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TGFβ$_1$ Prevents the Down-Regulation of Type I Procollagen, Fibronectin, and TGFβ$_1$ Gene Expression Associated With 3T3-L1 Pre-Adipocyte Differentiation

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Abstract Pre-adipocyte 3T3-L1 cells, after an appropriate induction stimulus, proceed through a defined change in morphology as differentiation progresses. Transforming growth factor β_1 (TGFβ$_1$) is able to block the morphological and biochemical changes which occur with differentiation of these cells if given within 36–40 h of induction [Ignotz and Massague (1985): Proc Natl Acad Sci USA 82:8530–8534]. To begin to elucidate the role of the extracellular matrix in adipogenesis, as well as the mechanism whereby TGFβ$_1$ inhibits differentiation, we examined the expression of two extracellular matrix genes, type I (α_1) procollagen and fibronectin, as well as endogenous TGFβ$_1$. Confluent cells were induced to differentiate by treatment with insulin, dexamethasone, and isobutylmethylxanthine in the presence or absence of TGFβ$_1$. Following 6 days of treatment, the cells in the differentiated group acquired the rounded shape of mature adipocytes; the cytosol of these cells also contained numerous lipid-filled vesicles, as demonstrated by oil red O staining. Cells treated with the differentiation compounds in the presence of TGFβ$_1$ maintained the fibroblast-like appearance of control cells and did not stain with oil red O. At the level of gene expression, both procollagen and fibronectin mRNAs were down-regulated during differentiation of 3T3-L1 cells. When cells from the control or differentiation groups were treated with TGFβ$_1$, there was a 2–5-fold induction of procollagen and fibronectin mRNAs throughout the 6-day time course. No change in type I procollagen transcription was observed by nuclear run-on analysis, suggesting that the increase in procollagen mRNA with TGFβ$_1$ treatment was due to a post-transcriptional process(es). However, both transcriptional and post-transcriptional components were observed in the regulation of fibronectin gene expression by TGFβ$_1$. In addition, TGFβ$_1$ was found to positively regulate its own expression, as treatment of the cells with TGFβ$_1$ enhanced endogenous TGFβ$_1$ expression and prevented the small decrease in TGFβ$_1$ mRNA levels which occurred early during the differentiation program. Thus, our data demonstrate that down-regulation of type I procollagen, fibronectin, and TGFβ$_1$ gene expression was prevented during TGFβ inhibition of 3T3-L1 differentiation. Taken together, these data suggest that TGFβ may inhibit differentiation of 3T3-L1 cells by maintaining the fibroblast-like extracellular matrix, thus preventing the changes in cell shape that accompany differentiation.

Key words: pre-adipocyte 3T3-L1 cells, TGFβ$_1$, collagen, fibronectin, insulin

Changes in the extracellular matrix often accompany and are functionally related to the onset and progression of differentiation [Elks and Manganiello, 1985; Form et al., 1986; Green and Kehinde, 1974, 1975; Heino and Massague, 1990]. 3T3-L1 pre-adipocyte cells serve as a useful model to study molecular mechanisms involved in differentiation because an established treatment regimen can induce the development of these cells into mature adipocytes which accumulate lipid [Hauner, 1990; Schmidt et al., 1990]. During differentiation 3T3 pre-adipocytes undergo a dramatic change in morphology, from a flat fibroblast-like form to a nearly spherical shape [Spiegelman and Farmer, 1982]. These changes in morphology are apparently "programmed" rather than a secondary consequence of lipid accumulation, because cell morphology changes even if lipid accumulation is blocked [Kuri-Harcuch and Green, 1978].

TGFβ is a multifunctional regulator of cellular proliferation and development and is known to induce morphologic changes in a variety of cell types [Sporn et al., 1987]. Additionally, differentiation of 3T3-L1 pre-adipocyte cells can be blocked by treatment within 36–40 h with TGFβ$_1$ [Ignotz and Massague, 1985]. We are using this

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observation as a tool to compare differences between cells which undergo differentiation and those inhibited from differentiation by treatment with TGFβ. TGFβ has pleiotropic effects on a variety of cell types, but one of its more general effects is to produce changes in the extracellular matrix [Ignotz and Massague, 1986; Roberts et al., 1986]. Because 3T3-L1 cells proceed through defined change in extracellular matrix composition as differentiation progresses [Ono et al., 1990; Weiner et al., 1989], as a first approach we studied the regulation of expression of two extracellular matrix genes, type I procollagen and fibronectin, as well as expression of the TGFβ1 gene.

MATERIALS AND METHODS

Cell Culture

The 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a 37°C incubator with 95% air/5% CO_2. Cells were allowed to become confluent for 3–4 days and re-fed on days 0 and 2 with Dulbecco's modified miminal medium containing 10% fetal calf serum supplemented with 1) *Control group*—no additions; 2) *TGFβ group*—100 pM TGFβ1; 3) *Differentiation group*—0.2 μM insulin, 0.4 μM dexamethasone, and 0.5 mM methyl isobutylxanthine (the differentiation "cocktail"); or 4) *Differentiation + TGFβ group*—differentiation "cocktail" plus 100 pM TGFβ1.

Oil Red O Staining

Cells were fixed in 50% methanol, then stained 15 min at room temperature with a 5% solution of oil red O in 40% isopropanol. Presence or absence of staining for lipid was determined by phase contrast microscopy.

RNA Analysis

At selected time points, cells were scraped in phosphate buffered saline with 10 μg/ml diethylpolyvinylcarbonate and total cellular RNA was isolated by the method of Baumbach et al. [1987]. Briefly, cells were lysed with 0.1% NP-40, digested consecutively with proteinase K and DNase I, followed by phenol/chloroform extraction and ethanol precipitation. Isolated RNA was bound to Zetaprobe nylon (BioRad Laboratory, Richmond, CA) with a slot blot apparatus (Schleicher & Schuell, Keene, NH). RNA was cross-linked to the nylon membrane by UV irradiation for 1 min. Probes were [^{32}P]-labeled by the random priming method [Feinberg and Vogelstein, 1983]. Gene probes were gel purified inserts isolated from plasmid DNA; inserts included type I collagen (α1R1) [Genovese et al., 1984], fibronectin [Schwarzbauer et al., 1983], and TGFβ [Derynck et al., 1986].

Nuclear Transcription Run-On Analysis

Nuclei were isolated [Greenberg and Ziff, 1984], frozen in liquid nitrogen, and stored at −70°C. Run-on analysis was by the method of Baumbach et al. [1987]. Labeled RNA transcripts (1–2.5 × 10^5 cpm/ml) were hybridized to type I (α_1) procollagen or fibronectin plasmids containing 2 μg of insert which was bound to Zetaprobe nylon with a slot blot apparatus. Plasmid containing 2 μg 28S rRNA was used to normalize the data.

RESULTS

Histochemical and Morphological Parameters of 3T3-L1 Pre-Adipocyte Differentiation

To induce the differentiation of fibroblast-like 3T3-L1 pre-adipocytes into mature adipocytes we followed an established treatment regimen [Green and Meuth, 1974; Green and Kehinde, 1975; Hauner, 1990; Schmidt et al., 1990]. Expression of the adipocyte phenotype was monitored by the following criteria. First, we used a lipid-specific stain (oil red O) to identify the cells which had developed lipid-containing vacuoles, a phenotypic characteristic of differentiated adipocytes [Green and Kehinde, 1974]. This histochemical assay provided a direct indication of both the time course of adipocyte differentiation and the percent cells which developed the adipocyte phenotype, as indicated in Table I. Only those cells treated with the differentiation "cocktail" stained for oil red O, and this occurred beginning on day 4. Six days following treatment the differentiation group exhibited >70% staining. The percent of cells which stained for lipid increased with time in culture up to day 10 (data not shown), at which time 75–80% of the cells stained positively by oil red O. There was a complete absence of oil red O stained cells in untreated control cultures and in cultures treated with TGFβ alone or TGFβ together with complete differentiation "cocktail."

3T3-L1 differentiation was additionally evaluated by morphological changes characteristic of the mature adipocyte. Figure 1A shows the morphology of control 3T3-L1 cells maintained for 6

TABLE I. Percent Cells Staining for Oil Red O in Control or Treated 3T3-L1 Cells Over a Time Course Spanning 0 to 6 Days (Treatments Began on day 0)

Treatment	Day	% oil red O positive cells
None (Confluent)	0	0
Control	2	0
TGFβ		0
Differentiation		0
Differ. + TGFβ		0
Control	4	0
TGFβ		0
Differentiation		6
Differ. + TGFβ		0
Control	5	0
TGFβ		0
Differentiation		48
Differ. +TGFβ		0
Control	6	0
TGFβ		0
Differentiation		72
Differ. + TGFβ		0

days in growth media only supplemented with fetal calf serum. The cells have maintained a fibroblast-like shape, and there are no lipid vacuoles characteristic of the differentiated adipocyte. Note that these cells are refractory to the oil red O lipid stain. The low level background staining is extracellular. Cells treated with $TGF\beta_1$ alone are fibroblast-like, but they exhibit a more elongated and "needle-like" morphology than the control cells (Fig. 1B); again there is no staining for intracellular lipid accumulation. Figure 1C shows that the 3T3-L1 cells given a treatment regimen to induce differentiation developed the characteristic phenotype of the mature adipocyte; i.e., the cells have become more rounded and have acquired numerous lipid vacuoles. These vacuoles contain lipid based on the strong staining by oil red O. It is interesting that the cells treated simultaneously with the differentiation-inducing "cocktail" and $TGF\beta_1$ (Fig. 1D) exhibit a composite morphology between the very elongated $TGF\beta_1$-alone cells and the spherical differentiated cells. Indeed, the cells in this group most closely resemble the control cells and, like control, do not stain for oil red O.

TGFβ Effects on Type I Procollagen and Fibronectin Gene Expression During Adipocyte Differentiation

We next determined a time course for the expression of type I procollagen and fibronectin genes in 3T3-L1 cells subjected to the 4 treatment regimens. Figure 2A shows that the control group maintained in growth media only supplemented with fetal calf serum exhibited less than a 30% change in cellular levels of type I procollagen mRNA during the 6 day culture period. When control cells were treated with $TGF\beta_1$, there was a 3-fold induction of collagen type I mRNA levels (relative to control) on days 1 and 2. On day 4 the mRNA levels were enhanced by ~50%, and by day 6 the control and TGFβ-treated cells exhibited similar levels of procollagen mRNA.

In contrast, the 3T3-L1 cells which were induced to differentiate showed a rapid and dramatic decrease in type I procollagen mRNA levels. This change apparently reflects the major modifications in morphology which accompany 3T3-L1 pre-adipocyte differentiation. However, these observed alterations in expression of genes encoding a principal extracellular matrix protein which are apparent by day 1, precede changes in cellular morphology which are not evident until ~day 3. The 3T3-L1 cells treated with the differentiation "cocktail" together with $TGF\beta_1$ showed a 3–5-fold increase in levels of procollagen mRNA compared to cells maintained under conditions that supported differentiation. This TGFβ-associated increase was evident on day 1 and was maintained throughout the 6-day time course. Thus, the addition of TGFβ not only prevents the decrease in procollagen mRNA levels associated with adipocyte differentiation, but also results in increased procollagen mRNA levels.

The 3T3-L1 cells from the control group exhibited a ~50% decline in levels of fibronectin mRNA over the 6-day time course (Fig. 2B). Those cells treated with TGFβ alone showed a marked enhancement of fibronectin mRNA levels (relative to control) which was evident by day 1. Fibronectin mRNA levels of TGFβ-treated cells remained elevated 2–3-fold throughout the 6-day time course.

The 3T3-L1 cells which were induced to differentiate showed a dramatic decrease in fibronectin mRNA levels, similar to the differentiation-related decline in type I procollagen mRNA. The initial downregulation of fibronectin mRNA was not as rapid as the decline in procollagen mRNA levels; however, the lowest level of fibronectin mRNA was observed on day 2, while the decline in procollagen expression continued for an additional 2 days. In contrast, the 3T3-L1 cells treated with both the differentiation "cocktail"

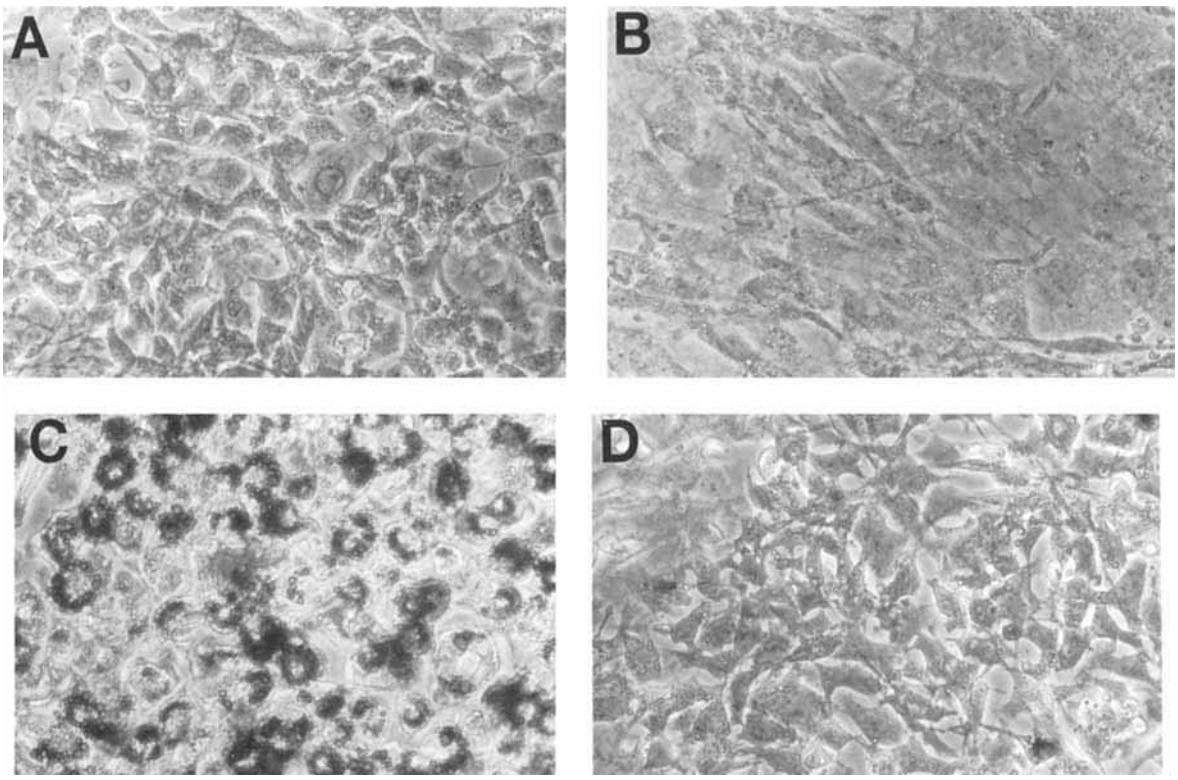

Fig. 1. Morphological changes and oil red O staining in 3T3-L1 cells following control, TGFβ, differentiation, or differentiation + TGFβ treatments. Sub-confluent 3T3-L1 cells were allowed to become confluent for 3–4 days and then treated on day 0. Cells from the 4 different groups were examined by phase contrast microscopy 6 days following treatment. Cells shown are from the following groups: **(A)** control, **(B)** TGFβ$_1$, **(C)** differentiation, and **(D)** differentiation + TGFβ$_1$. ×320. Staining is oil red O for lipid.

and TGFβ showed a 3–5-fold increase (relative to the differentiated cells) in the levels of fibronectin mRNA. This increase was maximal on day 2 and remained elevated through the 6-day time course. Interestingly, the cells treated with both the differentiation "cocktail" and TGFβ showed levels of fibronectin mRNA which paralleled those of the control cells. As noted in Figure 1, these two groups exhibited a similar morphology.

The slot blot analyses for determining the cellular levels of mRNAs for type I procollagen, fibronectin, and 28S ribosomal RNA in a representative time course experiment are shown in Figure 2C. Note that only small changes in the representation of 28S RNA were observed over the same time course and treatments for which dramatic changes in levels of type I procollagen and fibronectin mRNA were observed.

TGFβ Effects on Extracellular Matrix Protein Gene Transcription

Nuclei for run-on transcription analysis were isolated from parallel cultures of 3T3-L1 preadipocyte cells at the same time points that cells were harvested for RNA analysis. The levels of type I procollagen transcription in control cells declined over the 6-day time course (Fig. 3A). Cells treated with TGFβ alone maintained levels of procollagen transcription similar to those in control cultures over the 6 day time course. The 3T3-L1 cells which were induced to differentiate maintained an essentially constant level of procollagen transcription, despite the decline in procollagen mRNA (Fig. 2A) during the 6-day time course. The 3T3-L1 cells treated with both the differentiation "cocktail" and TGFβ exhibited levels of type I procollagen transcription similar to those in the differentiated cells throughout the 6-day time course. These results suggest that the TGFβ-mediated increases in cellular levels of type I procollagen mRNA observed in 3T3-L1 cells are post-transcriptionally regulated.

Nuclei from these treatment groups were also assayed for levels of fibronectin transcription by nuclear run-on analysis. The levels of fibronectin transcription in control cells were similar

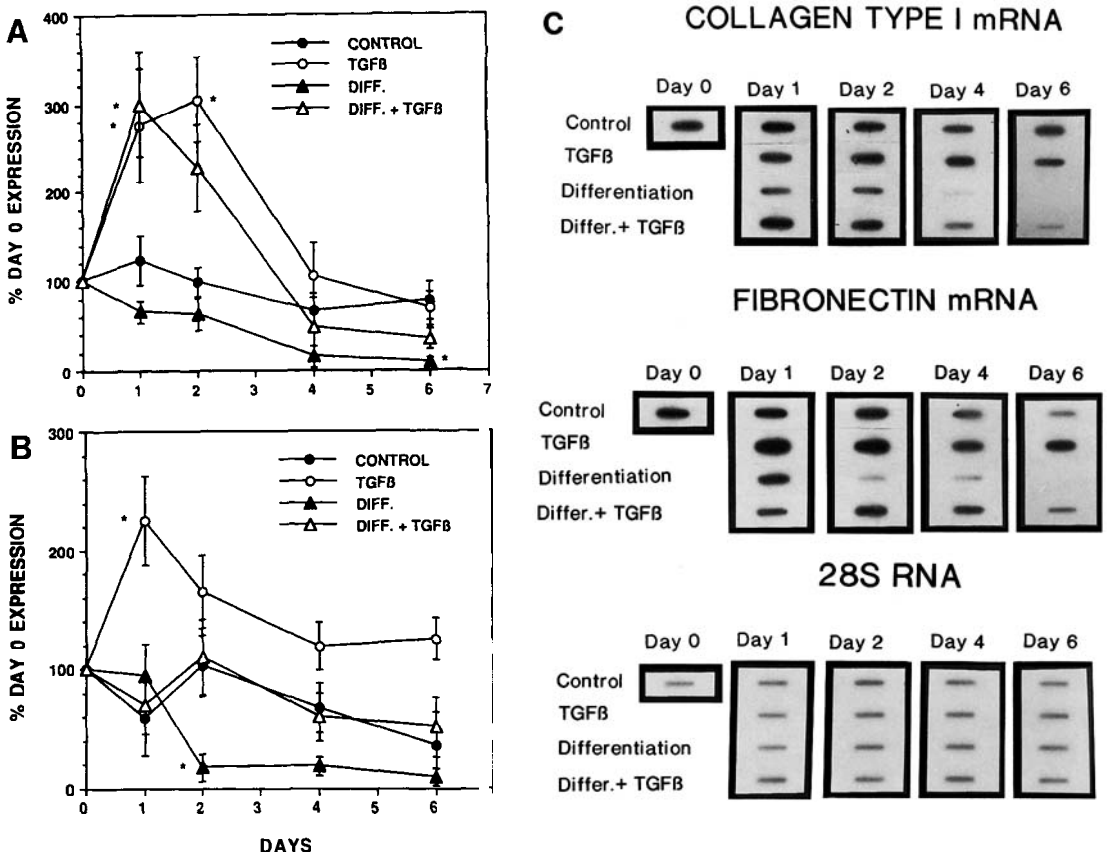

Fig. 2. Cellular levels of type I procollagen and fibronectin mRNAs during the time course of 3T3-L1 development. Day 0 indicates the initiation of control, TGFβ$_1$, differentiation (Diff.), or differentiation + TGFβ$_1$ (Diff. + TGFβ) treatments. Type I procollagen (**A**) or fibronectin (**B**) mRNA levels were assayed at days 0, 1, 2, 4, and 6. Data shown are the mean ± 1 S.D. of 4 independent time course experiments (* indicates statistically different from control [$P < .05$]). **C:** Slot blot analyses of cellular levels of mRNA for type I procollagen, fibronectin, and 28S rRNA during the time course of 3T3-L1 development. Data shown are slot blot analyses from a representative time course experiment.

over the 6-day time course (Fig. 3B). Also as observed for procollagen transcription, those cells treated with TGFβ alone showed no change in fibronectin transcription relative to control cultures over the 6-day time course. The 3T3-L1 cells which were induced to differentiate maintained a constant level of fibronectin transcription, despite the decline in fibronectin mRNA levels over the 6-day time course. However, in contrast to procollagen transcription, 3T3-L1 cells treated with both the differentiation "cocktail" and TGFβ showed increased levels of fibronectin transcription relative to the differentiated cells. While this increase was not dramatic, it was apparent by day 1 and was maintained throughout the 6-day time course.

Regulation of TGFβ Gene Expression During Adipocyte Differentiation

Because one of the well-documented effects of TGFβ treatment is on the extracellular matrix, we assayed TGFβ gene expression for the dual purpose of examining involvement in adipogenesis, as well as expression following exogenous treatment. Figure 4 shows that the TGFβ mRNA levels in 3T3-L1 adipocytes induced to differentiate were decreased relative to control cultures. This decrease was most apparent early during the time course of induction (days 1 and 2). Treatment of cells with exogenous TGFβ resulted in an increased expression of TGFβ mRNA at day 1, both in the TGFβ alone group and the differentiation + TGFβ group. This positive regulation of TGFβ expression was reversed by day 2 and not apparent later during the time course.

DISCUSSION

The 3T3-L1 pre-adipocyte cell line, after an appropriate induction stimulus, proceeds through a defined change in the extracellular matrix composition as differentiation progresses

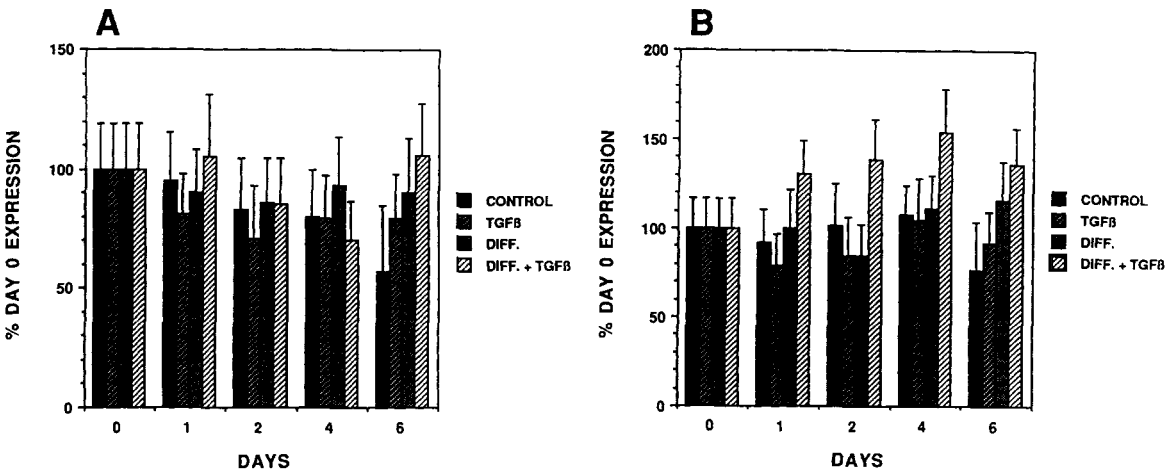

Fig. 3. Transcriptional activity of the type I (alpha I) procollagen and fibronectin genes during the time course of 3T3-L1 development. Day 0 indicates the initiation of control, TGFβ1, differentiation (Diff.), or differentiation + TGFβ1 (Diff. + TGFβ) treatments. Nuclei were isolated on days 0, 1, 2, 4, and 6 and analyzed by run-on assay for transcriptional activity of the procollagen (A) and fibronectin (B) genes. Data shown are the mean ± 1 S.D. of 3 independent time course experiments.

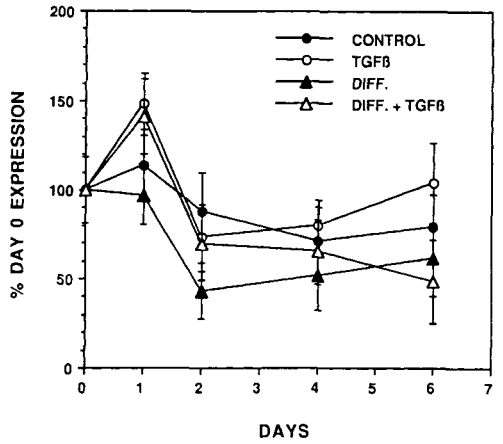

Fig. 4. Cellular levels of TGFβ mRNA during the time course of 3T3-L1 development. Day 0 indicates the initiation of control, TGFβ1, differentiation (Diff.), or differentiation + TGFβ1 (Diff. + TGFβ) treatments. Levels of endogenous TGFβ mRNA were assayed at days 0, 1, 2, 4, and 6. Data shown are the mean ± 1 S.D. of 2 independent time course experiments.

[Ono et al., 1990]. Type I collagen, a major component of the pre-adipocyte extracellular matrix, is down-regulated both at the mRNA and protein level following induction to differentiate [Weiner et al., 1989]. Our data also indicate a down-regulation of type I procollagen as well as fibronectin mRNA levels to $< 10\%$ of day 0 expression. Further, this decrease is initiated as early as 24 h after induction to differentiate. The decline in procollagen and fibronectin mRNAs precedes any change in morphology or in expression of many biochemical markers of the adipocyte phenotype [Bernlohr et al., 1984, 1985; Moustaid and Sul, 1991; Pairault and Green, 1979; Paulauskis and Sul, 1988; Spiegelman et al., 1983]. Further, we have shown by nuclear run-on transcription analysis that this down-regulation of procollagen and fibronectin mRNA following induction to differentiate is mediated by post-transcriptional mechanisms.

Sadowski et al. [1992] demonstrated that dexamethasone and insulin act synergistically to commit 3T3-L1 cells to differentiate 24–48 h after treatment. Further, these researchers observed five proteins which were elevated during commitment to differentiation and remained elevated in fully differentiated adipocytes, suggesting that one or more of these proteins may have a functional role in commitment to and/or expression of the adipocyte phenotype. TGFβ has previously been shown to inhibit the differentiation of 3T3-L1 cells if given within 36–40 h of the induction stimulus [Ignotz and Massague, 1985]. This again suggests an early event or "switch" in adipogenesis which is refractory to TGFβ after ~36 h. In our study TGFβ given simultaneously with the differentiation "cocktail" blocked development of differentiated adipocyte phenotype; the morphology of these cells resembled the controls. These morphologic observations are consistent with those of Ignotz and Massague [1985].

Because one of our objectives was to begin delineating, at the level of the extracellular matrix, how TGFβ might inhibit differentiation of 3T3-L1 cells, we compared the level of type I procollagen and fibronectin gene expression in control or differentiation-induced cells which were co-treated with 100 pM TGFβ1. The cells

treated with TGFβ showed a 2–5-fold increase in cellular levels of mRNA for these genes. Increased expression of type I collagen and fibronectin has also been reported in cultured fibroblasts and osteoblasts treated with TGFβ [Bortell et al., 1990; Breen et al., 1992; Centrella et al., 1992; Fine and Goldstein, 1987; Raghow et al., 1987; Roberts et al., 1986]. In addition, an increase in type I collagen expression has been reported in the inhibition of skeletal muscle myoblast differentiation by $TGF\beta_1$ [Heino and Massague, 1990], suggesting a general role for *modulation of* differentiation by TGFβ through its actions on the extracellular matrix.

Interestingly, the increased expression of procollagen and fibronectin mRNAs in the differentiation group treated with TGFβ ameliorated the 2–5-fold decrease in cellular mRNA levels of these genes following differentiation treatment alone. This TGFβ-associated abrogation in the down-regulation of extracellular matrix gene expression was particularly striking for fibronectin mRNA levels; i.e., the mRNAs were almost identical between the control and differentiation group treated with TGFβ. Also, these groups appeared remarkably similar in morphology as seen by phase contrast microscopy. Taken together, these data suggest that one way in which TGFβ may inhibit differentiation is by preventing the decline in fibronectin (and type I collagen) expression which accompanies development of the adipocyte phenotype. A similar mechanism has been proposed by Spiegelman and Ginty [1983], who observed decreased expression of lipogenic enzymes and inhibition of adipogenesis in 3T3-422A cells grown on fibronectin-coated culture dishes.

TGFβ has previously been reported to positively regulate its own expression in several different normal and transformed cells in culture [Van Obberghen-Schilling et al., 1988]. In this study we observed increased levels of TGFβ mRNA following TGFβ treatment of 3T3-L1 cells. Thus, the increased expression of extracellular matrix genes that we observed following TGFβ treatment may, in part, be attributed to the increased expression of endogenous TGFβ. It is interesting that the increased expression of TGFβ (and the most pronounced increases in procollagen and fibronectin expression) occurred one day following treatment with TGFβ, within the 36–40 h period after which 3T3-L1 cells are refractory to inhibition of differentiation by TGFβ.

To address the contribution of transcriptional activity to the changes in type I procollagen and fibronectin gene expression, we determined transcriptional activity by nuclear run-on analysis. For both procollagen and fibronectin, there were no differences in transcriptional activity between the control and TGFβ-treated cultures, despite the 2–5-fold increase in cellular levels of mRNA transcripts from these genes with TGFβ treatment. This result suggests that there is a post-transcriptional mechanism(s) operating to increase the levels of extracellular matrix-related gene transcripts following TGFβ treatment. Previous studies are in agreement with our findings that, at least for type I collagen mRNA up-regulation following TGFβ treatment of 3T3-L1 cells, control occurs at the post-transcriptional level [Raghow et al., 1987]. However, a TGFβ-inducible cis-acting element in the distal promoter of the rat type I (α_1) collagen gene has been reported [Ritzenthaler et al., 1991]. Several other studies have shown a transcriptional component to the increase in collagen type I mRNA, but in these studies a different subunit (α_2) of the type I gene was assayed which may be selectively regulated [Rossi et al., 1988].

In this study we observed a difference in the regulation of type I collagen and fibronectin gene expression in 3T3-L1 cells induced to differentiate in the presence of TGFβ. A small, but reproducible, transcriptional component was detected for the increased fibronectin mRNA levels in cells co-treated with TGFβ and the differentiation "cocktail." However, there was no increase in fibronectin transcription with TGFβ treatment of control cells. Because the only difference in these two groups is the addition of the differentiation "cocktail" (insulin, dexamethasone, and methyl isobutylxanthine), it is reasonable to consider that TGFβ alone does not alter transcriptional activity of the fibronectin gene, but acts only synergistically with the differentiation-inducing treatment. In contrast, TGFβ, either alone or with the differentiation "cocktail," did not change the rate of type I collagen gene transcription, although procollagen mRNA levels increased dramatically by an apparent post-transcriptional mechanism. These findings suggest that although procollagen and fibronectin mRNA levels exhibit similar changes in response to TGFβ treatment of 3T3-L1 cells, either when TGFβ is added to control or differentiation-induced cells, there are differences in the

involvement of transcriptional and post-transcriptional regulatory mechanisms that mediate type I procollagen and fibronectin gene expression.

Taken together these results demonstrate that there are multiple levels of control for the regulation of collagen and fibronectin gene expression during adipocyte differentiation. Although our findings do not distinguish the extent to which post-transcriptional regulation of collagen and fibronectin expression is similar, it is evident that there is a transcriptional component to the TGFβ-mediated regulation of the fibronectin gene, which is not operative in regulation of collagen. Despite variation in control of type I procollagen and fibronectin gene expression there is an equivalent downregulation in cellular levels of mRNA transcripts from these extracellular matrix genes which correlates with morphological and biochemical parameters of adipocyte differentiation. TGFβ appears to play a key role in mediating extracellular matrix modifications that inhibit expression of the adipocyte phenotype and additionally, by inhibiting 3T3-L1 differentation in a developmentally selective manner, can increase our understanding of the role of extracellular matrix-associated regulatory mechanisms in differentiation.

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