Changes in the expression of cyclin dependent kinase inhibitors during differentiation of immortalized fibroblasts into adipocytes

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ABSTRACT The mechanisms implicated in the differentiation of fibroblastic precursors into adipocytes can be analyzed in vitro using cell models, such as the 3T3-L1 cell line. Since cell differentiation involves an exit from the cell cycle, it is likely that molecules that inhibit proliferation participate in the control of adipogenesis. This study was aimed at determining the role, if any, of several cyclin-dependent kinase (CDK)-inhibitors and the transcription factor C/EBPα in the process of adipocyte differentiation. We analyzed by Western blot the expression of distinct cyclin-dependent kinase (CDK)-inhibitors and C/EBPα during various stages of differentiation of 3T3-L1 cells to adipocytes. We observed specific changes in the expression of CDK inhibitors and C/EBPα during the various phases of adipogenesis. Levels of p15INK4B were maximal in confluent cells prior to the induction of differentiation and minimal in differentiated cells. Maximal levels of p16INK4A were detected following 48 h of differentiation treatment. Highest levels of p18INK4C were measured during the phase of cell confluence prior to treatment and in differentiated cells. p21CIP1 was expressed during the exponential growth phase, during exit from clonal expansion, and in differentiated cells, while p27KIP1 was found above all in confluent and differentiated cells. The present results support the participation of CDK-inhibitors in the process of in vitro adipogenesis. Specifically, the proteins p18INK4C, p21CIP1 and p27KIP1 seem to play an outstanding role in the maintenance of the differentiated state of adipocytes. Understanding the molecular mechanisms involved in adipocyte differentiation will presumably facilitate the design of new drugs aimed at novel therapeutic targets.

KEY WORDS: adipocyte, cell culture, CDK inhibitors, p18INK4C, p21CIP1, p27KIP1, C/EBP alpha

Immortalized fibroblast cell lines with the potential to differentiate into adipocytes have allowed the dissection of different stages of the process and offered the possibility of determining the relevant molecular players, which dynamically interact in a sequential cascade. The 3T3-L1 cell line is a well-established preadipose cell line that, under appropriate conditions, can acquire an adipocyte-like phenotype (Green and Meuth, 1974, Green and Kehinde, 1975, Armani et al., 2010, Ruiz-Ojeda et al., 2016). Taking into account that terminal differentiation of most cell lineages involves withdrawal from the cell cycle, it is reasonable to suppose that some of the molecules involved in the control of cell proliferation may well play a role in the process of adipocyte differentiation.

Two families of transcription factors largely control adipocyte differentiation: CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) (Morrison and Farmer, 2000, Spiegelman et al., 2000). Ectopic expression of various C/EBPs has been shown to convert fibroblast cell lines into fully differentiated adipocytes (Lane et al., 1999), whereas C/EBP gene knockouts block adipocyte differentiation (Wang et al., 1995). These transcription factors act in a sequential cascade; the expression of C/EBPα and C/EBPβ induces the expression of PPARγ, which in turn induces C/EBPα expression. The latter slightly precedes the induction of most of the end product genes of fat cells. Although a relationship between adipocyte transcription factors and cell cycle control proteins is plausible, this has not yet been shown.

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Abbreviations used in this paper: CDK, cyclin-dependent kinase; CKi, cyclin-dependent kinase inhibitor; DM, differentiation medium.

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Cyclin-dependent Kinase Inhibitors (CKIs) represent one of the main blocks for the progression of the cell cycle (Vidal and Koff, 2000, Sherr and Roberts, 1999). Several molecules of this kind have been described and are classified into two groups, known as the Ink4 and Cip/Kip families. Proteins in the Ink4 family p16\(^{INK4A}\), p15\(^{INK4B}\), p18\(^{INK4C}\) and p19\(^{INK4D}\) inhibit cyclin D-associated kinase activity. Proteins constituting the Cip/Kip family p21\(^{CIP1}\), p27\(^{KIP1}\) and p57\(^{KIP2}\) block Cdk2-Cyclin E complexes and can also inhibit Cdk4-Cyclin D activity under certain circumstances but their action in this context seems more complicated since they are also required for the proper formation of Cdk-cyclin complexes (Cheng et al., 1999).

Post-transcriptional mechanisms involving the 26S proteasome regulate the cyclin-dependent kinase inhibitors (CKIs), p21\(^{CIP1}\) and p27\(^{KIP1}\) during preadipocyte proliferation. A phytochemical agent, Helenalin, inhibits ubiquitination and degradation of p21 and p27 during S phase progression and induces G1 arrest of synchronously replicating 3T3-L1 preadipocytes. It also increases p21 protein accumulation in both density-arrested and proliferating preadipocytes (Fernandes et al., 2008). Skp2 is necessary for p27 degradation and cell cycle progression during adipocyte differentiation. Another phytochemical agent, Curcumin, blocked Skp2 protein accumulation during early adipocyte hyperplasia, inducing p27 protein accumulation and G1 arrest of synchronously replicating 3T3-L1 preadipocytes (Ferguson et al., 2016).

Protein p27 was present in the cytosol and nucleus in density-arrested preadipocytes and decreased as preadipocytes synchronously re-entered the cell cycle during early phases of adipocyte differentiation. p27 is ubiquitylated and targeted for degradation in the 26S proteasome by distinct ubiquitin E3 ligases localized to the cytosol and nucleus during adipocyte hyperplasia (Auld and Morrison, 2006).

The current study shows the expression pattern of several CKIs during the process of adipocyte differentiation in 3T3-L1 cells, with a view to better understanding their role in this process. The fact that the expression of each protein varies in a distinct manner during adipocyte differentiation indicates that each of these proteins plays a distinct role in adipogenesis. Interestingly, the increased levels of p18\(^{INK4C}\), p21\(^{CIP1}\), and p27\(^{KIP1}\) associated with the differentiated state, support the contribution of these proteins to this condition.

Results

Cell cycle analysis of the differentiation process

To evaluate the degree of adipocyte differentiation of 3T3-L1 cells 6 days after treatment (see Materials and Methods), the percentage of differentiated cells was quantified by Red Oil O staining. As shown in Fig. 1, the number of differentiated cells was clearly superior (>80%) after differentiation treatment compared to control in which a small proportion of cells (8%) spontaneously differentiated into adipocytes.

Flow cytometry curves of DNA content are presented in Fig. 2. The profile of cells in clonal expansion phase (24 h in differentiation medium) shows a drastic reduction in the number of cells in G0/G1 and a predominance of cells in S-G2/M phases (Fig. 2C).

The proportion of cells in different cell cycle stages in the different phases of differentiation is shown in Table 1. An increase (p<0.05) in the percentage of cells in G1/G0 stage and a reduction in the other stages could be seen in 48 h confluent cells. In contrast, incubation for 24 h in the differentiation medium led to a reduction in the percentage of cells in the G1/G0 phase and an increase in the percentage of cells in the S and G2/M phases in the clonal expansion stage (p<0.01). Following 48 h in differentiation medium, the percentage of cells in G1/G0 increased whereas it decreased in the S and G2/M phases (p<0.01) coinciding with the completion of the mitotic cycles. On the sixth day of differentiation, we observed an increase in the percentage of cells in G1/G0 (p<0.05), which was higher than that observed in 48 h confluent cells. In contrast, the percentage of cells in G2/M decreased.

Expression of cell cycle regulatory proteins

Fig. 3A shows expression of members of CDK inhibitors from INK4 family. Namely, p15\(^{INK4B}\) was found to be moderately expressed in exponentially growing cells (day -3) and intensely expressed following 48 h confluence (day 0). Subsequently, the

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**TABLE 1**

PERCENTAGE OF CELLS IN EACH PHASE OF THE CELL CYCLE, DURING THE DIFFERENTIATION OF 3T3-L1 CELLS TOWARDS ADIPOCYTES

<table>
<thead>
<tr>
<th></th>
<th>A (%)</th>
<th>B (%)</th>
<th>C (%)</th>
<th>D (%)</th>
<th>E (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>50.4±3.44</td>
<td>59.9±0.66</td>
<td>26.1±7.07</td>
<td>58.2±3.77</td>
<td>67.47±1.93</td>
</tr>
<tr>
<td>S</td>
<td>7.2±0.26</td>
<td>5.4±0.19</td>
<td>11.3±2.6</td>
<td>4.86±0.11</td>
<td>5.6±0.23</td>
</tr>
<tr>
<td>G2/M</td>
<td>31.1±1.8</td>
<td>25.3±3.67</td>
<td>48.4±3.59</td>
<td>27.4±4.41</td>
<td>21.46±3.49</td>
</tr>
<tr>
<td>Polyploid cells</td>
<td>9.16±0.15</td>
<td>6.5±3.45</td>
<td>13.06±3.8</td>
<td>8.03±2.3</td>
<td>3.93±1.85</td>
</tr>
</tbody>
</table>

(A) Exponential growth; (B) 48 h confluent; (C) 24 h in differentiation medium; (D) 48 h in differentiation medium and (E) Sixth day after initiating the process of differentiation. Asterisks indicate statistical significance with respect to the previous state: *p<0.05 **p<0.01.

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**Fig. 1.** Photomicrographs (x100) of 3T3-L1 cells in culture, stained with Red Oil O to evidence lipid content. (A) Cells cultured in the usual medium (B) Cells incubated for 6 days in conditioned medium to induce their differentiation into adipocytes. The upregulation of lipid content following differentiation is evident.
level of expression of this protein gradually declined during the remaining phases of the process of differentiation. The expression of p16\textsuperscript{INK4a} gradually increased from the stage of exponential growth (day -3) to the phase of exit from clonal expansion (day 2), when its level of expression was found to be maximal. Expression of this protein dropped off noticeably when cells had already differentiated (day 6). The expression of p18\textsuperscript{INK4c} oscillated considerably during the process of differentiation. Thus, expression of this protein increased notably in 48 h confluent cells (day 0) in comparison to cells in the exponential proliferation phase (day -3). In the following phases (day 1 and 2), it gradually declined to almost undetectable levels. Finally, in the differentiated stage (day 6), moderate levels of expression were once again detected.

Expression of CDK inhibitors from CIP/KIP family is shown in Fig. 3B. Higher expression of p21\textsuperscript{CIP1} was found in cells which grew exponentially (day -3) than in 48 h confluent cells (day 0). During the following phases of differentiation, a gradual increase in the expression of this protein was observed, reaching maximal levels during the differentiated stage (day 6). The expression of p27\textsuperscript{KIP1} protein was expressed in exponentially growing cells (day -3). The expression of this protein rose considerably in 48 h confluent cells (day 0), but dropped off after incubation in differentiation medium during 24 h (day 1). Expression of this protein gradually increased during the subsequent phases of differentiation, and reached maximal levels during the differentiated phase (day 6).

Expression of the adipocyte transcription factor C/EBP\textalpha

As can be seen in Fig. 3C this protein was practically undetectable during the initial phases of the process of differentiation. Increased levels were detected on day 2 when cells exit from the cell cycle prior to differentiation at the end of clonal expansion.

Discussion

The present study shows changes in the expression of various cell cycle regulatory proteins during the process of differentiation from fibroblastic cells to adipocytes. The 3T3-L1 cell line reflects \textit{in vitro} many of the biological aspects of the process of \textit{in vivo} differentiation towards adipocytes. Using this tool, we have been able to figure out the role of these proteins in exit from the cell cycle and in the maintenance of the differentiated state.

In the process of differentiation there is growth arrest prior to the expression of differentiated characters and cells enter G\textsubscript{0}. Differentiation of 3T3-L1 cells to adipocytes is connected to events within the cell cycle. Thus, in the exponential growth phase (Fig. 2A) and in particular after 24 h of incubation in differentiation medium, i.e. clonal expansion phase (Fig. 2C), a high number of cells are in S and G\textsubscript{2}/M. In contrast, the majority of cells are in G\textsubscript{0}/G\textsubscript{1} when at confluence (Fig. 2B) and upon finishing clonal expansion (Fig. 2D). Cells in G\textsubscript{0}/G\textsubscript{1} also abound in the differentiated phase (Fig. 2E). Similar results were described by Simbulan-Rosenthal \textit{et al}. (1996) in their study of the DNA replication complex during differentiation.
In addition to changes in morphology and in the expression of certain genes, terminal differentiation is associated with loss of proliferative capacity, exit from G₀, and entry into G₀ quiescent stage. Since inhibitors of cyclin-dependent kinases play a main role in arresting the cell cycle, they may contribute to the exit from the cell cycle associated with differentiation and to the maintenance of the proliferative quiescence of differentiated cells. Thus, it would be reasonable to find higher levels of expression of CKIs in the phases of confluence, exit from clonal expansion and during the differentiated state. However, not all CKIs exhibited the same behavior. It is thus likely that in addition to their general function as inhibitors of proliferation, each of the CKIs plays specific roles within the process of differentiation of 3T3-L1 cells to adipocytes. According to our results, p15INK4b would play a role in proliferative arrest associated with confluence in agreement with Phelps and Xiong suggestion (1998). P16INK4a would be involved in the initiation of growth arrest after clonal expansion in contrast with these authors who observed a pattern of expression similar to that of p15INK4b, p18INK4c would participate in the maintenance of the G₀ state typical of differentiated cells as has been shown in other organs (Zindy et al., 1997), p21CIP1 has been implicated in the terminal differentiation of various cell lines of epithelial origin and organs (Halevy et al., 1995, Zabludoff et al., 1998). There have been some discrepancies about the expression of this protein during this process. Our results coincide with those of Morrison and Farmer (1999) and Phelps and Xiong (1998), with the exception that we did not detect a fall off at the end of the process, neither did Reichert and Eick (1999). Our finding of high levels of p21CIP1 in exponentially growing and clonal expansion cells is consistent with reports of increased expression of this protein when quiescent cells entered the cell cycle (Tian and Quaroni, 1999). Increased levels of p21CIP1 during proliferation may be due to members of the CIP/KIP family acting as activators of Cdk4-6/Cyclin D as well as inhibitors of Cdk2 (Cheng et al., 1999). p27KIP1 has been reported to participate in quiescence and in the terminal differentiation of different cells (Polyak et al., 1994; Tian and Quaroni, 1999) and the pattern of expression of p27KIP1 that we observed is consistent with this role and coincident with Morrison and Farmer (1999) and Phelps and Xiong (1998).

The cyclin dependent kinase inhibitor family is made up of seven members but it seems clear that the expression of only one CKI family member is sufficient to arrest the cell cycle. Since we observed the expression of various CKIs in each stage of the differentiation process, it seems likely that if one loses its function, this can be replaced by that of another member. Indeed, such a functional redundancy associated with important molecules is not a rare phenomenon in biological systems. Another possible explanation for the co-expression of CKIs during differentiation to adipocytes is their cooperation for growth arrest. This could take place between members of different CKI families, since those of the ankyrin family can only bind Cdk4-6/Cyclin D complexes, thus inducing the liberation of CIP/KIP proteins from these, favoring the subsequent inhibition of Cdk2-Cyclin E complexes (Reichert and Eick, 1999). After an analysis of our results, we propose the joint action of some of these CKIs. Thus, p15INK4b, p27KIP1 and p18INK4c, p27KIP1 would participate in cell cycle exit for cells in confluence. Regarding quiescence prior to differentiation, cooperation between p16INK4a-p21CIP1 and p16INK4a-p27KIP1 may occur. Finally, cooperation between p16INK4a-p27KIP1 and p18INK4c-p21CIP1 may occur in the quiescent maintenance of the differentiated state. Members of the same family of CKIs may also cooperate; in particular, those that inhibit the activity of the Cdk2-Cyclin E complex, which phosphorylates p27KIP1. This results in its degradation and as a consequence, the inhibition of Cdk2-Cyclin E maintains the activity of p27KIP1 (Sheaff et al., 1997). In this regard, p21CIP1 would cooperate with p27KIP1 by complexing with Cdk2-Cyclin E, inhibiting its activity and provoking arrest following clonal expansion and the quiescent maintenance of the differentiated state.

C/EBPα is a main transcription factor in adipocyte differentiation, since it activates the transcription of adipocyte-specific genes. We observed an increase in its expression following 48 h in differentiation medium. Maximal expression was detected when the cells had differentiated into adipocytes. Similar results have been reported by others (Phelps and Xiong, 1998, Reichert and Eick, 1999, Yeh et al., 1995). Our results thus corroborate the participation of this transcription factor in the process of differentiation. C/EBPα is capable of inducing the expression of p21CIP1 and of stabilizing this protein (Harris et al., 2001). This may explain the increased levels of p21CIP1 which were observed in exit from clonal expansion and in differentiated cells, coincident with the time expression of C/EBPα. Thus, the antiproliferative effect of C/EBPα reported by Timchenko et al., (1996) may be due to increased expression of p21CIP1. In addition to inducing the expression of p21CIP1 and to stabilizing this protein (Harris et al., 2001), C/EBPα can exert an antiproliferative effect via other mechanisms: a) activation of the Gadd45 gene (Reynisdóttir and Massagué, 1997), b) interaction with Rb and p107 which, when joined to C/EBPα, inhibit the activity of Cdk2 kinase (Timchenko et al., 1999), c) complexing with E2F transcription factors, inhibiting their transcriptional activity (Slo-miany et al., 2000) and d) association with Cdk4 which provokes its degradation (Wang et al., 2002). Our results show a correlation between the expression of C/EBPα and growth arrest in that C/EBPα is expressed on day 2, when the proportion of cells in the S phase is reduced following clonal expansion. These data thus support the role of C/EBPα in the arrest of the cell cycle for the establishment of the differentiated state.

Overall, the present results reveal evident changes in the expression of Cdk inhibitors in different phases of the process of differentiation of 3T3-L1 cells towards adipocytes. The observed changes are indicative of distinct roles played by the studied proteins in the time sequence of events associated with differentiation.

Materials and Methods

Induction of differentiation and cell cycle analysis of 3T3-L1 cells

The cell differentiation protocol was adapted from Student et al., (1980). Briefly, 3T3-L1 preadipocytes (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Biochrom) containing 10% fetal bovine serum (FBS). Confluent cells were then incubated for two days in differentiation medium (DM) containing 10% FBS-DMEM supplemented with 0.5 mM isobutylmethylxanthine, 1 µM dexamethasone and 2 µM insulin (all from Sigma). DM was then replaced by 10% FBS-DMEM with 1 µM insulin. After 48 hours, cells were changed to 10% FBS-DMEM, which was replaced every 48 hours until the cells were used for experimentation. Five distinct phases of the process of in vitro differentiation were considered: 1) exponential growth phase (day -3); 2) confluence (day 0); 3) the clonal expansion phase (day 1); 4) the cycle exit phase subsequent to clonal expansion (day 2) and 5) the differentiation phase (day 6). Cells in each of these phases were obtained for analysis
of cell cycle and protein expression. The accumulation of acetylglucereside was measured by means of Red Oil O staining (Fig. 1). At the previously mentioned phases of the differentiation process, the percentage of cells in each of the stages of the cell cycle was measured using propidium iodide.

**Western blotting**

Cells were washed in PBS and incubated in trypsin-EDTA. Then, they were resuspended in RIPA buffer and further dissociated by passing various times through G21 needle followed by incubation on ice for 45 min and centrifugation at 10,000 g for 10 min. Protein concentration was measured by bicinchoninic acid. Same quantity of protein was loaded into the different gel lanes. Proteins were separated under denaturing conditions and wet transferred to a nitrocellulose membrane (Protran 0.2 μm, Schleicher & Schuell). Non-specific binding was blocked by incubation in TBS-Tween 20 plus 10% (w/v) low-fat milk 3 h RT. Membrane was incubated with primary antibody 4°C ON gentle shaking. Three washes in buffer Tris 10 mM, pH 7.4 (Merck), NaCl 500 mM (Sigma) and Tween 20 0.1% (v/v, Sigma) were followed by incubation with peroxidase-conjugated secondary antibody 1 hour RT, 3 buffer washes and incubation in chemiluminescence substrate (ECL+, Amersham Pharmacia Biotech). Band densities were quantified by blot scanning (GS-710 Bio-Rad) and Quantity One (Bio-Rad) software.

Primary mouse monoclonal antibodies were IgG2a anti-p16INK4a clone F12 (Santa Cruz Biotechnology), IgG2b anti-p21Cip1 clone F5 (Santa Cruz Biotechnology), IgG1 anti-p27Kip1 clone 57, (Transduction). Primary polyclonal antisera were goat anti-p15INK4b (Santa Cruz Biotechnology), rabbit anti-p18INK4A (Biosource). Peroxidase-conjugated secondary antibodies were rabbit anti-mouse (Dako), rabbit anti-goat (Dako), pig anti-rabbit (Dako).

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**References**


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