Modulation of Adipocyte Differentiation by Tumor Necrosis Factor and Transforming Growth Factor Beta

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Abstract. Cultured TA1 adipocytes treated with tumor necrosis factor alpha (TNF) lose intracytoplasmic lipid and, over a period of days, come to resemble their predifferentiated progenitors (preadipocytes). To examine the extent to which this phenotypic reversion represents a return to a less differentiated cell, we examined three major characteristics that distinguish preadipocytes from adipocytes: (a) pattern of gene expression; (b) hormonal requirement for accelerated adipogenesis; and (c) pattern of protein synthesis. We found that within hours of TNF addition to adipocytes, mRNAs for genes whose expression is augmented during adipogenesis decreased to predifferentiated levels; in addition, like preadipocytes, TNF-treated adipocytes required exposure to hormones to accelerate adipogenesis. Further, the pattern of protein synthesis seen on polyacrylamide gels reverted to that seen before differentiation. Transforming growth factor-beta (TGF-β) also caused a rapid decrease in expression of adipose genes when added to fully differentiated cells, an effect that was achieved by treatment with either TGF-β1 or TGF-β2. These effects were seen in the absence of a demonstrable proliferative response to either TNF or TGF-β. Thus characteristics that define the "terminally" differentiated state in adipocytes are subject to modulation by environmental influences.

The acquisition of a specialized phenotype, which characterizes the latter stages of differentiation of cells and tissues, is associated with the coordinate expression of new genes and the concomitant loss of expression of genes specific to the less differentiated cell. Although it is generally believed that this process is unidirectional, the differentiated state of a cell can be altered under experimental situations. "Reprogramming" experiments, which can be accomplished, for example, by fusing cells of different lineages in culture (6), presumably reflect the presence of diffusible factors that regulate the state of differentiation. In recent experiments in nonfusing myogenic cell lines, fibroblast growth factor has been shown to reverse muscle-specific gene expression independent of cell proliferation (14). In the experiments described below we consider the possibility that some differentiated events in adipocytes may be, at least in part, reversible and regulated by two products of activated macrophages: tumor necrosis factor alpha (TNF) and transforming growth factor beta (TGF-β).

TNF, initially described as an agent with necrotic effect on tumors (8), has recently been shown to have a wide variety of effects on normal cells as well. For example, TNF has been shown to affect endothelial cells, modifying the endothelial surface to accelerate the transport of phagocytes across the capillary wall (18, 28). In addition, TNF stimulates endothelial cells to produce granulocyte/macrophage colony stimulating factor (7) and augments cellular expression of HLA-A,B antigens (15). TNF inhibits myogenesis in human muscle cells (26) and augments expression of ferritin heavy chain in both human muscle cells and adipocytes (44). TNF also mediates the fever and vascular instability (shock) which accompany endotoxemia (45), and may play an important role in the cachexia of chronic infections and cancer (3, 30, 43). TGF-β also exhibits potent, but generally quite different, effects on a wide variety of cell types. It inhibits the proliferation of epithelial cell lines while stimulating the proliferation of some mesenchymal cells; some of these effects may be achieved through elevated expression of components of the extracellular matrix (25). In addition, TGF-β has profound effects on the expression of specific phenotypes in cells that differentiate: TGF-β promotes chondrogenesis and epithelial cell differentiation while blocking adipogenesis, myogenesis, and hematopoesis in vitro (42).

Among the best characterized tissue culture models of differentiation are sublines of fibroblasts that, upon growth arrest and appropriate hormonal manipulation, exhibit the characteristics of adipocytes (10, 11, 19, 23, 33). We have recently observed that cachectin, a monokine produced by endotoxin-stimulated macrophages, specifically inhibits the...
expression of several differentiation-specific genes whose expression is characteristic of adipocytes (43). By sequence homology (4) and function (27, 43) TNF and cachectin have proven to be the same molecule. In light of the close morphological similarity observed in our earlier experiments between preadipocytes and cachectin-treated adipocytes, we were led to examine whether recombinant TNF might be capable of modulating the differentiated state in these cells. Further, since TGF-β has recently been shown to be a product of activated macrophages (2) and has been reported to inhibit adipocyte differentiation (20, 41), we examined whether TGF-β might also evoke a pattern of gene expression characteristic of the predifferentiated adipocyte.

Materials and Methods

Cell Culture and Hormonal Treatment
TAI cells (10), an adipogenic cell line derived from 5-aza-cytidine treatment of 10T1/2 C18 cells (33), were grown in Eagle's basal medium (Gibco Laboratories, Grand Island, NY), supplemented with 10% FBS (Gemini Bio-products, Inc., Calabasas, CA) heat inactivated for 30 min at 56°C. In certain experiments FBS was replaced with defined supplemented calf serum (HyClone Laboratories, Logan, UT). Cells were maintained at 37°C in a humidified incubator in 5% CO₂. Cultures were fed every 3 d. The day at which cells reach confluence is referred to as day 0. Differentiation was accelerated either by the addition of media supplemented with 10⁻⁶ M dexamethasone plus 1.25 × 10⁻⁴ M indomethacin from day 0 to day 3 or by the addition of indomethacin (at 0.3–1.25 × 10⁻⁴ M) alone. After cells had been induced to differentiate they were maintained in growth medium without hormones. Human recombinant tumor necrosis factor (specific activity = 10¹⁷ U/mg rTNF (Cetus Corp., Emeryville, CA) was added at a final concentration of 1 U/ml. TGF-β (unfractionated), TGF-β₁, and TGF-β₂, purified from porcine platelets, were purchased from R & D Systems, Inc. (Minneapolis, MN).

RNA Isolation
Total RNA was prepared from adipocyte cultures as described by Chirgwin et al. (13). Cells were washed with PBS and lysed by the addition of a solution of 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.2% N-lauryl sarcosine, and 0.2 mM 8-mercaptoethanol applied directly to the culture dishes. The cell lysate was layered on 5.7 M cesium chloride and centrifuged at 80,000 g for 19–24 h. The RNA pellet was resuspended in 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 0.5 mM MnCl₂, 2 mM DTT, 0.1 mM EDTA, 0.4 mM each of ATP, CTP, and GTP, 10% glycerol, and 10 µg/ml BSA. Nuclei were incubated with alpha ³²P-UTP (5,200 Ci/mmol; ICN Radiochemicals, Irvine, CA) at a concentration of 2 mCi/ml for 40 min at 25°C with gentle shaking.

RNA was harvested from nuclei as described by Smith et al. (40) and modified by Knight et al. (23), and hybridized to linearized cDNAs that had been applied to nitrocellulose filters and baked for 2 h at 80°C in a vacuum oven. Filter prehybridization and hybridization reactions were those of Friedman et al. (17). Hybridizations were performed for 4 d at 42°C with ~15 × 10⁶ cpm per reaction mixture in 200 µl vol.

Protein Analysis
Day 3 TAI adipocytes (differentiated with 1.25 × 10⁻⁴ M indomethacin) were incubated for 4 h in methionine-deficient basal Eagle's medium without serum. 100 µCi [³⁵S]methionine (Bethesda Research Laboratories) was then added per milliliter tissue culture media for 1 h. Total cellular proteins were isolated and subjected to electrophoresis under denaturing conditions in 10% polyacrylamide gels.

Hybridization Conditions
Prehybridizations and hybridizations were performed at 42°C with 50% formamide, 5× SSPE (1× SSPE = 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA, 2× Denhardt's reagent, (1× = 0.02% each of Ficoll, polyvinylpyrrolidone, and BSA), 200 µg/ml denatured Herring sperm DNA. Prehybridizations were from 2–25 h, hybridizations for 48 h. cDNA clones were nick translated (34) or labeled by the random primer method (16), and 1–5 × 10⁶ cpm of denatured ³²P-labeled cDNA was added for each milliliter of hybridization mix. Filters were washed in 2× SSPE, 0.1% SDS at room temperature, followed by two 30-min washes at 50°C. When hybridization to more than one cDNA was carried out, filters were washed between hybridizations in boiling 0.1% SDS/0.1% SSPE until no residual radioactivity remained. Autoradiography was carried out at ~70°C using XAR-5 film (Eastman Kodak Co., Rochester, NY) with one intensifying screen. Autoradiographs were analyzed using a scanning densitometer (Schleicher & Schuell, Inc., Keene, NH) attached to a reporting integrator (Hewlett-Packard Co., Palo Alto, CA).

cDNA Clones
cDNA clones of genes whose expression was enhanced in TAI adipocytes, isolated from a library of TAI adipocytes by differential screening (10), were used to assess the effects of TNF and TGF-β on adipocyte gene expression. In addition, a cDNA clone for glycerophosphate dehydrogenase (GPD) derived from 3T3-L1 cells was provided by Dr. Bruce Spiegelman. β-actin was obtained from Dr. Larry Kedes and pyruvate kinase from Dr. Keith Webster.

Results

Effect of Recombinant TNF in Adipocytes and Preadipocytes
As seen in Fig. 1, 5 d of exposure to recombinant TNF reverses lipid-laden TAI cells to preadipocyte morphology with concomitant loss of lipid droplets. Preceding this reversal in differentiated morphology of TAI adipocytes with...
TNF, there is a rapid and coordinate decrease in the expression of adipocyte-inducible genes, both those expressed only upon differentiation (class I: clones 47, GPD) and those expressed before differentiation but augmented during differentiation (class II: clones 20, 27) (Fig. 2). Cell counts and measurements of thymidine incorporation showed that these effects were obtained without any demonstrable proliferative effects on this cell line (data not shown). Thus TNF not only reversed the differentiated cellular phenotype, but caused a return to predifferentiated levels of gene expression of an entire set of genes specifically inducible during adipogenesis in tissue culture.

This effect of TNF on both class I and II adipocyte genes is seen in preadipocytes as well as adipocytes. Fig. 3 demonstrates that a gene actively transcribed before differentiation, but whose expression is further augmented during adipogenesis (clone 27) maintains expression at predifferentiated levels with TNF treatment, just as class I genes (clones 1, 28, GPD) mRNAs are completely inhibited by TNF. Thus, TNF inhibits expression of both transcriptionally active and quiescent genes in preadipocytes as well as repressing their expression in fully differentiated adipocytes.

In most developmental systems, terminal differentiation is associated with coordinate transcriptional activation of genes for the major cellular products of the particular differentiated cell. We therefore tested whether the decreased mRNA levels we observed after TNF treatment of TAI adipocytes is a result of transcriptional inhibition of those genes. We had pre-

Figure 3. TNF inhibits type I and type II adipose gene expression in preadipocytes. TAI cells were grown in Eagle's basal medium supplemented with 10% heat inactivated FBS. 10^{-8} M dexamethasone and 1.25 \times 10^{-4} M indomethacin were present from day 0 to day 3. More than 90% of the control cells developed a typical adipocyte morphology under these conditions. TNF at 10 ng/ml was added to preadipocytes 2 d before they reached confluence and maintained in the medium until harvest. Total RNA was isolated and 10 ug of each sample used to prepare Northern blots. Blots were probed with nick-translated cDNAs corresponding to adipose-inducible clones 1, 28, 27, and GPD. Lanes A contain RNA from control preadipocytes; lanes B from control adipocytes harvested at day 3; and lanes C from TAI cells treated with TNF from day 2 to day 3.
Figure 4. TNF inhibits clone 28 gene expression at a transcriptional level. $^{32}$P-labeled RNA synthesized in isolated nuclei (see Materials and Methods) was hybridized to 3 μg immobilized cDNA of clone 28 (28), β-actin (A), or a pEMBL plasmid control (E). Results are shown for nuclei obtained from cells before differentiation (PC), at day 3 when TNF was added 2 d before differentiation (TNF-PC), in adipocytes (CONTROL), and when TNF was added to already differentiated (day 3) adipocytes for 24 h (24).

Effect of TGF-β on Adipocytes and Preadipocytes

We next compared the effects of TNF on adipocyte gene expression and on adipocyte morphology with another agent known to inhibit adipocyte differentiation, TGF-β. TGF-β has been previously reported to inhibit differentiation when added to 3T3-L1 preadipocytes during the early stages of hormonal exposure which triggers differentiation (20). Treatment during the latter stages of this period had no effect on either development of adipocyte morphology or appearance of GPDH activity. In our experiments, we added TGF-β to actively proliferating TA1 preadipocytes and maintained it in the medium continuously until control cells had achieved maximal levels of adipocyte gene expression. In confirmation of previous experiments (20, 41), we found that this treatment prevented the appearance of lipid droplets and expression of adipose genes (not shown). We also added TGF-β to fully differentiated adipocytes and measured its effects on morphology and gene expression. Unexpectedly, we found that TGF-β could exert an effect on TA1 adipocytes as well as preadipocytes, resulting in the slow disappearance of lipid-laden cells from the population over the course of several days. We examined whether this morphological change was accompanied by changes in gene expression by treating adipocytes with TGF-β or TNF and analyzing expression of clone 28 after 4, 24, and 48 h. Fig. 5 demonstrates that TGF-β reduces expression of this gene as rapidly and effectively as TNF, with a decrement in expression evident as early as 4 h after treatment. Expression of the adipose gene clone 1 was also reduced by exposure to TGF-β, although the control gene pyruvate kinase was unaffected (not shown). These effects were achieved in the absence of any proliferative response to TGF-β (not shown).

These experiments were performed with TGF-β purified from porcine platelets, a preparation now known to contain multiple forms of TGF-β, including TGF-β1 and TGF-β2 (12). Although TGF-β1 and TGF-β2 have generally been found to exert similar effects, there are examples of biologi-
TGF-β, we measured the dose response of clone 28 expression after comparable exposure to TGF-β~ or TGF-β~. Cells were harvested, RNA isolated, and dot blots prepared. These were hybridized with clone 28 cDNA, washed, and then hybridized with pyruvate kinase (a gene whose expression is unaffected by TGF-β). Autoradiograms were scanned with a densitometer. Units represent the ratio of signal intensity obtained with clone 28 to that obtained with pyruvate kinase. (b) Adipocytes were grown, exposed to 5 ng/ml TGF-β~ or 5 ng/ml TGF-β~ for various periods of time, and clone 28 expression was analyzed as in a.

Protein Synthetic Pattern in TNF-treated Adipocytes

There are at least two ways to interpret the effects of TNF and TGF-β on mature adipocytes. These agents may selectively inhibit the expression of a subset of genes, some of which we have the ability to measure, without affecting the differentiated state per se. Alternatively, they may interfere with the regulatory processes that commit TAI cells to differentiate and thereby cause them to return to the predifferentiated state. We have taken advantage of the capability of glucocorticoid hormones such as dexamethasone (11) and the drug indomethacin (23) to precociously trigger the differentiation of TAI preadipocytes in an effort to address this issue. Using dexamethasone and indomethacin, induction of adipocyte-inducible mRNAs occurs earlier but, in general, not to a greater extent than in control cultures (11, 23). In addition, it appears that the effect is a triggering phenomenon, since only transient exposure to the hormone or drug is required to accelerate differentiation. We first reversed the differentiated phenotype with TNF and obtained a population of cells, like those in Fig. 1, indistinguishable at a morphologic level from preadipocytes. Then we asked whether the kinetics of reinduction of adipocyte-inducible mRNAs followed a pattern of drug-exposed or drug-naive cells. The results with indomethacin (Fig. 7) and dexamethasone (not shown) indicated that TAI cells which were once fully differentiated revert, upon TNF treatment, to a state indistinguishable from the preadipocyte, in that drug or hormonal treatment is again required to accelerate reexpression of adipocyte-inducible genes.

**Table I. The Effect of TGF-β on Clone 28 Expression Is Serum Dependent**

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<thead>
<tr>
<th>Treatment</th>
<th>FBS</th>
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<tr>
<td>Control</td>
<td>100</td>
<td>104</td>
</tr>
<tr>
<td>TGF-β~</td>
<td>6</td>
<td>32</td>
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<tr>
<td>TGF-β~</td>
<td>9</td>
<td>43</td>
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TAI cells were grown and differentiated in the presence of indomethacin from day 0 to day 3 in media supplemented either with 10% FBS or 10% calf serum (CS). After withdrawal of indomethacin for 24 h, cells were refed medium containing either serum alone (Control); 5 ng/ml TGF-β~ or 5 ng/ml TGF-β~. After an additional 24 h, cells were harvested. RNA was isolated and dot blots prepared. These were hybridized with radiolabeled clone 28 cDNA and pyruvate kinase as described in the legend to Fig. 6. The ratio of signal intensities for these two probes in adipocytes differentiated in the presence of FBS was defined as 100%.
tion that disappear or are greatly reduced on differentiation and pattern of newly synthesized high molecular mass proteins in day Figure 8. Protein synthetic pattern in TNF-treated adipocytes. The pattern of newly synthesized high molecular mass proteins in day 3 adipocytes 24 h after the addition of 10 ng/ml TNF (TNF) is compared to proteins synthesized in preadipocytes (pre) and untreated adipocytes (fat). The arrows indicate bands present before differentiation that disappear or are greatly reduced on differentiation and return with TNF treatment. ● indicates bands present in the differentiated TA1 adipocyte which disappear with TNF treatment. Molecular masses of marker proteins that were subjected to coelectrophoresis are shown at right.

Discussion

From the RNA analyses and within the resolution of one-dimensional protein gels, it appears that the overall pattern of gene expression in TNF-treated adipocytes is strikingly similar to that of the TA1 preadipocyte. Thus, by the criteria which have been traditionally used to define differentiation in these cultures, the preadipocyte and TNF-treated adipocyte cannot be distinguished. Yet are these cells truly dedifferentiated?

It appears that TNF is not simply acting as a lipolytic agent on these cells. For example, among the large number of new proteins that characterize the differentiated adipocyte are certain enzymes which have been used to define the differentiated phenotype. The appearance of these enzymes is independent of lipid accumulation in the adipocyte, since they are expressed in differentiated cells in which lipid accumulation is inhibited. One such enzyme is GPD, which is fully expressed in adipocytes differentiated in biotin-deficient media, conditions that completely prevent intracytoplasmic lipid accumulation (24). Such enzymes can serve as markers to distinguish between catabolic states where lipid accumulation might be suppressed from a predifferentiated precursor, the preadipocyte. As seen in Fig. 2, the mRNA for GPD was completely inhibited by TNF; strongly suggesting that TNF treatment does not simply prevent morphologic evidence of differentiation, but inhibits those early steps in gene transcription that characterize the differentiated state.

However, although changes in gene expression engendered by TNF appear different from those seen in an adipocyte exposed to lipolytic stimuli, it is unlikely that the TNF-treated (or TGF-β-treated) adipocyte is identical in every respect to a preadipocyte. We have recently observed that the expression of ferritin heavy chain is stimulated by TNF to levels exceeding those found in the preadipocyte (44); thus there exists at least one gene that can distinguish control preadipocytes from TNF-treated adipocytes. In addition, adipocytes acquire characteristics specific to the cytokine with which they were treated; for example, TGF-β, like TNF, inhibits differentiation when added to preadipocyte cultures (20) and causes decreases in lipid accumulation and reduction in expression of adipose-inducible genes (Fig. 5), but does not induce expression of ferritin heavy chain (F. Torti, unpublished observations). As more markers of TNF- and TGF-β-treated cells are characterized, further distinctions among the preadipocyte, TNF-, and TGF-β-treated adipocyte will likely become evident.

The ability of TGF-β to reduce expression of adipose genes in differentiated adipocytes has not been previously described. Ignotz and Massague (20) showed that 3T3L1 preadipocytes could be inhibited from differentiating into adipocytes by TGF-β. However, TGF-β added during the last 16 h of hormonal induction failed to prevent differentiation. They therefore proposed that fully committed adipocytes become refractory to TGF-β, although they do not lose TGF-β receptors. In our experiments, TA1 adipocytes exposed to TGF-β for prolonged periods of time (6 d) gradually evidenced a loss of lipid. The reduction in adipose gene expression was even more rapid, occurring in several hours in the presence of either TGF-β, or TGF-β₂. The difference between our results and those obtained in 3T3L1 cells (20) may reflect differences in the cell lines used, or be related to the serum dependence of the TGF-β effect.

We found the degree of decrease in gene expression mediated by TGF-β to be affected by serum components. This is perhaps not surprising in view of the highly interactive na-
tecture of TGF-β. For example, TGF-β synergizes with EGF in promoting anchorage-independent growth of NRK cells (1), and in the presence of PDGF, can stimulate colony formation in myc-transfected fibroblasts (35). Maximal inhibition of adipose gene expression in adipocytes may therefore require the synergistic assistance of an additional serum factor(s), which may be variably represented in different sera. Alternatively, factor(s) present in some sera may inhibit the action of TGF-β on fully differentiated adipocytes. Possible candidates include a TGF-β binding protein secreted by activated macrophages and capable of preventing binding of TGF-β to its receptor (2); or growth factors, which have been shown to be capable of reversing some effects of TGF-β (e.g., its mitogenic action on osteoblasts, which can be reversed by EGF, PDGF, and TNF [9]). Although the response of preadipocytes to TGF-β appeared less dependent on serum factors than that of adipocytes, this may simply reflect an altered dose responsiveness to serum components in these two cell types. Alternatively, since TGF-β effects can be altered and even reversed depending on the state of differentiation of the target cell (37, 38), it is possible that TGF-β-dependent inhibition of differentiation is mechanistically independent from its ability to inhibit adipose gene expression in adipocytes. Clarification of these issues will require the use of defined growth factors in combination with TGF-β; these experiments are currently in progress. Nevertheless, although the mechanism of action of TGF-β on adipose genes may be more complex or more sensitive to environmental influences than that of TNF, it is clear that TGF-β, like TNF, can affect the expression of adipose genes in fully differentiated adipocytes.

The ability of TNF and TGF-β to alter the state of adipocyte differentiation and gene expression may have physiological relevance. There is, for example, marked involution of adipocytes in mammary tissue during lactation (39), perhaps reflecting a reduced need for storing lipids and an increased requirement for lipid production in milk. The uncontrolled metabolic wasting associated with the cachexia of cancer or chronic infections is associated with a progressive loss of adipose tissue and a pattern of metabolic changes similar to those associated with cachectin/TNF production (5, 21, 22, 30, 31). Whether TNF is a mediator of any of these processes in vivo is unknown. Nevertheless, the ability of TNF to convert mature adipocytes to ostensibly normal preadipocytes may be associated with physiological states in which both mobilization of energy stores and prevention of lipid storage are involved. Interestingly, TGF-β has also been implicated in the response to stress and tissue damage (25, 42) and has recently been shown to inhibit mammary ductal growth in vivo (39). Like TNF, the secretion of TGF-β is accelerated in activated macrophages (2). These observations, in conjunction with the similarity of effects of TNF and TGF-β on adipose cells which we report here, make it tempting to speculate that these two disparate molecules, whose known functions show little overlap, may collaboratively affect tissue differentiation and in the pathophysiology of cachexia.

Regardless of any hypothesis concerning the physiological role of TNF and TGF-β the effects on adipocytes we have described here are striking. By many criteria, including the ability to rapidly and coordinately inhibit the expression of several adipose-inducible genes, TNF appears to revert differentiated TA1 cells to a state characteristic of preadipocytes. TGF-β also is capable of affecting expression of differentiated functions in adipocytes, although its mechanism of action may be more complex. Elucidation of the mechanisms by which TNF and TGF-β exert their effects may provide insight into the events required for triggering the so-called "terminal," but largely reversible, differentiation of this cell line.

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