and MDI-treated cells for expression of the type 1 FGFR (Fig. 1C). MDI treatment resulted in a slight decrease in FGFR expression in some experiments (e.g., Fig. 1C, lane 2). However, the differentiated adipocytes still expressed high amounts of FGFR mRNA (Fig. 1C, lanes 2 and 4), that were usually comparable to levels seen in pre-adipocytes (Fig. 1C, lanes 1 and 3) and Balb/c-3T3 cells (Fig. 1C, lane 5). Transcripts for the adipocyte differentiation marker GPD were detected in RNA samples from the MDI-treated 3T3-L1 cells (Fig. 1D, lanes 2 and 4), but not in samples from pre-adipocytes (Fig. 1D, lanes 1 and 3) or Balb/c-3T3 cells (Fig. 1D, lanes 5 and 6), confirming that specific hormonal induction of adipose conversion had occurred in the 3T3-L1 cells. Each of the components of the MDI differentiation mixture used in the above experiments (methylisobutylxanthine, dexamethasone, and insulin) are known to effect gene expression in fibroblasts, yet none are unable to induce differentiation individually. We tested the individual and combined effects of methylisobutylxanthine, dexamethasone, and insulin on PDGF\(\beta\)R expression in 3T3-L1 cells. As shown in Fig. 2, stimulation with the MDI mixture resulted in large decreases in PDGF\(\beta\)R mRNA expression (lane 3). However, when used individually, methylisobutylxanthine, dexamethasone, and insulin failed to elicit a similar repression of PDGF\(\beta\)R expression. In some experiments methylisobutylxanthine alone induced slight decreases in PDGF\(\beta\)R levels (see, for example, Fig. 2, lane 5); however, maximal repression was always induced in the presence of dexamethasone and insulin (Fig. 2, lane 3). These data establish a good correlation between down-regulation of PDGF\(\beta\)R mRNA levels and the terminal differentiation process. To confirm that the reduced levels of PDGF\(\beta\)R mRNA following MDI treatment resulted in decreased expression of PDGF\(\beta\)R protein, membrane preparations from control and MDI-treated 3T3-L1 cells were subjected to SDS-PAGE. Following transfer of the separated proteins to nitrocellulose, antiserum to the PDGF\(\beta\)R detected a 180-kDa band (corresponding to the expected apparent molecular mass of the mature PDGFR) in membranes from control pre-adipocytes (Fig. 3A, lane 1), but not from MDI-treated (Fig. 3A, lane 2) fibroblasts. To more accurately quantify the reduction in the number of PDGFRs following adipose conversion, we performed ligand binding studies, using iodinated PDGF-BB. As shown in Fig. 3B, MDI treatment resulted in large decreases in the PDGF-binding capacity of 3T3-L1 cells. Scatchard analysis (not shown) of these data showed that pre-adipocytes bound approximately 158,000 PDGF-BB molecules/cell. This number is comparable to the number of PDGF-binding sites on other murine fibroblast cell lines (11). However, MDI-treated 3T3-L1 cells possessed only approximately 27,000 PDGF-binding sites/cell. Since PDGF-BB binds to both PDGFR isotypes, the decreased binding is likely to reflect loss of functional PDGF\(\alpha\)Rs and PDGF\(\beta\)Rs. Kinetic studies were carried out to determine the rate of loss of PDGF\(\beta\)R. As shown in Fig. 4A, decreases in levels of
Fig. 3. Changes in cell surface expression of PDGFRs after differentiation of 3T3-L1 cells. A, Western blot showing reduced expression of PDGFR in membranes from MDI-treated cells. Cultures of fibroblasts were differentiated as described in the legend to Fig. 1. 100-μg samples of membrane protein preparations from control (lane 1) and MDI-treated (lane 2) cells were separated on a 7.5% polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody to the extracellular region of the PDGF βR. B, PDGF-BB binding to control and MDI-treated cultures of 3T3-L1 fibroblasts. PDGF-BB binding to control (C, -MDI) and differentiated (○, + MDI) cultures of 3T3-L1 fibroblasts was determined as described under "Materials and Methods."

PDGF βR mRNA were evident as early as 3 h after MDI treatment. The PDGF R mRNA was down-regulated with a \( t_{1/2} \) of approximately 6 h and was almost completely undetectable after 9 h of MDI treatment (Fig. 4A). The rate of decay of the PDGF βR mRNA was similar to the \( t_{1/2} \) of the PDGF βR message in fibroblasts observed following inhibition of RNA polymerase II-dependent transcription with actinomycin D (Fig. 4B). Thus, down-regulation of PDGF R expression is an early event in 3T3-L1 adipocyte differentiation.

Recent reports described increased expression of the cyclin-dependent kinase inhibitor, p21/cip/waf, during terminal differentiation of myogenic cells (20, 21), and induction of p21 expression was suggested to be a major mechanism for arresting the cell cycle machinery following terminal differentiation of myoblasts. To investigate cyclin-dependent kinase inhibitor induction in differentiating 3T3-L1 cells and its temporal relationship to PDGF R down-regulation, we examined the expression of p21/cip/waf, as well as that of the related gene p27kip (hereafter referred to as p21 and p27, respectively), during the course of adipose conversion. As shown in Fig. 5B, p21 mRNA expression was slightly repressed in differentiating adipoblasts after 2 days of MDI treatment and returned to levels similar to those present in postconfluent pre-adipocytes by day 4 of MDI treatment. In contrast, p27 mRNA was undetectable in samples of total RNA from postconfluent 3T3-L1 pre-adipocytes, but was induced severalfold after 2-4 days of MDI treatment (Fig. 5C). Therefore, induction of the cyclin-dependent kinase inhibitor p27 does occur during the 3T3-L1 differentiation program, but is a relatively late event that is preceded by large decreases in PDGF R expression (Fig. 5A).

Mechanism of Repression of PDGF R Expression in Differentiated 3T3-L1 Cells—In order to study the mechanism of regulation of PDGF βR expression, we have obtained genomic sequences upstream of the 5′-untranslated region of the murine PDGF βR gene. Sequences from the 5′-flanking region of the PDGF βR gene contain consensus sites for transcription factor binding and drive expression of reporter genes in a manner that is similar to the regulated expression of the endogenous PDGF R mRNA (data not shown). These studies are described in more detail elsewhere. The plasmid we have designated pGL2K81 contains 1.4 kilobase pairs of PDGF βR promoter sequence inserted upstream of a luciferase reporter gene. pGL2K81 was transfected into control postconfluent 3T3-L1 fibroblasts, or into parallel cultures of cells that had been induced to differentiate with MDI. As shown in Fig. 6A, the PDGF βR promoter drove luciferase expression in the pre-adipocytes, but not in 3T3-L1 cells undergoing differentiation. A control vector, pGL2b (lacking the PDGF βR promoter sequences), failed to promote significant expression of the luciferase gene in the pre-adipocytes, or in the differentiated adipose cells. The decreased luciferase activity in extracts from the MDI-treated pGL2K81 transfectants specifically reflected decreased activity of the PDGF βR promoter, since a plasmid containing Rous sarcoma virus (RSV) promoter elements linked to a CAT reporter gene drove strong expression of CAT activity, in both the control and differentiating cells. In fact, we routinely observed slightly stronger expression of CAT activity in extracts from the MDI-treated RSV-CAT transfectants undergoing differentiation. This appears to result from the gen-

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eral increase in transcriptional and translational activity in differentiating cells.

We have been unable to determine whether the decreased expression of the PDGFαR during differentiation results from transcriptional down-regulation, since we fail to detect strong signals from the PDGFαR transcript using nuclear run-off assays. This reflects the relatively low constitutive expression of the PDGFαR gene in murine 3T3 fibroblasts. However, studies are underway using PDGFαR promoter reporter constructs to address this question.

PDGFαR-mediated Signal Transduction Is Impaired in Differentiated Adipocytes—It is commonly observed that higher doses of ligand are required to attain maximum receptor occupancy than are necessary to elicit full biological responsiveness to the ligand. That is, cells often possess receptors in excess of what is required to achieve maximum activation of a particular signaling pathway. Therefore, we considered it important to determine whether the decreased expression of PDGFαRs during 3T3-L1 differentiation merely reflected loss of redundant PDGFαRs, or indeed resulted in sufficient down-regulation of PDGFαRs to significantly desensitize cellular responses to PDGF.

Since differentiated 3T3-L1 cells expressed fewer PDGFαRs than pre-adipocytes, higher doses of PDGF were required to achieve the same degree of receptor occupancy. As illustrated in Fig. 3B, 3 ng/ml PDGF was required for MDI-treated cells to attain the same receptor occupancy elicited by 0.8 ng/ml ligand in control cultures. This suggested that higher concentrations of PDGF might be necessary to elicit a given PDGF-dependent signaling activity in MDI-treated cells than in pre-adipocytes. To test this, PDGF-stimulated MAP kinase activity was assayed in control and MDI-treated fibroblasts. PDGF activation is thought to activate MAP kinase activity via a phosphorylation cascade involving sequential activation of c-ras, c-raf, MAP kinase kinase, and MAP kinase. In assays containing Ca2+ chelators, MBP phosphorylation can be used to quantify MAP kinase activity. As shown in Fig. 7, PDGF stimulation resulted in a dose-dependent appearance of MBP kinase activity in extracts from postconfluent 3T3-L1 fibroblasts. Half-maximal stimulation of MBP kinase activity occurred at 0.3–1 ng/ml, and maximal stimulation was attained at 3 ng/ml. In MDI-treated cells expressing at least 5-fold fewer PDGFαRs than MDI-untreated cells, stimulation required 3 ng/ml ligand, with maximal stimulation attained at 3 ng/ml. In MDI-treated cells expressing at least 5-fold fewer PDGFαRs than MDI-untreated cells, stimulation required 3 ng/ml ligand, with maximal stimulation attained at 3 ng/ml. In MDI-treated cells expressing at least 5-fold fewer PDGFαRs than MDI-untreated cells, stimulation required 3 ng/ml ligand, with maximal stimulation attained at 3 ng/ml. In MDI-treated cells expressing at least 5-fold fewer PDGFαRs than MDI-untreated cells, stimulation required 3 ng/ml ligand, with maximal stimulation attained at 3 ng/ml.

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reason for the decreased sensitivity of differentiated adipocytes to PDGF-stimulated MAP kinase activity is the large decrease in PDGFR expression.

PDGF is the only fibroblast mitogen known to possess chemotactic factor activity (9), and chemotactic responses to PDGF are mediated only by the PDGFR (22). Terminally differentiated 3T3-L1 adipocytes are known to be insensitive to the mitogenic actions of growth factors, including PDGF. Chemotactic assays were carried out to determine whether PDGFR down-regulation during the course of 3T3-L1 adipoblast differentiation was also associated with decreased migratory responsiveness toward PDGF. As shown in Fig. 8, pre-adipocytes showed a strong chemotactic response to PDGF-BB. Maximal migration was attained at 5–10 ng/ml of PDGF-BB. As noted previously (9), high concentrations of PDGF (>10 ng/ml) resulted in reduced chemotaxis, due to more rapid dissipation of the PDGF concentration gradient toward which the cells migrate. In contrast to pre-adipocytes, 3T3-L1 cells undergoing differentiation displayed markedly reduced chemotactic responsiveness to PDGF. At maximally effective doses of 5–10 ng/ml of PDGF-BB, the chemotactic responsiveness of MDI-treated cells was reduced by greater than 70%. As discussed above, mechanisms other than PDGFR down-regulation may contribute to the decreased sensitivity of differentiating cells to PDGF-BB. Nevertheless, our data show a good correlation between loss of PDGFRs and reduced biological responsiveness to PDGF.

DISCUSSION

We have demonstrated large decreases in the expression of PDGFR mRNA and cell surface protein levels, following hormonal induction of differentiation in 3T3-L1 fibroblasts. With the exception of ectopically expressed transforming oncogenes (11), no agents are known to cause such dramatic changes in the transcription of the PDGFR in fibroblasts. The specific changes in PDGFR expression that accompany 3T3-L1 differentiation differ from oncogene-induced changes, however; transforming oncogenes result in down-regulated expression of the PDGFR, but not the PDGFR. By contrast, expression of both the PDGFR and the PDGFR decreased during adipocyte differentiation.

We have shown greatly reduced activity of the PDGFR gene promoter in differentiating cells. It is likely that the reduced transcription of the PDGFR gene occurs very rapidly following induction of differentiation, and that the decrease in PDGFR mRNA levels reflects decay of preexisting message, as PDGFR transcripts decline to very low levels by 9 h following the onset of adipocyte differentiation. This rapid loss of PDGFR expression is noteworthy in that it precedes most other transcriptional changes known to occur during adipocyte differentiation. For example, expression of the adipocyte-specific transcription factors PPARγ and C/EBPα is detectable no sooner than 2–3 days post MDI treatment and peaks as late as days 5–9 after initiation of differentiation (15, 16). Similarly, adipocyte morphology is not acquired until after 6–7 days of the initial MDI treatment. Thus, loss of PDGFR expression is a very early event in the 3T3-L1 differentiation program, and may precede commitment to differentiation.

Although both the α and β PDGFR isotypes are down-regulated following induction of adipocyte differentiation, this does not appear to reflect a general decrease in expression of growth factor receptors. Expression of the FGFR mRNA is not significantly changed, and the number of epidermal growth factor receptors are also reported to remain constant following differentiation (23). Insulin receptor expression is reported to increase following adipocyte differentiation, but this is a relatively late event; the number of insulin-binding sites doubles after day 4 of the differentiation program (24). Such changes in receptor expression are likely to reflect changes in growth factor requirements and/or responsiveness following differentiation, and may be essential for the new physiological role of the differentiated cell. For example, increased expression of insulin receptors correlates with increased sensitivity of hexose transporters to insulin (25) in adipocytes, reflecting the role of adipocytes as a regulator of energy stores. It is likely to be of physiological significance that mature adipocytes exhibit reduced numbers of PDGFRs relative to precursor cells; decreased sensitivity to the actions of PDGF (mitogenesis, che-
motaxis, survival) may be of importance for the biological function of mature adipocytes.

Induction of signals leading to proliferation is an important function of PDGF. Proliferation and differentiation are thought to be mutually exclusive events. Indeed, proliferative agents are frequently observed to prevent terminal differentiation. For example, PDGF stimulates proliferation and inhibits differentiation of 3T3-L1 cells (26) and smooth muscle cells (27). Likewise, PDGF is a potent mitogen for myogenic C2C12 cells and inhibits their differentiation into myotubes (28). Similarly, tumor promoters with mitogenic activity, such as 12-O-tetradecanoylphorbol-13-acetate (18) and the indole alkaloid dihydroteichoicvin B (23) inhibit adipose conversion of 3T3 fibroblasts. Thus, the balance of expression of genes that are required for proliferation or differentiation is likely to govern a cell’s decision to proliferate or instead to undergo growth arrest and terminal differentiation. For example, overexpression of c-myc can prevent differentiation of 3T3-L1 cells (17), and this block may be overcome by ectopic overexpression of the fat-specific transcription factor C/EBPα (29). Since the ligand-activated PDGFR transduces important proliferative signals, such as induction of the c-myc proto-oncogene (30), it is possible that loss of PDGFRs contributes to reduced sensitivity of the differentiating cells to mitogenic effects of PDGF. Desensitization to PDGF may be important in enabling continuation of the differentiation program in the face of local production of growth factor, since signals transduced by the PDGFR are known to inhibit terminal differentiation in 3T3-L1 fibroblasts as well as other cell lines (26–28). Down-regulation of mitogen receptors following induction of differentiation may be a more general phenomenon, and does indeed occur in cells other than pre-adipocytes. For example, Jin et al. (31) noted decreased expression of PDGFRs following differentiation of rat myoblasts, and NGF-induced differentiation of PC12 cells is accompanied by down-regulation of cell surface receptors for epidermal growth factor (32), which is a mitogenic factor for this cell line.

Terminal growth arrest has been shown to be associated with induction of the cyclin-dependent kinase inhibitor p21 (20, 21). Induction of p21 is likely to be important in arresting the cell cycle apparatus, and may contribute to maintaining the growth-arrested state following terminal differentiation. We did not observe large increases in p21 expression following induction of the differentiation program in 3T3-L1 cells, yet increased expression of the related cyclin-dependent kinase inhibitor p27 did occur after adipocyte conversion. This was a late event, however, and was preceded by transcriptional down-regulation of the PDGFR. These results suggest that loss of PDGFR expression may be important in preventing activation of mitogenic signaling pathways after the onset of a differentiation program, and may, in conjunction with other biochemical events such as induction of cyclin-dependent kinase inhibitors, contribute to maintaining an effective block to cellular proliferation. Clearly, however, loss of PDGFRs does not provide the sole block to mitogenesis following differentiation; we have been unable to induce proliferation of differentiated 3T3-L1 adipocytes in response to FGF, although these cells continue to express the PDGFR (this report).

Inappropriate expression of genes that promote proliferation over differentiation may cause neoplasia. For example, the cyclin-dependent kinase inhibitor p16 is deleted in certain human tumors (33) and may contribute to the malignant state. If, as is suggested by our data, loss of PDGFRs is important for the terminally differentiated phenotype, it is possible that failure of the PDGFR to down-regulate may result in increased propensity for malignancy. Since tumorigenesis appears to be a multistep process, failure of the PDGFR to down-regulate may, in conjunction with other lesions, contribute to human malignancy. This idea is consistent with numerous reports showing inappropriate or overexpression of PDGFRs (frequently co-expressed with PDGF ligand) in human tumor cells (for reviews, see Refs. 34 and 35, and references therein).

Knowledge of the proteins and DNA sequences involved in down-regulating the PDGFR gene during adipose conversion should further our understanding of transcriptional changes that occur during terminal differentiation. The promoter-reporter gene studies described herein localize at least one such differentiation-responsive cis-acting element of the PDGFβR gene promoter to within 1.4 kilobase pairs of the transcriptional start site. Further analysis of the promoter region of the PDGFβR gene is underway.

Acknowledgments—We thank Claire Stepan and Deb Dobson for the GFP cDNA probe. We are also grateful to Randy Pooy and Tony Hunter for providing the murine p27 cDNA.

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