Tumor necrosis factor-induced c-myc expression in the absence of mitogenesis is associated with inhibition of adipocyte differentiation

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ABSTRACT Tumor necrosis factor (TNF) inhibits and reverses differentiation of mouse adipogenic TA1 cells. We have found that TNF induces c-myc in a sustained manner in both preadipocytes and adipocytes; in contrast, serum induces c-myc transiently and only in preadipocytes. This TNF-mediated c-myc induction is not coupled with cell proliferation but is correlated with TNF-mediated inhibition of adipocyte differentiation. We prepared an inducible c-myc transformant of TA1 cells by transfection of the mouse c-myc gene under the control of the metallothionein-I promoter. These cells are unable to differentiate to adipocytes in the presence of Zn²⁺/Cd²⁺, and in differentiated TA1 cells, Zn²⁺/Cd²⁺ causes reduction of adipocyte-specific gene expression as does TNF. Lastly, exposure of TA1 cells to antisense c-myc oligonucleotide partially blocked the TNF-mediated reduction of adipocyte-specific gene expression. Thus, TNF-mediated c-myc expression is distinct in character from that involved in mitogenic responses but appears to play an important role in inhibition and reversal of adipocyte differentiation.

Tumor necrosis factor (TNF) has a wide variety of biological activities depending on the cell type and the growth state of cells, including cytotoxicity and modulation of cell growth and differentiation (1). TNF initiates its functions through two distinct TNF receptors: type I (55–60 kDa) and type II (75–80 kDa) and stimulates intracellular events, including activation of protein kinase C (2) and release of arachidonic acid (3). Through activation of transcription factors AP1, NfXB, and possibly other DNA-binding proteins, TNF induces expression of many genes including metalloproteinases, cytokines ( interleukin 1 and interleukin 6), and immediate-early genes of growth stimulation (c-fos, c-jun, and c-myc) (1). Despite extensive study of cellular responses to TNF, it remains uncertain which events are important for particular actions of TNF.

In mouse adipogenic cell lines such as TA1 and 3T3-L1, TNF completely inhibits the process of adipocyte differentiation and reverts the fully differentiated adipocyte to the preadipocyte (4, 5). Addition of TNF prevents expression of adipocyte-specific genes, such as p2, GPD1 (glycerol phosphate dehydrogenase), and FSP27, in differentiating adipogenic cells and reverses expression in fully differentiated cells (5, 6). It has recently been shown that TNF reduces the level of the CCAAT/enhancer-binding protein α (C/EBP-α) in fully differentiated adipocytes (7–10), which has been implicated as a major regulator of adipocyte-specific gene expression (11–14). Immediate-early genes such as c-fos and c-jun are transiently induced by TNF in TA1 cells; however, their induction has not been associated with inhibition of adipocyte differentiation (3).

Expression of c-myc is known to be important for promotion of cell growth (15). When resting cells are stimulated to proliferate with serum or other mitogens, c-myc message increases rapidly and decreases 8–12 hr after stimulation (15, 16). In contrast, we have found that TNF induces c-myc message for at least 48 hr in TA1 cells but does not induce cell proliferation in either preadipocytes or fully differentiated adipocytes. Many studies have shown that ectopic expression of c-myc interferes with differentiation of various cell types, including adipogenesis in 3T3-L1 cells (17). Here we demonstrate that: (i) c-myc expression is correlated with TNF-mediated reduction of adipocyte-specific gene expression; (ii) c-myc expression under the control of the metallothionein-I promoter is able to mimic the effect of TNF; (iii) antisense oligonucleotides to c-myc partially block TNF action; (iv) the induction of c-myc by TNF, unlike that by serum, is independent of mitogenesis. These results strongly suggest that the unusual pattern of c-myc induction by TNF is central to its ability to interfere with adipocyte differentiation.

MATERIALS AND METHODS

Cells and Plasmids. TA1 cells (18) were cultured in Eagle’s basal medium (BME Earle’s salts medium) supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO₂. TA1 cells were differentiated spontaneously by the continuous maintenance of confluent cells; ≈70% of the population was detected as adipocytes 6–10 days after reaching confluence. The developmental process was defined by referring to the day cells reach confluence as day 0. Purified recombinant TNF-α was provided by Cetus.

pHygMT-c-myc plasmid contains the hyg gene from pHyg (19) and mouse c-myc cDNA (provided by Naoko Arai, DNAX) under the control of metallothionein-I promoter. A 1.9-kb EcoRI–Bgl II fragment containing metallothionein-I promoter region (20) was subcloned into EcoRI–BamHI sites of pBluescript II SK(+) (Stratagene) and recovered on a HindIII–Xba I fragment containing the promoter region. The SRE promoter was removed from pHygSRα vector (21, 22) by HindIII and Xba I digestion and replaced with the HindIII–Xba I metallothionein-I promoter fragment to yield pHygMT plasmid. The mouse c-myc cDNA was inserted into the BamHI site downstream of the metallothionein-I promoter in pHygMT, and the plasmid containing c-myc cDNA in the sense orientation was used in this study.

Northern Blotting. Total cellular RNA was prepared by the method of Chirgwin et al. (23). Ten micrograms of total RNA was separated by electrophoresis on 1.1% agarose/6% formaldehyde gel, followed by blotting onto nylon filter (Hybond-N*, Amersham). The fragments of mouse c-myc, mouse c-fos (from Naoko Arai), human c-JUN (3), human ferritin light chain gene (24), mouse thymidine kinase gene (ATCC 37556), p2 cDNA (18), and FSP27 cDNA (6) were labeled by using

Abbreviations: TNF, tumor necrosis factor; C/EBP-α, CCAAT, CCAAT/enhancer-binding protein α; FCS, fetal calf serum.

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random primers to specific activity of $2 \times 10^8$ cpm/µg. Hybridization and washing was done as described (24).

Antisense Oligonucleotide to c-myc. Oligodeoxynucleotides, antisense (a/s) c-myc CACGTTGAGGCCAT and sense (s) c-myc ATGCCCTCAACGTG (25), were synthesized with an Applied Biosystems DNA synthesizer. Oligonucleotides were added to culture medium at 200 µg/ml.

c-Myc Protein Analysis. Cells were harvested 24 hr after addition of TNF and oligonucleotide and lysed in 1 mM phosphate buffer, pH 7.25/1% Triton X-100/0.5% deoxycholate/0.1% SDS/0.2% sodium azide. One milligram of cell lysate was preadsorbed with protein A-agarose and then incubated with 6E10 anti-c-myc antibody (Cambridge Research Biochemicals) (26) and protein A at 4°C overnight. Immunoprecipitated c-Myc protein was separated on SDS/polyacrylamide gels and electrophoreted onto Immobilon-P (Millipore). The filter was incubated for 1 hr in phosphate-buffered saline/0.2% Tween 20 (PBS-T)/5% nonfat dried milk and then incubated for 1 hr in the same solution containing 6E10 antibody to 5 µg/ml, washed three times for 5 min in PBS-T/1.8% Nonidet P-40/0.2% SDS and incubated for 45 min with anti-mouse IgG conjugated with peroxidase. p67-69-myc protein was visualized by the enhanced chemiluminescence system (Amershram).

RESULTS

Prolonged Induction of c-myc mRNA by TNF in TA1 Cells. As reported (3), TNF induces c-fos and c-jun mRNA transiently in preadipocyte TA1 cells at day −1 (Fig. 1a). The time course of induction of these mRNAs in TNF-treated cells is very similar to that observed in serum-stimulated cells (Fig. 1a); this pattern of expression is also seen in fully differentiated cells (Fig. 1b). In contrast, c-myc mRNA is induced by TNF in both preadipocytes and adipocytes in a sustained manner, lasting at least 24–48 hr after stimulation, whereas serum stimulation results in transient induction of c-myc message only in preadipocytes (Fig. 1a and b). TNF does not induce cell division in either preadipocytes or adipocytes, whereas significant increase of mitotic cells is seen after serum stimulation in preadipocytes (Table 1). DNA synthesis, as monitored by incorporation of 5-bromodeoxyuridine (BrdUrd), is induced by serum in preadipocytes but is not induced by TNF (Table 1). In addition, thymidine kinase mRNA is induced in serum-stimulated preadipocytes, but no induction is observed in any TNF-stimulated cells or in serum-stimulated adipocytes (Fig. 2).

TNF treatment dramatically reduces both aP2 and FSP27 (adipocyte-specific genes) expression in adipocytes as reported (5), concomitant with increased abundance of c-myc mRNA (Fig. 1b). When TNF was removed from the culture medium, c-myc message decreased, and the levels of aP2 and FSP27 messages began to increase (Fig. 1c). These results suggest that prolonged induction of c-myc by TNF is not coupled with cell proliferation but is correlated with inhibition of adipocyte differentiation.

Exogenous c-myc Expression Mimics the Effect of TNF. Deregulated c-myc expression has been reported to abolish differentiation in 3T3-L1 adipogenic cells; however, morphological transformation of these cells may have contributed to the observed effects (17). To test whether c-myc is involved in the TNF signal-transduction pathway leading to inhibition

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<th>DNA synthesis and cell division</th>
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<td>Control</td>
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<tr>
<td>BrdUrd positive, %</td>
<td>5.1</td>
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<td>Mitotic index, %</td>
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TA1 cells (day −1 and day 8) were incubated with 150 µM BrdUrd for 18–24 hr after stimulation with 10% serum or TNF at 30 ng/ml. The S-phase index was measured by staining cells with a monoclonal antibody to BrdUrd (Oncogene Science), according to the supplier. Results represent the percentage of BrdUrd-positive cells determined by counting ~1000 cells in a fluorescence microscope. Mitotic cells were counted (500–1000 total cells in each experiment) at 24 hr after stimulation. Results represent the average (±SEM) of four experiments.
and reversal of adipocyte differentiation, we isolated inducible c-myc transformants of TA1 cells. TA1 cells were transfected with pHygMT-c-myc plasmid, which contains a mouse c-myc cDNA downstream of the metallothionein-1 promoter. Inducibility of c-myc message was tested in 16 independent clones. Two clones initially showed good induction of exogenous c-myc mRNA by addition of Zn\textsuperscript{2+}/Cd\textsuperscript{2+}. One clone (MTmyc-2) that retained c-myc inducibility over 3 months was used for the following study. In MTmyc-2 cells, addition of Zn\textsuperscript{2+}/Cd\textsuperscript{2+} induces exogenous c-myc message within 2.5 hr, and the level of message remains elevated for at least 24–48 hr; removal of Zn\textsuperscript{2+}/Cd\textsuperscript{2+} from the culture medium reduces the level of c-myc mRNA within 24 hr (Fig. 3 a and b). This time course of exogenous c-myc induction with Zn\textsuperscript{2+}/Cd\textsuperscript{2+} is very similar to that of endogenous c-myc induction mediated by TNF (Fig. 1).

We examined whether regulated expression of c-myc in MTmyc-2 cells could mimic the effect of TNF on preadipocytes and adipocytes. First, three independent control transformants (vector alone) and MTmyc-2 cells were incubated with Zn\textsuperscript{2+}/Cd\textsuperscript{2+} or TNF from preconfluence (day −1) to day 5, and expression of adipocyte-specific genes was monitored. Consistent with prior experiments (6, 7), expression of aP2 and FSP27 was not detected in the presence of TNF in all clones (Fig. 4a, lanes 3). Expression of aP2 and FSP27 was reduced in the presence of Zn\textsuperscript{2+}/Cd\textsuperscript{2+} in MTmyc-2 cells but was not reduced in control transformants (Fig. 4a, lanes 2), suggesting that Zn\textsuperscript{2+}/Cd\textsuperscript{2+} inhibits differentiation of MTmyc-2 cells via exogenous c-myc expression. In fully differentiated (day 9) MTmyc-2 cells both Zn\textsuperscript{2+}/Cd\textsuperscript{2+} and TNF reduce levels of aP2 and FSP27 mRNA 48 hr after addition (Fig. 4b, lanes 1, 2, and 4). Removal of Zn\textsuperscript{2+}/Cd\textsuperscript{2+} or TNF from the culture medium results in recovery of aP2 and FSP27 mRNA (Fig. 4b, lanes 3 and 5). These results demonstrate that induction of c-myc expression can mimic TNF with respect to inhibition and reversal of adipocyte differentiation and that continued expression of c-myc is required to elicit these effects.

**Antisense c-myc Oligonucleotide Interferes with TNF Action.** To test whether inhibition of c-myc expression blocks TNF action, we attempted to reduce c-Myc protein levels with a 15-base antisense oligodeoxynucleotide complementary to codons 1–5 of the mouse c-myc message. Fully differentiated TA1 and MTmyc-2 cells were incubated for 24 hr with TNF and the c-myc antisense oligonucleotide or with TNF and sense oligonucleotide as a control. As anticipated based on studies in other systems (27), the antisense c-myc oligonucleotide reduces the level of c-Myc protein significantly, although it was not reduced to control levels in TA1 cells (Fig. 5a). Fig. 5b shows that TNF-mediated reduction of aP2 expression was partially reversed by exposure of cells to the antisense but not to the sense c-myc oligonucleotide. These results suggest that c-myc expression is important for TNF-mediated reduction of expression of adipocyte-specific genes.

**DISCUSSION**

Growth stimulation by various mitogens and cytokines, including TNF, has been reported to be accompanied by...
Fig. 5. Antisense c-myc oligonucleotide inhibits c-Myc protein and interferes with TNF-mediated reduction of aP2 expression. Differentiated TAl cells or MTMyC-2 cells (days 7–10) were refed and left untreated (control) or incubated for 24 hr with TNF at 30 ng/ml and sense c-myc oligonucleotide (s-myc) or antisense c-myc oligonucleotide (a/s-myc) at 200 μg/ml. (a) The c-Myc protein was immunoprecipitated by anti-c-Myc antibody. The intensity of the c-Myc protein bands in two independent experiments was measured by scanning densitometry. (b) The amount of aP2 mRNA was measured by scanning densitometry of RNA blots. The aP2 mRNA level from control cells (no TNF/no oligonucleotide) was defined as 100%. Results represent the average of five experiments ± SEM.

induction of proto-oncogenes, such as c-fos, c-jun, and c-myc (16, 28–30). Their mRNAs are induced immediately after stimulation and decline to low or undetectable level by 60 min (c-fos), 4–12 hr (c-jun), or 10–24 hr (c-myc). In contrast to growth-coupled c-myc induction, TNF induces c-myc mRNA for prolonged periods of at least 24–48 hr in TAI cells (Fig. 1 a and b) and, as described here, is not coupled with cell proliferation but, instead, is associated with TNF-mediated inhibition and reversal of adipocyte differentiation. The amount of c-myc mRNA in TNF-treated cells is lower than that at peak in growth-stimulated preadipocytes by serum (Fig. 1a), suggesting that a critical level of c-myc may be necessary to promote cell growth. Alternatively, transient rather than continuous increase of c-myc mRNA may be essential for progression in the cell cycle.

It remains likely that TNF-mediated inhibition of adipocyte differentiation is transduced through multiple pathways (including c-myc induction). Indeed, c-myc antisense DNA could block only a portion of the TNF-mediated reduction of adipocyte-specific gene expression. Furthermore, TNF exhibited more pronounced effects on adipocyte differentiation than did Zn2+/Cd2+ in MTMyC-2 cells (Fig. 3), despite the fact that Zn2+/Cd2+ induces c-myc to equivalent or even greater extent than does TNF in MTMyC-2 cells (data not shown).

TNF reduces the level of C/EBP-α (7–10), which acts as a transactivator of many adipocyte-specific genes and appears to play a critical role in differentiation of 3T3-L1 and TAI cells (11–14). Because c-myc message increases within 30 min after TNF addition (data not shown) and a decrease in C/EBP-α mRNA is seen only after 3–6 hr in TAI cells (10), it is possible that c-myc expression leads to inhibition of C/EBP-α gene expression; this hypothesis remains to be proven, however, by investigating the time course of TNF effects on C/EBP-α gene transcription. Because the promoter of C/EBP-α contains a consensus c-myc-binding se-quence (31), we speculate that c-myc might participate directly to inhibit expression of the C/EBP-α gene.

The protein product of the retinoblastoma susceptibility gene, Rb, regulates c-myc transcription through an E2F (transcription factor)-binding site (32, 33). The complex of underphosphorylated Rb and E2F represses transcription, whereas phosphorylation of Rb by mitogens causes dissociation of the Rb-E2F complex, resulting in activation of E2F-mediated transcription (34, 35). We have recently obtained results indicating that TNF stimulates phosphorylation of Rb in TAI cells (J.N.-T., personal observation) and, therefore, activate c-myc transcription via an E2F-dependent pathway. These and future studies will help elucidate the details of this TNF-signaling pathway, which appears to involve a regulatory action for c-myc that is distinct from its well-described role in cell proliferation.

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