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Tumor Necrosis Factor Inhibits Human Myogenesis In Vitro

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We examined the effects of human recombinant tumor necrosis factor-alpha (TNF) on human primary myoblasts. When added to proliferating myoblasts, TNF inhibited the expression of alpha-cardiac actin, a muscle-specific gene whose expression is observed at low levels in human myoblasts. TNF also inhibited muscle differentiation as measured by several parameters, including cell fusion and the expression of other muscle-specific genes, such as alpha-skeletal actin and myosin heavy chain. Muscle cells were sensitive to TNF in a narrow temporal window of differentiation. Northern (RNA) blot and immunofluorescence analyses revealed that human muscle gene expression became unresponsive to TNF coincident with myoblast differentiation. When TNF was added to differentiated myotubes, there was no effect on muscle gene expression. In contrast, TNF-inducible mRNAs such as interferon beta-2 still responded, suggesting that the signal mediated by TNF binding to its receptor had no effect on muscle-specific genes after differentiation.

Tumor necrosis factor (TNF) is a monokine secreted by activated macrophages. It was first described as a factor in the serum of endotoxin-treated mice which caused necrosis in certain transplanted tumors and cell lines (6). The recent availability of recombinant TNF-alpha (hereafter referred to as TNF) has permitted broad evaluation of its functions. Recombinant TNF, in addition to being rapidly cytotoxic to certain animal tumor cell lines, is curative in a number of murine tumors (8). Clinical trails with TNF as an antitumor agent in humans have recently been initiated.

It has become evident, however, that the cytotoxic properties of TNF represent only one aspect of the functions of this protein. Although cytotoxic effects of TNF on tumor cell lines of many species have been known for more than 10 years, only recently has a role for TNF (cachectin [3]) in normal cellular function been identified. We described the effects of TNF on a adipocyte cell line, TA1 (33), identifying a nontransformed cell as a target for TNF action. The addition of TNF to adipocytes in tissue culture causes mobilization of lipid and a reversible decrease in expression of adipose-inducible genes, effects mediated by selective changes in gene transcription. Other actions of TNF have been identified since: TNF enhances the phagocytic abilities of leukocytes (29) and modifies the endothelial surface to accelerate transport of phagocytes across the capillary wall (11), and it mediates the fever and vascular instability (shock) which accompanies endotoxemia (34). Together, these studies suggest that TNF, secreted by macrophages and monocytes in response to certain infectious or immunologic insults, plays a pivotal role in the organism's response to injury and stress.

We were thus led to examine the effects of TNF on muscle cells by two sets of observations: one that indicated that TNF mediates some responses to stress in which muscle (as well as adipose tissue) plays a prominent role, and another suggesting more general effects of TNF on cells of the mesenchymal lineage (19). Muscle cells in culture represent a well-characterized in vitro differentiation system in which morphological changes that occur as proliferating myoblasts differentiate into myotubes (26) are accompanied by well-described alterations in the expression of a number of proteins, including those of the myosin (30) and actin (1) gene families. Furthermore, methods for cultivating human primary muscle cells in quantity have recently been developed (38). This has allowed us to assess the effects of human recombinant TNF on a normal human target cell.

We show here that TNF, in a defined period during differentiation of human muscle cells in culture, specifically and reversibly inhibits differentiation of these myogenic cells.

MATERIALS AND METHODS

Human muscle culture. The isolation of human myoblasts has been described by Webster et al. (38). Briefly, myoblasts from sample XXV were separated from nonmuscle cells on the basis of their reactivity with the monoclonal antibody 5.1H11, which recognized a human muscle-specific cell surface antigen (36). Labeled cells were analyzed by a fluorescence-activated cell sorter, and myoblasts were collected. These sorted cells were expanded, and frozen stocks were used for subsequent experiments. Human muscle myoblasts were grown on collagen-coated tissue culture dishes in F-10 medium supplemented with 15% fetal calf serum (FCS) and 0.5% chicken embryo extract (CEE); this mitogen-rich medium is referred to as growth medium. To stimulate muscle fusion and differentiation, cultures were incubated in starvation medium (Dulbecco modified Eagle with 1 μM insulin and 2.5 μM dexamethasone) for approximately 18 h as previously described (37). Cultures were then shifted to fusion medium (same as starvation medium except supplemented with 2% horse serum), and the medium was replaced daily. To study the effect of TNF on human myogenesis, 25 ng of human recombinant TNF (Cetus Corp., Emeryville, Calif.) per ml was typically added to cultures (see individual figure legends for details).

The growth of human myoblasts in the presence of TNF (25 ng/ml) was monitored by determining the number of cells per dish in triplicate. A total of 6×10^4 cells were added to collagen-coated 35-mm dishes. Cultures were maintained in growth medium (F-10, 15% FCS, 0.5% CEE) with and without TNF, and the medium was replaced every other day. Triplicate samples were harvested by trypsin-EDTA treatment, and cell number was determined daily with a

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Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.) for 6 days.

cDNA probes. The interferon beta-2 (IFN-β_2) cDNA was provided by P. Sehgal (Rockefeller University, New York, N.Y.); it specifically recognizes a 1.3-kilobase (kb) mRNA (22). All muscle-specific cDNAs were provided by L. Kedes (Stanford University): α-cardiac actin (pHMcA-3'uT-DB), α-skeletal actin (pHMalphaA-3'uT-Fnu) (13), and myosin heavy chain (pHMsMHC-1; P. Gunning and L. Kedes, personal communication). The TNF-inducible message Xgs has been characterized in our laboratory (S. Torti et al., manuscript in preparation) and was originally provided by Gordon Ringold (Syntex, Palo Alto, Calif.). The β-actin probe (14) was used to determine whether equal amounts of RNA were transferred to nitrocellulose filters.

RNA analysis. RNA samples were isolated from human muscle cultures by lysis in guanidine thiocyanate and centrifugation through a 5.7 M CsCl cushion (7). RNA was size-fractioned through a 1.4% agarose gel in 2.2 M formaldehyde and MOPS (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) buffer. All hybridizations of Northern (RNA) blots were done in hybridization buffer (50% formamide, 4× SSC, 1× Denhardt solution, salmon sperm DNA (10 μg/ml), 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesol-fonic acid, pH 7.4]; 1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate). ^{32}P-labeled cDNA probes were prepared by the random primer method (10). Signals on autoradiograms were quantitated with a Hoeffer scanning densitometer (model GS 300). Fold inductions were calculated with the β-actin probe to normalize for RNA application.

Immunofluorescence analysis. The immunofluorescence analysis of myosin heavy chain was done by methods described by Silbertstein et al. (30). Briefly, cultures were fixed in phosphate-buffered saline containing 1% formaldehyde for 15 min at 37°C. The fixation solution was aspirated, and cells were permeabilized by adding cold (−20°C) methanol for 20 min at room temperature. Cultures were then incubated with first (4A.1025) and second antibody conjugated to fluorescein, and immunofluorescence was visualized with a Leitz epifluorescence Ortholux microscope. The monoclonal antibody 4A.1025 recognizes all muscle-specific isoforms of myosin heavy chain (Blau and Silberstein, unpublished observations).

RESULTS

TNF inhibits muscle-specific gene expression. Our first experiments analyzed the response of cultured human cells to the addition of TNF. Since our previous experiments demonstrated that adipocytes respond to TNF by an altered pattern of gene expression, we measured the ability of TNF to modulate the expression of muscle-specific genes in these cultured human cells. To determine whether TNF would affect muscle gene expression, we added TNF (25 ng/ml) to proliferating myoblasts. RNA was isolated from cultures at various time points before and after the shift to medium conditions necessary for human muscle differentiation (Fig. 1). Northern blot analysis of RNA samples from control and TNF-treated cultures revealed that TNF treatment inhibited the expression of muscle-specific mRNAs. TNF treatment inhibited the expression of myosin heavy chain and α-skeletal actin, genes which are expressed in differentiated muscle cells but not in myoblasts (12) (Fig. 1). TNF also affected the expression of α-cardiac actin. This isoform of actin has been shown to be expressed in human myoblasts

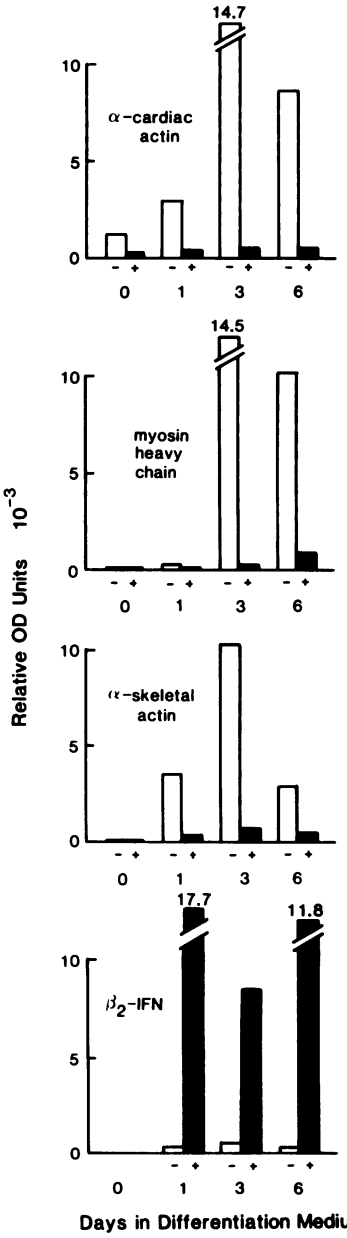

FIG. 1. TNF inhibits muscle gene expression. Cells (10^6) were added to 100-mm collagen-coated dishes on day −3. Fresh growth medium with and without TNF (25 ng/ml) was added to cultures on day −1. On day 0, the medium was aspirated and replaced with starvation medium with or without TNF for overnight incubation for approximately 18 h. From this point on, cultures were maintained in differentiation medium. After overnight incubation in starvation medium, cultures received fusion medium with or without TNF. Fresh fusion medium was added daily. Samples were harvested at the indicated intervals, and the mRNAs were analyzed by Northern blot analysis. Autoradiograms were scanned by a densitometer at the strongest signal to get full-scale response. Values shown represent relative values as determined by integration of peak areas. Open bars, Control samples; solid bars, TNF-treated samples. The results shown are typical and were confirmed by multiple experiments.

(12) prior to shifting to culture conditions which favor differentiation. The expression of α-cardiac actin increases when human myoblasts are shifted to medium conditions that induce muscle differentiation (12, 15). TNF treatment inhibited α-cardiac actin gene expression both prior and subsequent to the induction of differentiation (Fig. 1).

To determine whether this inhibition of muscle gene expression resulted from a mitogenic actin of TNF, we added TNF (25 ng/ml) to growing cells. Cultures were inoculated at low density (see Materials and Methods) in growth medium. This experimental design permitted the growth of myoblasts with minimal cell fusion. This was necessary because, as shown below, TNF treatment inhibited the ability of myoblasts to fuse and form myotubes. Samples were taken daily for determination of the number of cells per dish. TNF was added every day with refeeding. Both control and TNF-treated cultures decreased their rate of growth with increasing cell density. TNF neither stimulated nor inhibited the growth of human myoblasts maintained in growth medium, even after 6 days of treatment (data not shown).

IFN-β_2 has been shown to be TNF inducible in human fibroblasts (18). To determine whether this IFN was also induced by TNF in human muscle cells, RNA samples from the previous time course were examined by Northern blot analysis. With an IFN-β_2 cDNA probe, IFN-β_2 mRNA was detected only in TNF-treated samples (Fig. 1). The expression of IFN-β_2 was markedly stimulated by TNF treatment throughout this time course. Thus, the ability of TNF to induce IFN-β_2 is not unique to fibroblasts, but also a property of both dividing myoblasts and mitotically quiescent myotubes (Fig. 1 and below).

The inhibition of α-cardiac actin expression in myoblasts by TNF is dose dependent. To determine the sensitivity of human muscle cells to TNF treatment, we made use of our observation that the expression of α-cardiac actin in myoblasts was inhibited by TNF (Fig. 1). Proliferating myoblast cultures were treated for 24 h with increasing doses of TNF.

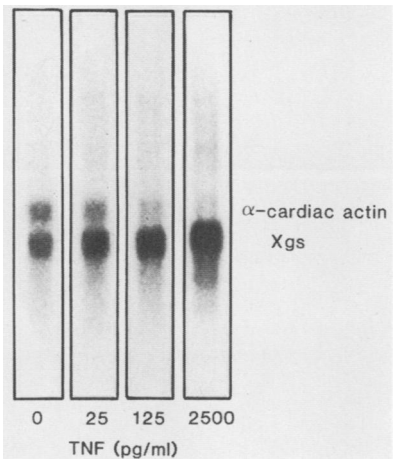

FIG. 2. TNF inhibits the expression of α-cardiac actin and stimulates the expression of a TNF-inducible gene, Xgs, in a dose-dependent manner. Proliferating myoblasts were incubated with TNF at the indicated concentrations for 24 h, and RNA was isolated and examined by Northern blot analysis. ^{32}P-labeled random primed probes for α-cardiac actin and Xgs were added together for hybridization. The filter was exposed to Kodak X-ray film overnight at −70°C. β-Actin probing of this filter confirmed that equal amounts of RNA were transferred (data not shown).

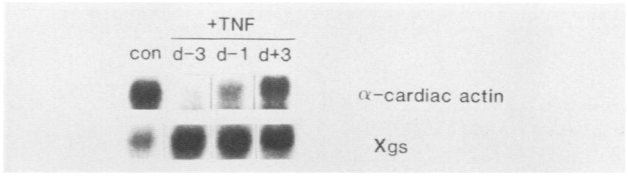

FIG. 3. α-Cardiac actin expression becomes unresponsive to TNF treatment as myoblasts become committed to differentiate. Cultures were inoculated at 5×10^5 cells per 100-mm dish on day −4 in growth medium. One set received TNF (25 ng/ml) on day −3, another on day −1, and a third set received TNF on day +3. Cultures were replenished with fresh medium with or without TNF every other day up to day 0 (day starvation medium was added). On day +1, all cultures were shifted to fusion medium, which was replaced daily. All samples were harvested together on day +6. RNA was isolated and analyzed as described in the legend to Fig. 2. con, Control (no TNF).

RNA was isolated and analyzed for expression of α-cardiac actin. The results shown in Fig. 2 demonstrate that there was a detectable effect on α-cardiac actin mRNA level with TNF (25 pg/ml), and the level of inhibition increased progressively in a manner dependent on TNF dose. In concert with the ability of TNF to decrease the expression of a muscle gene, there was a dose-dependent increase in the level of Xgs, an mRNA which is TNF inducible (Fig. 2 and below), as well as IFN-β_2 (data not shown). Autoradiograms were quantitated by scanning densitometry to determine the magnitude of the effect of TNF on gene expression. The 2,500-pg/ml dose of TNF increased the level of Xgs approximately 5.5-fold and decreased α-cardiac actin expression more than 5-fold. In other experiments, we have shown that 2,500 pg of TNF per ml had maximal effects on both the inhibition of α-cardiac actin and induction of Xgs (data not shown).

TNF acts on myoblasts to block muscle differentiation. To determine whether the effects of TNF on muscle cells occurred at all stages of differentiation or were limited to a more narrow temporal window, we added TNF to cultures of myoblasts at various times prior to and after shifting the culture to conditions which promote differentiation. Two sets of samples were taken on day 6: one set was analyzed for muscle gene expression by Northern blot analysis, and the other was examined for myosin heavy-chain expression by immunofluorescence. The results from Northern analysis are shown in Fig. 3. Myoblasts which were treated with TNF 3 days prior to the shift to differentiation conditions (day −3 sample) showed the strongest inhibition of α-cardiac actin expression. Samples which received TNF on day −1 showed a lesser but still marked effect (decreased approximately 3.5-fold relative to the control level), while only a slight decrease in α-cardiac actin expression (approximately 1.6-fold) was observed when TNF was added 3 days after the shift to differentiation conditions. The data suggested that as myoblasts fused and differentiated into myotubes, they became resistant to TNF's ability to inhibit muscle gene expression (Fig. 3).

To determine whether these effects on muscle gene expression were associated with an inhibition of differentiation, we evaluated these cells by light microscopy and immunofluorescence (Fig. 4). Figure 4a demonstrates the normal morphology found in differentiated cultures 6 days after differentiation, with extensive fusion and clustering of nuclei in myotubes. The corresponding fluorescence image due to the expression of myosin heavy chain is shown in Fig. 4b. The effects of TNF treatment are shown in the remaining panels of the figure. In agreement with the RNA analysis,

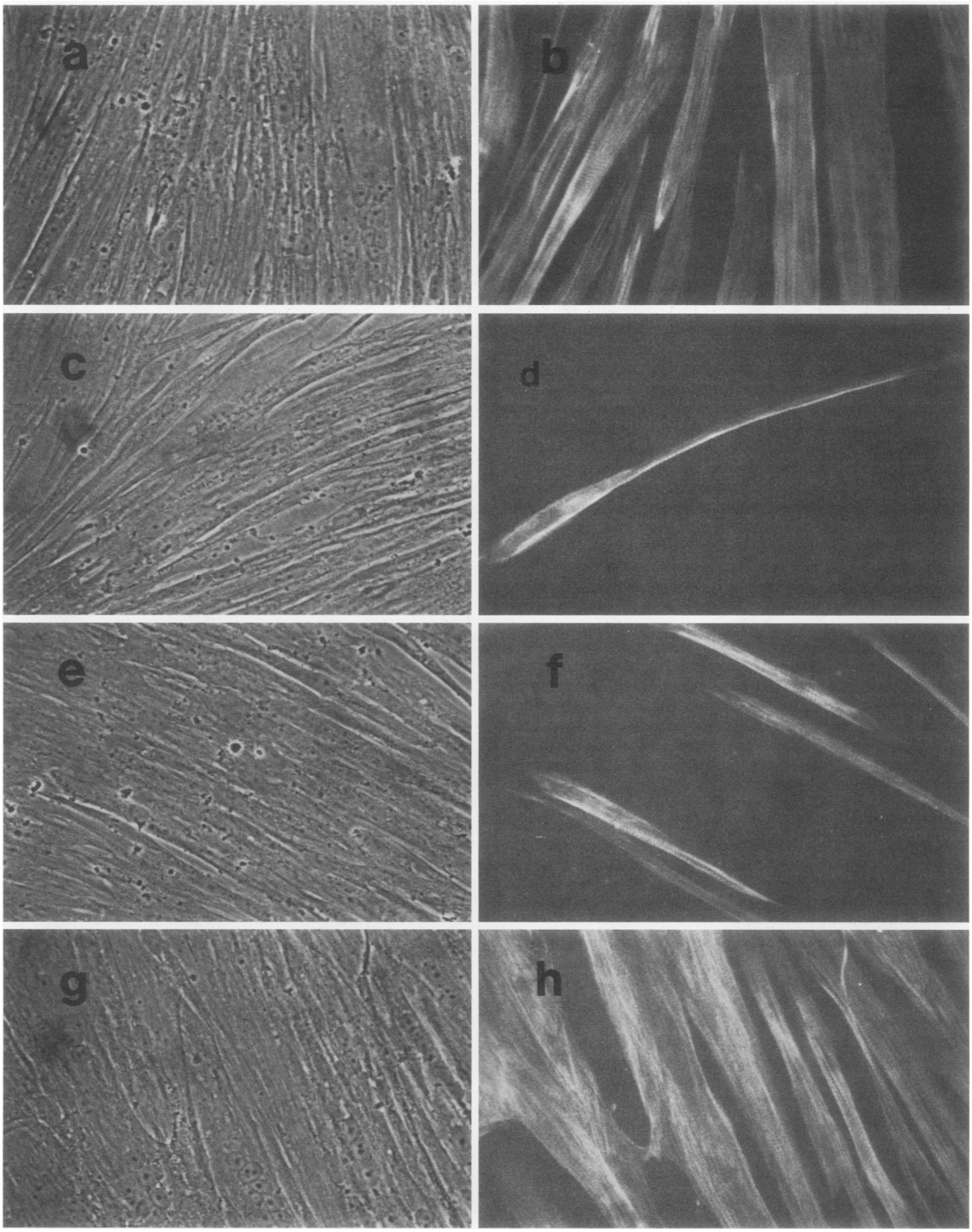

FIG. 4. Myoblasts become unresponsive to TNF as they fuse and differentiate into myotubes. Parallel cultures were treated as described in the legend to Fig. 3 and analyzed for myosin heavy-chain expression by immunofluorescence (see Materials and Methods). (a, c, e, g) Phase-contrast pictures; (b, d, f, h) corresponding immunofluorescence of myosin heavy chain. Control (a, b) cultures and cultures treated with TNF on day −3 (c, d), day −1 (e, f) and day +3 (g, h) were photographed on a Leitz Ortholuz epifluorescence microscope with a 25× H_2O immersion objective and Kodak 400 ASA Ektachrome film. The photos shown represent typical fields, except for the field shown in c and d (see text).

adding TNF on day −3 resulted in dramatic inhibition of fusion and myosin expression (Fig. 4c and d). The field shown in Fig. 4d contains a single myosin-positive cell; the majority of fields were negative for myosin. However, as myoblasts differentiated, they became unresponsive to TNF's ability to block differentiation, as indicated in Fig. 4e and f, where cells received TNF on day −1. Cells treated with TNF on day +3 showed little effect of TNF (Fig. 4g and h), in agreement with the Northern results on α-cardiac actin, suggesting that it is the increasing population of differentiated cells which are resistant to TNF.

These effects of TNF on muscle gene expression were reversible. We inhibited muscle differentiation maximally by treating cultures from day −3 to day +3 with TNF under our standard differentiation conditions (see Materials and Methods). TNF was removed from cultures and fresh medium was added daily to day +12. These TNF-treated cultures on day +12 demonstrated a return to normal levels of expression of a human muscle-specific cell surface antigen, 5.1H11, and myosin heavy-chain expression by immunofluorescence staining (data not shown). Cultures which received TNF continuously until day +12 showed rare evidence of differentiation, as determined by 5.1H11 and myosin heavy-chain expression.

TNF does not affect muscle gene expression after differentiation. Adding TNF to progressively more differentiated cultures suggested that only myoblasts and not fully differentiated myotubes respond to TNF by decreased expression of muscle-specific genes. To test this directly, we asked whether TNF treatment would inhibit muscle gene expression if the monokine was added to differentiated multinucleated myotubes in the absence of residual myoblasts. Human myoblast cultures were differentiated as described in the legend to Fig. 1. To remove any myoblasts which might remain among the differentiated myotubes, we added cytosine arabinoside (Ara-c, 10^{-5} M), which is selectively cytotoxic for dividing cells, to half of the cultures on day +3. Medium was replaced daily, and TNF was added on days +5 and +6. All cultures were harvested on day +7 (48 h of TNF treatment) and analyzed for gene expression by Northern analysis and myosin heavy-chain expression by immunofluorescence. Results were identical with and without Ara-c treatment. The Northern analysis of α-cardiac actin expression shown in Fig. 5 demonstrated that TNF treatment of differentiated myotubes had no effect on α-cardiac actin mRNA. Further, the expression of myosin heavy chain, as determined by Northern blots and immunofluorescence (data not shown), showed no effect of TNF treatment after differentiation was induced. Thus, muscle genes sensitive to TNF in myoblasts became resistant to TNF in myotubes.

Expression of nonmuscle genes remains sensitive to TNF in both myoblasts and myotubes. One possible explanation for the loss of TNF responsiveness after muscle differentiation is the loss of TNF receptors or receptor function. We used the TNF-inducible mRNA IFN-β_2 to allow us to discriminate between changes in gene regulation versus receptor function. In the experiment described above, pure populations of myotubes derived from Ara-c treatment of differentiated muscle cells still responded to TNF by an induction of IFN-β_2 (Fig. 5). Myoblasts also responded to TNF with the induction of IFN-β_2 (Fig. 1). Another TNF-inducible gene is Xgs, which has recently been characterized in our laboratory. As shown in Fig. 2, proliferating myoblasts expressed Xgs, and the level of expression was increased after TNF treatment in a dose-dependent manner. Although the expression of α-cardiac actin became unresponsive to TNF after

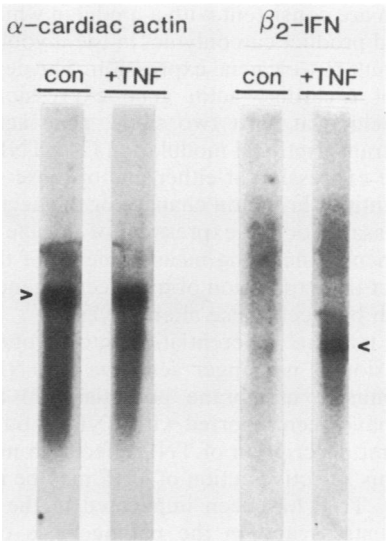

FIG. 5. IFN-β_2 is induced and α-cardiac actin expression is not inhibited by TNF treatment of differentiated myotubes. The same blot was analyzed first for IFN-β_2 expression in control and TNF-treated cultures, then stripped and hybridized to detect α-cardiac actin expression in control and TNF-treated samples. con, Controls. Arrows indicate hybridizing mRNA to α-cardiac actin (left) and IFN-β_2 (right).

muscle differentiation (Fig. 3 and 5), TNF still induced the expression of Xgs (Fig. 3). Thus, the ability to respond to TNF was maintained throughout differentiation; the selective TNF response of muscle genes at different points during differentiation cannot be explained by a loss in myotubes of TNF-mediated signal transduction.

DISCUSSION

Events involved in myogenesis, particularly in the terminal differentiation of myoblasts to myotubes, have been successfully modeled in cultured muscle cells (12, 15, 23, 25). The identification of muscle-specific products and genes has allowed studies on the regulation of gene expression during myogenesis (24). The experiments reported here suggest that cultured muscle cells may also be useful in elucidating the action of monokines on specific target tissues.

In these experiments, TNF was added to cultured human myoblasts and myotubes. Our data demonstrate that TNF is capable of discriminating between these two states. Thus, TNF added to myoblasts can prevent both the expression of muscle genes (α-skeletal and -cardiac actin and myosin heavy chain) and the fusion which characterize terminally differentiated myotubes. However, TNF cannot reverse the expression of these genes when applied to fully differentiated myotubes (Fig. 3). This is true even though we have shown these cells to be fully competent to respond to TNF in other ways: IFN-β_2 expression and Xgs expression, for example, were as inducible in myotubes as they are in myoblasts (Fig. 1 and 5). Even more striking, the ability of TNF to affect muscle gene expression is gradually lost as myoblasts approach terminal differentiation. Thus, TNF applied at day −3 was more effective in preventing expression of myosin heavy chain and α-cardiac actin than TNF applied at day −1 (Fig. 3 and 4). Thus, there is a temporal correlation between commitment to differentiation and resistance to TNF which suggests that these events may be related.

These data are consistent with a model in which TNF or a TNF-induced product can only act in the myoblast environment to prevent muscle gene expression. The developmental regulation of α-cardiac actin gene expression has been shown to include at least two steps, gene activation and subsequent transcriptional modulation (24). TNF may act to prevent gene expression at either one of these steps, possibly by preventing chromatin changes or the accumulation of factors necessary for the expression of muscle genes. Further experiments, including measurements of the ability of TNF to affect the expression of transfected genes, may help to distinguish between these alternatives.

Following terminal differentiation into myotubes, muscle gene expression is no longer sensitive to TNF. Although effects on muscle membrane potential (35) and glucose uptake (20) have been reported with TNF, to our knowledge this is the first description of TNF effects on muscle differentiation. This selective action of TNF may be related to its in vivo role. TNF has been implicated in the mammalian response to stress and in the pathogenesis of endotoxic shock (4, 17, 34). The primary human muscle cells we studied here were derived from a normal 7-year-old child; the cells of origin are presumably satellite cells, which have the potential to proliferate under conditions of muscle injury. This suggests, albeit indirectly, that a target of TNF action may be the satellite cell in muscle tissue. Thus, under conditions of muscle injury, TNF may function to inhibit satellite cell differentiation while sparing differentiated muscle tissue.

A number of quite disparate agents have been shown to inhibit differentiation of cultured myoblasts and primary myoblast cultures. Some examples are dimethyl sulfoxide (5), fibroblast growth factor (31), and metalloendoprotease inhibitors (2, 9). Our studies with TNF are similar to other recent reports (21, 27) which demonstrate that transforming growth factor-β (TGF-β) inhibits muscle differentiation and muscle cells become unresponsive to the inhibitory action of TGF-β after differentiation. In studies in progress, we are comparing the effects of TGF-β and TNF on human myogenesis to establish whether these agents are acting to inhibit myogenesis through similar mechanisms or are acting at discrete steps in the myogenic pathway.

In addition to these more global effects on muscle differentiation, the evidence that TNF inhibits α-cardiac actin expression in human myoblasts establishes a system to study the effect of TNF on a well-defined muscle-specific gene without modifying the differentiated state. As shown by the results in Fig. 1 and 2, TNF potently inhibits the expression of α-cardiac actin prior to and independent of any modification of the differentiated state of these myoblasts.

Three mesenchymal cells have now been shown to be affected by TNF, each differently. TNF not only inhibits the differentiation of adipocytes but, consistent with its role in mobilizing lipid (16, 28, 33), also reverses aspects of the differentiated phenotype. In hematopoietic cell lines, TNF stimulates differentiation of phagocytic cells (32). In this manuscript, we show that TNF inhibits differentiation of human muscle cells if applied before, but not after, the cells begin to differentiate. At an organismal level, these disparate effects of TNF might be explained by the requirements to mobilize lipid, increase phagocytosis, and prepare satellite cells to respond to injury. However, to understand the role of TNF in these processes, it will be important to learn what is different about either the cellular environments or the target genes themselves that allows TNF to modulate gene expression in adipose, hematopoietic, and muscle cells in such disparate ways.

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