Dexamethasone reverses TGF-β-mediated inhibition of primary rat preadipocyte differentiation

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Received 27 February 2003; revised 7 April 2003; accepted 7 April 2003

First published online 28 April 2003

Edited by Veli-Pekka Lehto

Abstract Dexamethasone and transforming growth factor-β (TGF-β) show contrary effects on differentiation of adipocytes. Dexamethasone stimulates adipocyte differentiation whereas TGF-β inhibits it. In the present study, we investigated whether dexamethasone could reverse the TGF-β-mediated inhibition of preadipocyte differentiation. Primary rat preadipocytes, obtained from Sprague–Dawley rats, were pretreated with dexamethasone in the presence or absence of TGF-β, prior to the induction of differentiation. Co-treatment of dexamethasone and TGF-β before inducing differentiation reversed the TGF-β-mediated inhibition of preadipocyte differentiation. In order to elucidate the mechanism by which dexamethasone reversed the effect of TGF-β on the inhibition of preadipocyte differentiation, the expression of CCAAT/enhancer binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) was examined. Dexamethasone increased C/EBPα and PPARγ expression in the absence of TGF-β and also recovered the TGF-β-mediated suppression of C/EBPα expression in preadipocytes. Its effect was sustained in differentiated adipocytes as well. However, those effects were not observed in 3T3-L1 preadipocytes or differentiated adipocytes. These results indicate that dexamethasone reverses the TGF-β-mediated suppression of adipocyte differentiation by regulating the expression of C/EBPα and PPARγ, which is dependent on the cellular context.

Key words: Dexamethasone; Rat preadipocyte; Transforming growth factor-β; CCAAT/enhancer binding protein-α; Peroxisome proliferator-activated receptor γ

1. Introduction

Adipogenesis is a multistep process that fulfills the need for the storage of excess energy. Experiments using various cell culture systems, which include 3T3-L1 [1], PAZ6 [2] and a primary preadipocyte culture [3], revealed that several transcriptional factors play major roles in adipocyte differentiation. CCAAT/enhancer binding proteins (C/EBPs), nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPARγ), and adipocyte determination and differentiation factor-1/sterol regulatory element binding protein-1c are all major transcription factors associated with the adipocyte differentiation process.

Transforming growth factor-β (TGF-β) exhibits potent effects on a wide variety of cell types. It inhibits the proliferation of epithelial cell lines [4], while stimulating the proliferation of some mesenchymal cells [5,6]. The effects of TGF-β on the differentiation of various tissues of mesenchymal and epithelial origin include both the prevention and induction of expression of specific phenotypes. TGF-β is able to block the differentiation of preadipocyte cell lines, such as 3T3-L1 [7,8], TA1 cells [9] and primary cultures of preadipocytes [10,11]. It inhibits the differentiation of 3T3-L1 preadipocytes by decreasing C/EBPα and PPARγ, which are critical for adipogenesis [12,13].

Dexamethasone and isobutylmethylxanthine, which are both important adipogenic inducers, induce the expression of C/EBPδ and C/EBPβ, respectively [14]. The expression of both C/EBPδ and C/EBPβ is known to increase that of C/EBPα and PPARγ [14,15], which in turn stimulates the differentiation of 3T3-L1 preadipocytes. The effect of dexamethasone on the enhancement of adipocyte differentiation is dependent on the cellular context. For example, it inhibits the differentiation of 3T3-F442A preadipocytes [16], although dexamethasone is routinely used for the differentiation of preadipocyte cell lines, such as 3T3-L1 and TA1.

In this study, we investigated the effects of the co-treatment of dexamethasone and TGF-β on primary rat preadipocyte differentiation, as dexamethasone stimulates the differentiation of adipocytes and TGF-β inhibits it.

2. Materials and methods

2.1. Animals and cells

Male Sprague–Dawley (SD) rats (Dae Han Bio Link), 8–12 weeks of age, were allowed to acclimatize to handling for 4 days under standard conditions of 12 h light per day, prior to the experiments. Food and water were provided ad libitum. 3T3-L1 preadipocytes were obtained from ATCC, and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Life Technologies).

2.2. Preparation and differentiation of primary rat preadipocytes

The preadipocytes were prepared from adipose tissue using a modified method [17,18]. The epididymal adipose deposits from male SD rats, aged 8–10 weeks, were quickly removed. The epididymal fat depots were washed with phosphate-buffered saline (PBS), cut into 2-mm pieces, and then mixed with 10 ml of collagenase buffer (2 mg/ml, type II; Sigma). After incubating for 50 min at 37°C in a rocking platform shaker, the 15-ml conical tubes, containing digested tissues, were centrifuged at 500 rpm for 1 min to remove the floating mature

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Abbreviations: TGF-β, transforming growth factor-β; C/EBP, CCAAT/enhancer binding protein; PPARγ, peroxisome proliferator-activated receptor γ; aP2, fatty acid binding protein

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00371-5

adipocytes. For the preparation of the preadipocytes, the infranatants were centrifuged at 2000 rpm for 5 min. The resulting pellet was resuspended in a hemolytic buffer (Sigma), incubated at room temperature for 3 min, washed twice with PBS, and then resuspended in DMEM supplemented with 10% FBS. The prepared preadipocytes, or 3T3-L1 preadipocyte cell line, were plated at a concentration of 1×10^6 cells per well, in 6-well plates, and cultured for 3 days. Differentiation was induced by the addition of DMEM supplemented with 0.5 M isobutylmethylxanthine, 2 μM dexamethasone, 1.7 μM insulin and 10% FBS. The induction medium was removed 2 days after incubation. After an additional 2 days incubation with DMEM supplemented with 10% FBS and 1.7 μM insulin, the medium was replaced every 2 days with DMEM supplemented with 10% FBS. At the time of the experiments more than 70% of the primary culture cells, and 90% of the 3T3-L1 cells, were filled with multiple lipid droplets. The lipid accumulations in the differentiated adipocytes were assessed through staining of the neutral fats and cholesterol esters using Oil Red O dye (Sigma). In brief, the differentiated adipocytes were fixed in 4% formaldehyde for 10 min, and then stained for 30 min with the Oil Red O dye dissolved in isopropanol. The stained adipocytes were washed with PBS, and then the quantification of the lipid staining was measured by the addition of 1 ml isopropanol to extract the dye, the absorbance was measured at 420 nm [19].

2.3. Dexamethasone and TGF-β treatment

The prepared preadipocytes were incubated with 2 μM dexamethasone, in the presence or absence of TGF-β (1 ng/ml), for 3 days. The medium was then removed and the total RNA was isolated. For preadipocyte differentiation, the preadipocytes treated with dexamethasone, in either the presence or absence of TGF-β, were subjected to differentiation. On day 8, the total RNA was isolated and subjected to Northern blot analysis.

2.4. Preparation of cDNA fragment

To prepare cDNA probe for Northern analysis, the following primer pairs were designed and used in the reverse transcription polymerase chain reaction, using rat adipocyte cDNA as a template: C/EBPα, 5′-GAATCTCCTAGTCCTGGCTC-3′ and 5′-GATGAGAACAGCAACGAGTAC-3′; PPARγ, 5′-TGGGGATGTCTCACAATGCCA-3′ and 5′-TTCCTGTCAAGATCGCCCTCG-3′; fatty acid binding protein (aP2), 5′-GACCTGGAAACTCGTCTCCA-3′ and 5′-CATGACACATTCCACCACCA-3′. The resulting bands were cloned into a pGEM-T vector (Promega) and sequenced.

2.5. Northern blot analysis

The total RNA from the primary rat adipocytes or 3T3-L1 cells was prepared using RNAzol B (Tel-Test), electrophoresed in a 3% formaldehyde gel, and transferred onto a nylon membrane using a turbo blotter (Schleicher and Schuell). To estimate the amount of RNA loading, the transferred membrane was photographed. ^{32}P-labeled cDNA probes were prepared using a High Prime DNA labeling kit (Roche Applied Science) and used for sequential hybridizations. After each hybridization, the membrane was washed with 0.1×SSC, 0.05% SDS at 50°C and exposed to X-ray film for 18 h at −80°C using an intensifying screen. The membrane was stripped thoroughly by boiling in 0.01% SDS for 5 min following the hybridization with each probe.

2.6. Statistical analysis

The data are presented as the means ± S.E.M., and were statistically analyzed using the unpaired *t*-test. A difference was considered significant with a P value of <0.05.

3. Results

3.1. TGF-β enhances adipocyte proliferation and suppresses differentiation

The preadipocytes isolated from the epididymal adipose deposits of SD rats were treated with TGF-β prior to the induction of differentiation. After incubation for 3 days, the number of preadipocytes was observed to increase compared with control (Fig. 1A), indicating that TGF-β stimulates the proliferation of preadipocytes. We also examined the effect of dexamethasone on TGF-β-stimulated proliferation of preadipocytes. Dexamethasone suppressed the proliferation of preadipocytes induced by TGF-β (Fig. 1B).

To examine the roles of TGF-β in the differentiation of preadipocytes, the isolated preadipocytes were incubated with TGF-β for 3 days. The culture medium was replaced with the differentiation medium, and the differentiation process was initiated as described in Section 2. On day 8 after the induction of differentiation, the differentiated adipocytes were stained with Oil Red O dye or photographed. The pretreatment with TGF-β significantly suppressed the differentiation of the preadipocytes (Fig. 1A).

3.2. Dexamethasone reverses TGF-β-mediated suppression of preadipocyte differentiation

In an effort to find factors that can reverse the TGF-β suppressing activity of preadipocyte differentiation, preadipocytes were treated with linoleic acid or dexamethasone, in the presence or absence of TGF-β, prior to the induction of differentiation, as they are known to enhance preadipocyte differentiation. After 3 days of treatment differentiation was in-

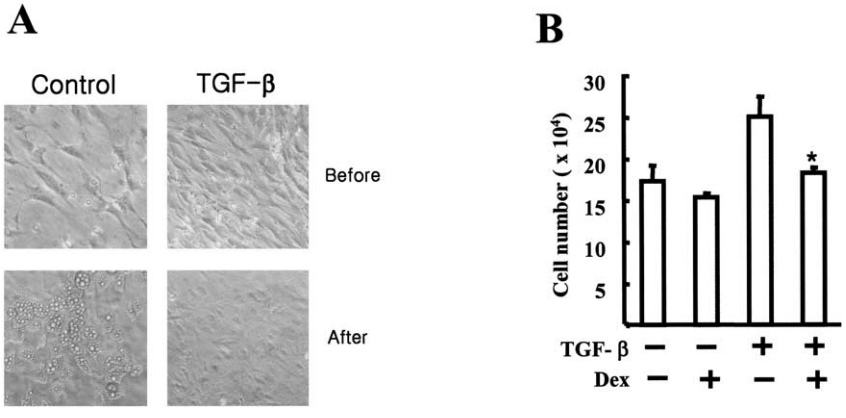

Fig. 1. The effects of TGF-β on the proliferation and differentiation of primary rat preadipocytes. A: Preadipocytes isolated from epididymal adipose deposits were plated in 6-well plates (1×10^6 cells per well), treated with 1 ng/ml of TGF-β for 3 days, and then photographed. Differentiation was induced after the treatment with TGF-β for 3 days. On day 8 after the initiation of differentiation, the adipocytes were photographed. B: Preadipocytes isolated from epididymal adipose deposits were plated in 12-well plates (1×10^5 cells per well), treated with 1 ng/ml of TGF-β in the presence or absence of dexamethasone (10^{-7} M) for 3 days, and then cell number was counted. All values are represented as the means ± S.E.M. of triplicates. *$P<0.05$ compared to the TGF-β-treated sample.

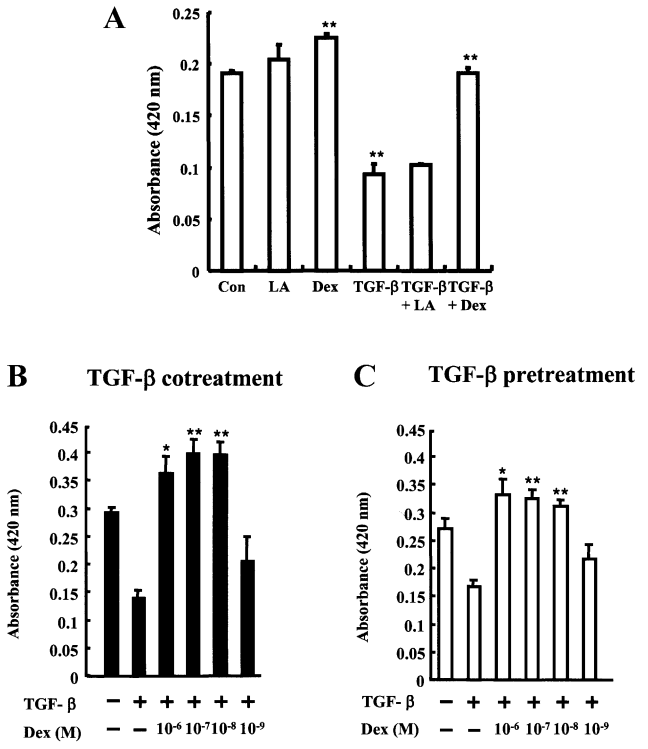

Fig. 2. The effects of dexamethasone and TGF-β on preadipocyte differentiation. A: Preadipocytes isolated from epididymal adipose deposits were plated in 12-well plates (2×10^5 cells per well), treated with 30 μM linoleic acid, 10^{-7} M dexamethasone, and 1 ng/ml TGF-β for 3 days, and then differentiation was induced for 8 days. Oil Red O staining was used to evaluate the differentiation, and the absorbance was measured. $^{**}P<0.01$ compared with the control. LA, linoleic acid; Dex, dexamethasone. B: Dexamethasone reverses the TGF-β-mediated suppression of preadipocyte differentiation. Preadipocytes isolated from epididymal adipose deposits were plated in 12-well plates (2×10^5 cells per well), treated with 1 ng/ml of TGF-β in the presence or absence of various concentrations of dexamethasone for 3 days (co-treatment) or (C) treated with 1 ng/ml TGF-β for 1 day, with various concentrations of dexamethasone added, and incubated for an additional 2 days (pretreatment). After 8 days of the differentiation process, Oil Red O staining was used to evaluate the differentiation, and the absorbance was measured. All values are represented as the means ± S.E.M. of duplicates. Data shown are representative of one of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared to the TGF-β-treated sample.

duced. The differentiated adipocytes were evaluated with Oil Red O staining and the stained dye was extracted with isopropanol. Dexamethasone was found to significantly increase the differentiation of the preadipocytes ($P<0.01$), whereas linoleic acid mediated an increase of differentiation, but this was not significant statistically ($P<0.1$) compared with the control. Pretreatment with TGF-β completely inhibited the differentiation of the preadipocytes. Interestingly, co-treatment with dexamethasone and TGF-β reversed the TGF-β-induced inhibition of differentiation ($P<0.01$) compared to TGF-β treatment alone, but co-treatment with linoleic acid did not (Fig. 2A). This implies that the signal pathway associated with TGF-β-mediated inhibition of preadipocyte differentiation can be affected by dexamethasone during the preadipocyte differentiation process.

To determine the optimal concentration of dexamethasone for reversing the TGF-β-mediated suppression of preadipocyte differentiation, various concentrations of dexamethasone were used in the treatments in the presence of TGF-β. As shown in Fig. 2B, dexamethasone (10^{-6}–10^{-8} M) significantly reversed the TGF-β-mediated inhibition of preadipocyte differentiation compared to TGF-β alone ($P<0.01$), but dexamethasone did not reverse the effects of TGF-β at the concentration of 10^{-9} M ($P<0.2$). This means that a physiological concentration of dexamethasone is able to reverse the TGF-β-mediated suppression of preadipocytes.

In order to find if the dexamethasone is able to inhibit the TGF-β effect, even if the TGF-β is added 1 day before the treatment with dexamethasone, TGF-β was incubated for 1 day and the dexamethasone added without changing the medium. On day 8 of the differentiation, the differentiated adipocytes were measured by Oil Red O staining. Although TGF-β was added 1 day before the dexamethasone treatment, it still effectively reversed the TGF-β-mediated suppression of the differentiation, with the exception of a concentration of 10^{-9} M (Fig. 2C). Both results indicate that dexamethasone is effective enough to the reverse TGF-β-mediated suppression of differentiation, regardless of pretreatment or co-treatment with TGF-β, and that the TGF-β effect is not reversible, as the differentiation was also inhibited when the differentiation was induced in the absence of TGF-β.

3.3. *Dexamethasone enhances expression of C/EBPα in preadipocytes and adipocytes*

To understand the molecular mechanism of the dexamethasone effect on TGF-β-induced suppression of adipocyte differentiation, the expression of C/EBPα and PPARγ was analyzed using Northern blot analysis, as C/EBPα and PPARγ are known to be powerful transcription factors involved in the regulation of adipocyte differentiation. Preadipocytes, isolated from SD rats, were incubated with dexamethasone in the presence or absence of TGF-β for 3 days. The total RNA was isolated from the preadipocytes before the induction of differentiation. Dexamethasone increased the levels of C/EBPα and PPARγ mRNA, and co-treatment with dexamethasone and TGF-β recovered the level of C/EBPα mRNA expression suppressed by TGF-β alone. This means that dexamethasone was able to reverse the TGF-β-mediated suppression of C/EBPα mRNA expression. Meanwhile, the co-treatment with dexamethasone and TGF-β suppressed dexamethasone-induced PPARγ mRNA expression at a concentration of 1 ng/ml TGF-β (Fig. 3A).

Next, we wondered whether this effect could be sustained during the differentiation process. Therefore, we performed the same treatment on preadipocytes for 3 days, after which the differentiation was initiated as described in Section 2. On day 8 of the differentiation, the total RNA was isolated. Dexamethasone alone increased the C/EBPα mRNA expression, and the co-treatment with TGF-β reversed the TGF-β-mediated inhibition in differentiated adipocytes. The expression patterns for PPARγ mRNA were similar to those of C/EBPα (Fig. 3B). These results indicate that pretreatment with dexamethasone and TGF-β affects the whole process of adipocyte differentiation through the induction of C/EBPα and PPARγ mRNA expression. We also examined the expression of aP2 mRNA as a differentiation marker. Interestingly, dexamethasone increased aP2 mRNA expression, even in preadipocytes where no lipid filling was observed.

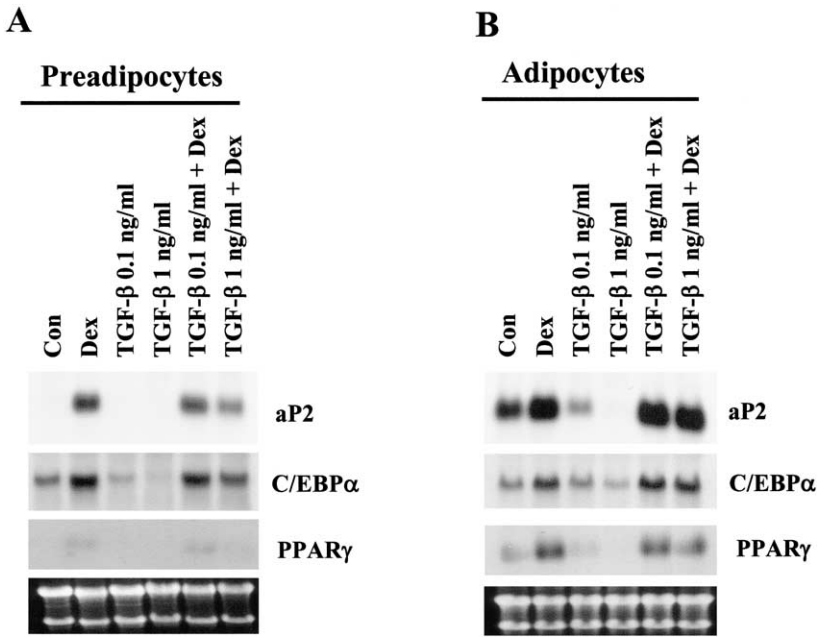

Fig. 3. The effect of dexamethasone on the TGF-β-mediated suppression of preadipocyte differentiation. A: Preadipocytes isolated from epididymal adipose deposits were plated in 6-well plates (6×10^5 cells per well), treated with TGF-β in the presence or absence of 10^{-7} M dexamethasone for 3 days. The total RNA was isolated and subjected to Northern blot analysis. B: For the analysis of differentiated adipocytes, treated preadipocytes were differentiated for 8 days, and the total RNA was isolated. The transferred blot was photographed and used as an equal loading control. Data shown are representative of one of two independent experiments.

3.4. Dexamethasone did not enhance expression of C/EBPα in 3T3-L1

Next, we examined whether dexamethasone could reverse the TGF-β-suppressed 3T3-L1 preadipocyte differentiation. TGF-β- and/or dexamethasone-treated 3T3-L1 preadipocytes were differentiated. The degree of differentiation was evaluated with Oil Red O staining. Unlike in the primary rat adipocytes, the co-treatment with dexamethasone and TGF-β did not reverse the TGF-β-mediated suppression of the 3T3-L1 preadipocyte differentiation compared to the control or the dexamethasone-treated 3T3-L1 preadipocytes, although TGF-β alone inhibited the differentiation of the 3T3-L1 preadipocytes (Fig. 4A). Thus, we examined the expression of C/EBPα mRNA. The 3T3-L1 preadipocyte cell line was treated with TGF-β and/or dexamethasone, and incubated for 3 days. The total RNA was isolated from the preadipocytes before the induction of differentiation, and subjected to Northern blot analysis. The treatments induced no expression of C/EBPα mRNA in 3T3-L1 preadipocytes, with no expression of aP2 mRNA, as a differentiation marker (Fig. 4B). To determine the expression of C/EBPα mRNA in differentiated 3T3-L1 adipocytes, TGF-β- and/or dexamethasone-treated 3T3-L1 preadipocytes were differentiated. On day 8 of differentiation, the total RNA was isolated, and Northern analysis performed. Although TGF-β suppressed the expression of C/EBPα and aP2 mRNA, co-treatment with dexamethasone and TGF-β did not recover the TGF-β-mediated inhibition of either expression (Fig. 4C). Taken together with the primary rat preadipocyte data, the TGF-β-induced suppression of adipocyte differentiation is mediated by down-regulation of C/EBPα, and dexamethasone reverses the TGF-β effect through its induction.

4. Discussion

Glucocorticoids and TGF-β play an important role in determining the differentiation and function of adipocytes. The effect of glucocorticoids on the promotion of adipocyte differentiation has been studied in several cell culture systems [20–23], although dexamethasone inhibits the differentiation of the 3T3-F442A preadipocyte cell line [16]. TGF-β is known to inhibit the adipogenic conversion of 3T3-L1 cells [7,8], rat perirenal [24] and human preadipocytes [11]. Therefore, we thought that glucocorticoids might interact with TGF-β in the process of preadipocyte differentiation. However, no detailed analyses of the effects of glucocorticoids on TGF-β-inhibited adipogenic differentiation have been performed. In this study, we addressed this issue.

Two families of transcription factors, C/EBPs and PPARs, play major roles in the differentiation of preadipocytes, and are induced by various adipogenic inducers, including dexamethasone, isobutylmethylxanthine, long-chain fatty acids and prostanoids [14,25,26]. The effects of dexamethasone on adipocyte differentiation vary depending on the cell line. For instance, dexamethasone promotes differentiation in 3T3-L1 preadipocytes and is required during the early stages of differentiation [14]. In contrast, the differentiation of the 3T3-F442A preadipocyte cell line is inhibited by dexamethasone [27]. In our system, dexamethasone enhanced the differentiation of primary rat preadipocytes isolated from the epididymal fat depots of SD rats. In addition, treatment with dexamethasone reversed the TGF-β-inhibited adipogenic differentiation.

In 3T3-L1 cells, dexamethasone increases the expression of C/EBPδ involved in the early stages of adipocyte differentia-

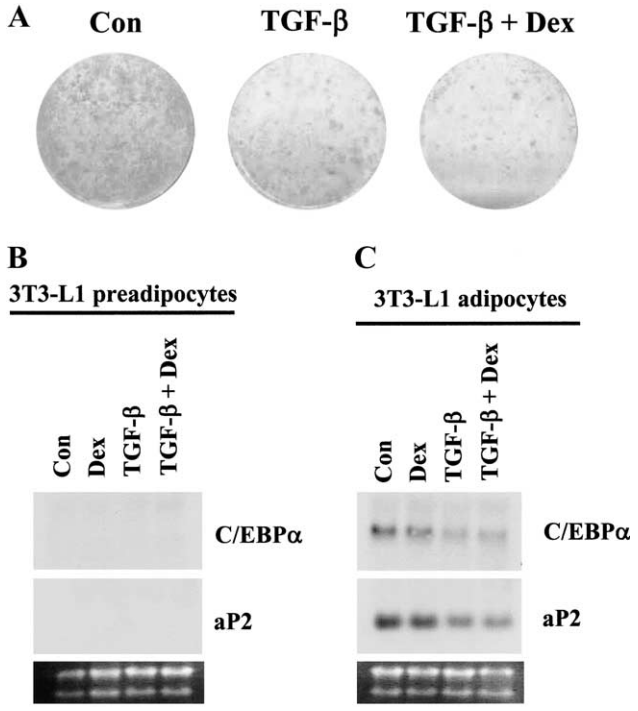

Fig. 4. The effect of dexamethasone and TGF-β on 3T3-L1 preadipocyte differentiation. A: 3T3-L1 preadipocytes were plated in 6-well plates (6×10^5 cells per well), treated with 1 ng/ml TGF-β in the presence or absence of 10^{-7} M dexamethasone for 3 days, then differentiation was induced. On day 8 of differentiation, the differentiated adipocytes were stained with Oil Red O and then photographed. B: 3T3-L1 preadipocytes were plated in 6-well plates (6×10^5 cells per well), treated with 1 ng/ml TGF-β in the presence or absence of 10^{-7} M dexamethasone for 3 days. The total RNA was isolated and subjected to Northern blot analysis. C: For the analysis of the differentiated adipocytes, treated preadipocytes were differentiated for 8 days, then the total RNA was isolated. The transferred blot was photographed and used as an equal loading control.

tion. The expression of either C/EBPβ or C/EBPδ increases the expression of C/EBPα, which in turn stimulates the final process of differentiation through the induction of differentiation marker genes [14,28]. This means that the expression of C/EBPα begins after the induction of differentiation. In rat preadipocytes, however, C/EBPα expression was observed in preadipocytes, and was induced by treatment with dexamethasone before the induction of differentiation, whereas neither the control nor the treatment with dexamethasone showed C/EBPα expression in 3T3-L1 preadipocytes. These data are in line with those for porcine preadipocytes [29], where dexamethasone increases the C/EBPα-positive cells before the induction of differentiation [3]. This implies that the expression of C/EBPα could begin before the induction of differentiation, and is dependent on the cellular context and that C/EBPα could play some roles, at least, in primary rat preadipocytes prior to the initiation of differentiation.

The adipocyte-specific aP2 is known as a marker for differentiation-dependent adipocyte proteins [30] and is increased during the differentiation process of porcine stromal-vascular cells [31], but we showed that aP2 expression was induced by dexamethasone in primary rat adipocytes, and the induced level was sustained until after differentiation. It is known that promoters from adipocyte-specific genes, such as GLUT4 or SCD1, and the aP2 genes in 3T3-L1 cells are transactivated by C/EBPs, including C/EBPα [32,33]. Therefore, the dexamethasone-induced C/EBPα may enhance the expression of aP2 mRNA in primary rat preadipocytes. In addition, PPARγ may also be involved in the increase of aP2 in dexamethasone-treated primary rat preadipocytes, as PPARγ is known to induce the expression of aP2 mRNA in primary human monocytes and the monocytic cell line THP-1 [34], and human adipocytes [35]. This is the first report detecting aP2 mRNA induced by dexamethasone in primary rat preadipocytes. Although aP2 is known as an adipocyte differentiation marker, and functions as a carrier for the cellular uptake and intracellular transport of fatty acids, the function of the dexamethasone-induced aP2 in primary rat adipocytes remains to be elucidated.

TGF-β causes a rapid decrease in the expression of adipose genes when added to fully differentiated adipocytes in TA1 preadipocytes [9]. Exposure to TGF-β, prior to the induction of differentiation, blocks the differentiation of 3T3-L1 preadipocytes, but not after the induction of differentiation [8]. In our primary rat preadipocyte culture system, TGF-β inhibited the differentiation of preadipocytes through the down-regulation of C/EBPα and PPARγ, and dexamethasone induced the expression of both genes, and also recovered the TGF-β-mediated suppression of C/EBPα and PPARγ mRNA expression. In turn, dexamethasone reversed the TGF-β-mediated block of preadipocyte differentiation through the increase of C/EBPα and PPARγ mRNA expression suppressed by the TGF-β.

There is a possible mechanism by which dexamethasone reverses TGF-β-mediated suppression of C/EBPα and PPARγ mRNA expression. Glucocorticoid receptor (GR) binds to the activation domain of Smad3 and blocks the transcription of Smad3-mediated target genes [36]. It may be possible that interaction between GR and Smad3 might block recruitment of transcriptional co-repressors such as HDAC and TGIF [37]. Thus, GR could reverse Smad3-mediated suppression of target gene transcription by TGF-β. However, this effect is dependent on cellular context, since with 3T3-L1, dexamethasone failed to induce C/EBPα mRNA expression in the absence of TGF-β and recover the C/EBPα mRNA expression that had been inhibited by TGF-β. Since primary cells may reflect an in vivo context better than aneuploid cell lines, the fact that dexamethasone reversed the TGF-β-mediated block of primary rat preadipocyte differentiation has significant physiological implications. That is, TGF-β mRNA and protein, the inhibitor of adipocyte differentiation, are paradoxically increased in the adipose tissue of two different strains of genetically obese mice, *ob/ob* and *db/db*, compared with their lean controls [38], but elevated TGF-β may not inhibit the differentiation of adipocytes, since adult *ob/ob* mice show an increased secretion of corticosteroids [39], which could inhibit the TGF-β-mediated block of adipocyte differentiation. Meanwhile, an increase in TGF-β stimulates the proliferation of preadipocytes, leading to the expansion of the adipose mass in the obese model.

Acknowledgements: This research was supported by Grant CBM-0200212 from the Center for Biological Modulators of the 21st Century Frontier R&D Program, the Ministry of Science and Technology, Korea.

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