Bimodal effects of TNF-α on differentiation and hypertrophy of skeletal muscle cell cultures

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Abstract

In order to investigate the mechanisms regulating skeletal muscle homeostasis and the balance between atrophic and hypertrophic signals, and based on the fact that often factors with a positive (or negative) role in *in vitro* myogenesis have the same role in muscle homeostasis *in vivo*, we have exposed muscle cell cultures to Tumor Necrosis Factor-α (TNF-α), Insulin-like Growth Factor I (IGF-I) or their combination in two different experimental sets: L6 myogenic cells were induced to differentiate either in the absence or presence of these factors; alternatively, the same cells were differentiated with standard methods (by lowering the serum concentration) and then exposed to the above factors. With this approach we could confirm that TNF-α and IGF-I have opposite effects on skeletal muscle differentiation. However, we showed that both TNF-α and IGF-I induce muscle hypertrophy to the same extent when delivered to a promiscuous culture of myotubes and single cells. This striking observation may contribute to explain why muscle hypertrophy often involves muscle damage (and a consequent cytokine-mediated cycle of inflammation). On the other hand, we extended previous observations on IGF-I hypertrophic effects on skeletal muscle cell obtained by using transgenic cell lines: here we show that IGF-I retains its ability to positively affect myogenesis even on cell cultures which have already reached a significant fusion index. Taken together our observations indicate that TNF-α has differential effects on myogenic differentiation and trophysm depending on the time it has been added to the cell culture. Both TNF-α and IGF-I can promote significantly myotube growth when added to muscle cell culture previously induced to differentiate.

Keywords: Myogenic cell lines; Cachexia; Atrophy Hypertrophy; Cytokine.

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INTRODUCTION

Cell culture studies have allowed the characterization of many specific effects played on skeletal muscle cells by a wide number of factors (reviewed in [4]). Skeletal muscle cells are exposed *in vivo* to a plethora of cytokines, growth factors and hormones in a complicate and finely tuned network regulating cell survival, proliferation and differentiation. *In vitro* studies allow dissecting the pathways involved in these responses by analytically exposing muscle cells to specific stimuli in experimentally controlled conditions. Nonetheless, often conflicting results are reported even when using cell cultures and experimentally defined conditions.

It is well established, for instance, that TNF-α is an inhibitory cue for skeletal muscle differentiation [8, 25]. However, myoblasts do express TNF-α and still are able to differentiate into myotubes in culture [10, 32]. Seemingly conflicting reports exist on the role TNF-α plays on muscle regeneration, a process which recapitulates myogenesis during adult life. It is still controversial whether TNF-α receptor expression is necessary or not for a proper muscle regeneration [5, 9]. TNF is expressed in regenerating muscle and is involved in the repair process of this tissue [37, 38]. However, low but chronic levels of circulating TNF negatively affect regeneration after experimentally induced injury [6].

Similar paradoxical effects can be noticed when studying the effects triggered by myogenesis promoting factors, such as IGF-I. Its role in inducing muscle differentiation and hypertrophy is well established [18, 27, 28]. Still, in a synthetic medium deprived of serum, IGF-I was reported to have a modest effect on skeletal muscle differentiation of L6 cells [26].

To try to reconcile strikingly different observations on skeletal myogenesis, we performed cultures of myogenic cells which were exposed to TNF-α and/or

IGF-I in different moments of their differentiation process. Here we report that TNF-α can exert differential effects on the size of myotubes depending on the time the culture was exposed to this cytokine.

MATERIALS AND METHODS

Cell cultures

L6 myogenic cell line [39], subclone L6C5 [30, 34], was used throughout this study. This cell line was maintained in culture as described by Nervi [30]. For the experiments the cells were seeded at a density of 15.000 cells /cm^2 in DMEM + 10% FBS (Sigma, St. Louis, MO) (growth medium, GM). The following day, the cells were induced to differentiate by shifting the medium to DMEM supplemented with 1% FBS (differentiation medium, DM). TNF-α (Roche Diagnostics, Mannheim, D) and IGF-I (purchased from Chemicon, Temecula, CA) were added to the culture medium at a concentration of 5 ng/ml and 1 nM, respectively. TNF-α, IGF-I or their combination were added to the culture medium every day, PBS was added to controls and the media were fully replenished every two days. For the first set of experiments, the day after seeding the cells were cultured in GM for 8 h in the absence or presence of TNF-α and/or IGF-I, then cultured in DM in the absence or presence of TNF-α and/or IGF-I for 5 days.

To study the effects on pre-differentiated myotubes, L6 cultured in DM for 5 days (in the absence of other stimuli) were then exposed to TNF-α and/or IGF-I in DM for 4 days. Additions and media changes were performed as described above. For the second set of experiments, the total culture time resulted 9 days from the day after cell seeding.

Immunoflurescence analysis

Differentiated L6 cell were identified by sarcomeric myosin heavy chain (MHC) staining (clone MF20, hybridoma supernatant, DSHB, University of Iowa, IO) as described elsewhere [11]. Nuclei were counterstained with Hoechst dye 33342 (5 μg/ml) as described previously [7]. Photomicrographs were obtained using Axioskop 2 plus system equipped with an Axiocam HRc (Zeiss, Oberkochen, GE) at standard 1300x1030 pixel resolution. Quantitative analysis of differentiation was performed by determining the number of nuclei in MF20 positive cells over total nuclei in at least ten randomly chosen microscopic fields per sample (% differentiation). The values shown represent the mean ± SEM of at least three independent experiments performed in triplicate.

RESULTS

In the first set of experiments, L6 cells were exposed to TNF-α, IGF-I or their combination while still in GM and allowed to differentiate by shifting the medium to a low serum one (DM) in the continuous presence of the treatments above. After 5 days in DM, differentiation was assessed by both MHC expression and morphological analysis of the cell fusion process (Fig. 1). As known, myogenic cells respond to low serum concentrations by forming multinucleated myotubes which accumulate myosin in their cytoplasm. IGF-I stimulated this phenomenon by significantly increasing the percentage of cells recruited to myotubes (Fig. 1b). By contrast, TNF-α hampered this response to such an

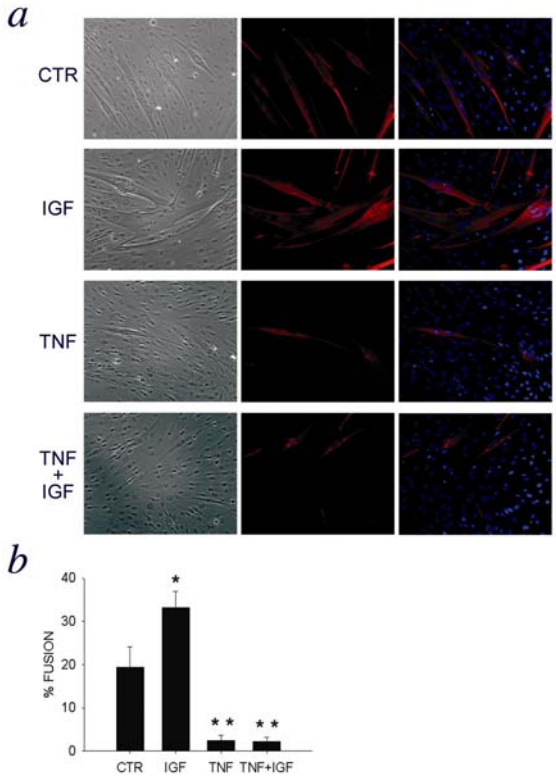

***Figure 1**. Immunofluorescence analysis of differentiating muscle cell cultures. L6C5 cells were treated with IGF-I and/or TNF-α for 8 hrs in GM, then shifted to DM in the continuous presence of the treatments as indicated for 5 days.*

***a**) representative photomicrographs of phase contrast images (*left panels*), MHC immunolocalization (in red,* center panels*) and the latter merged with Hoechst-stained nuclei (in blue,* right panels*).*

***b**) fusion index (percentage of cells fused into myotubes) calculated and shown as mean ± SEM of at least three independent experiments. The medium degree of differentiation shown by L6C5 cells is suitable to highlight the opposite effects of IGF-I and TNF-α on myogenic differentiation: IGF-I significantly increases both the percentage of fusion and the dimension of each myotube; TNF-α negatively affects both phenomena and counteracts IGF-I effects on differentiation.*

** = p<0,05; ** = p<0,005 by Student's* t *test vs. control.*

extent that even IGF-I was not able to counteract the inhibition of muscle differentiation observed in the presence of TNF-α (Fig. 1b).

In the second set of experiments, treatments with the recombinant factors were started only after the occurrence of muscle differentiation, i.e. on day 5 of culture in DM when multinucleated myotubes were already evident. The same concentrations of IGF-I and TNF-α were used in the two sets of experiments. Based on the first set of experiments, after 5 days of culture in DM it is possible to notice the presence in culture of an heterogeneous population of myotubes of various sizes as well as of numerous cells that underwent cell cycle arrest but failed to fuse into myotubes. When these populations were further cultured in DM for 4 additional days the percentage of cells which fused increased and both the number and the size of myotubes increased (Fig. 2). IGF-I exerted its positive effects on myogenesis and hypertrophy to a full extent (Fig. 2) by: a) significantly increasing the fusion index of the culture, i.e. recruiting additional cells to fuse; b) inducing the appearance of large myotubes with hypertrophic features, such as nuclear rings and high cytoplasm/nuclei ratio [29]. Strikingly, we noticed similar effects when differentiated myotubes were treated with TNF-α (Fig. 2). In this case, the relatively thin myosin expressing myotubes observed in control conditions in the first set of experiments had become large myotubes containing dozens of nuclei. It is worth recalling that, only if administered after the first wave of myogenic differentiation, TNF-α had this unexpected output at the same concentration which had been proven to be inhibitory for myogenic differentiation. The combined treatment with IGF-I and TNF-α did not further affect both myotube architecture and fusion index, as compared with single treatments (Fig. 2).

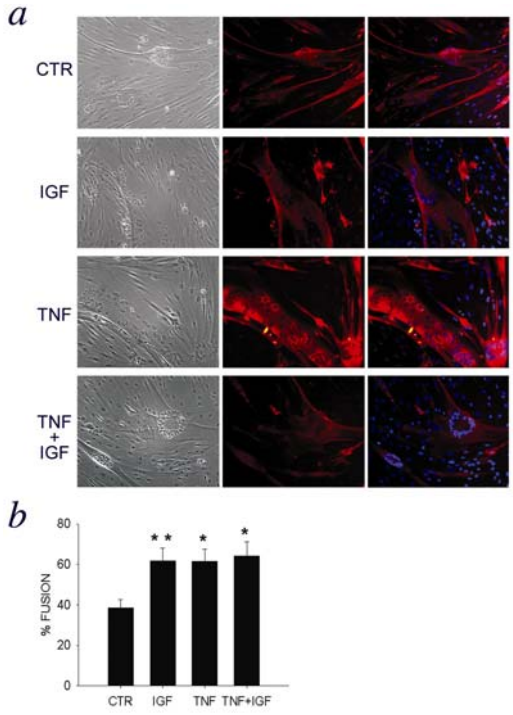

***Figure 2**. Immunoflurescence analysis of differentiating muscle cell cultures. L6C5 cells were cultured in DM for 5 days. Only then, the cell cultures were treated with IGF-I and/or TNF-α in DM for additional 4 days. Controls were maintained in DM for the same additional period of time.*

***a**) representative photomicrographs of phase contrast images (*left panels*), MHC immunolocalization (in red,* center panels*), and the latter merged with Hoechst-stained nuclei (in blue,* right panels*).*

***b**) fusion index (percentage of cells fused into myotubes), calculated and shown as mean ± SEM of at least three independent experiments. When cultured for a prolonged period of time, L6C5 myotubes grow, and appear to be formed by a higher number of cells. When added to cultures of myotubes, both IGF-I and TNF-α promote hypertrophy, as shown by the occurrence of nuclear rings, and significantly increase the percentage of fusion of the cell culture. Their effects are not additive. * = p<0,05; ** = p<0,005 by Student's* t *test vs. the control.*

DISCUSSION

Here we report a paradoxical effect of TNF-α on muscle cell cultures, whereby this cytokine added to differentiating myoblasts hampers their differentiation whereas, when administered to a culture of pre-differentiated myotubes, it induces hypertrophy. IGF-I treatment is used as the gold standard for the induction of muscle differentiation and hypertrophy. TNF-α effects on muscle cells are similar to those of IGF-I in terms of hypertrophy, while opposite to those of IGF-I during differentiation.

It is a well known phenomenon that a factor may elicit differential effects on muscle cells, and this may be not quite surprising in the case of TNF-α and IGF-I, which both exert pleiotropic actions on cell survival, proliferation and differentiation [14, 36]. For instance, IGF-I can either inhibit or induce L6 cell differentiation at different concentration, with a maximal effect on differentiation at 1 nM [16, 26]. Depending on its concentration the effects of TNF-α on protein content of muscle cells also show a bimodal behaviour: at low concentrations (1U/ml or less) or high concentrations (100 U/ml or more) TNF-α decreases or increase the protein content of murine C2C12 myotubes, respectively [2]. Exactly the opposite effects were reported by using the rat L6 cell line, i.e. a different cell type of myogenic cells [13]. As a term of comparison, we treated L6 cell cultures with TNF-α 5 ng/ml (equivalent to 2000 U/ml). When adding TNF-α at such high concentration to previously differentiated L6 cell

culture we observed induction of hypertrophy. Noteworthy, we inhibited myogenic differentiation when we exposed L6 cells to the continuous presence of TNF-α (at the same concentration) from the beginning of the culture. The main difference accounting for the differential outputs of TNF-α in terms of biological effects relies on the fact that TNF-α is given either to undifferentiated myoblasts or to a promiscuous culture of differentiated and undifferentiated cells. We thus highlight a novel time-dependence of the effects this important cytokine plays on muscle hypertrophy. The kinetics of muscle exposure to TNF-α appear to be important *in vivo* as well. Inflammation following muscle damage is an important component for tissue repair. Lymphocytes invading the damaged area produce several cytokines, including TNF-α, that are necessary for muscle repair to occur. In healthy individuals, these repeated cycles of damage, inflammation and repair can lead to muscle hypertrophy [19]. In contrast, if TNF-α levels are chronically elevated above a threshold, muscle regeneration is negatively affected [6], an event that may well occur in atrophy / cachexia.

In this paper, we also report that TNF-α blocks the positive effects of IGF-I on myogenic differentiation, thus confirming previous reports [15]. It has been reported that TNF-α decreases endogenous expression of IGF-I in C2C12 , providing a possible mechanism for TNF-α mediated inhibition of differentiation for this specific cell line [17]. However, TNF-α retains this feature even when exogenously administered with IGF-I, which demonstrates that it is dominant over the latter independently from IGF-I expression [22] [15]. Experimental evidence show that TNF-α does not abrogate IGF-I effects by blocking the PI 3-kinase pathway [15]. We have recently reported a novel mechanisms whereby TNF-α inhibits muscle differentiation, i.e. by activating non-apoptotic caspase pathways [8]. Whether this pathway is important in mediating TNF-α effects even in the presence of IGF-I has yet to be addressed.

IGF-I-induced hypertrophy is an heavily-studied phenomenon due to its possible therapeutic application for a wide range of pathologies [3,12]. While it is known that proliferation precedes differentiation in IGF-I stimulated myogenesis [14] the two effects of IGF-I have been elegantly dissected [28,29]: Musarò has demonstrated that post-mitotic expression of IGF-I is still sufficient to promote maturation of the myogenic program leading to hypertrophy. In his works the expression of the transgene is driven by the MLC promoter and occurs as early as 1 day after the cell cycle withdrawal of the myogenic cells [29]. Here we extend these observations on IGF-I effects on skeletal muscle hypertrophy and we show that IGF-I is still significantly active on muscle cells even when added much "later" in the course of differentiation, i.e. on day 5 of culture in differentiating condition, a time when myotubes are already well developed. In these conditions IGF-I seems to be able to recruit the remaining single cells to participate in myogenesis, as demonstrated by the significantly increase in the fusion index of myotubes treated with IGF-I from day 5 to day 9 of culture. Our findings likely mirror the *in vivo* situation, where differentiated muscle fiber coexist with undifferentiated myogenic cells and may become exposed to increased levels of IGF-I.

The most striking result reported here is the TNF-α hypertrophying effect on pre-differentiated myotubes. While this is the first report of an hypertrophying effect of TNF-α on skeletal muscle , it is well know that this cytokine induces (pathological) hypertrophy of cardiac muscle [31]. Indeed, TNF-α provokes an hypertrophic growth in cardiomyocytes by activating ROS-mediated NF-kappaB activation [20, 21, 40]. We show that when skeletal muscle cell cultures with a percentage of fusion of about 20% are exposed for some days to TNF-α they end up with a 3-fold increase in the percentage of fusion (more than 60%). In control conditions (i.e. when cultured for the same additional time in the absence of TNF-α) the number of cells newly added to myotubes increased only 2-fold. Taken together, these evidence suggest that the myoblasts which failed to fuse during the first days of culture in differentiating conditions were actively recruited to new and/or previously differentiated myotubes. TNF-α is known to be both a mitogen and a scatter-factor for myoblasts [33, 35]. These features may contribute to mobilize unfused cells and induce their participation in the growth of myotubes. The fact that TNF-α and IGF-I are not synergistic in their hypertrophic effects may imply that either a plateau of fusion capacity is reached in the cell cultures after 9 days of culture or that the two factors actually share signalling pathways that are already triggered to a maximum extent.

Myotube cultures have been used as *in vitro* models of muscle fibers to elucidate the atrophying effects of cachectic cytokines such as TNF-α [23, 24]. Our results are in disagreement with experiments by Guttridge and co-workers, where loss of MHC occurs in C2C12 myotubes treated with 10 ng/ml TNF-α [1]. The different cell type and TNF-α concentration used may explain the apparent discrepancies.

Abbreviations

DM, differentiation medium; DMEM, Dulbecco's Modified Eagle Medium; FBS, Foetal Bovine Serum; GM, growth medium; IGF-I, Insulin-like Growth Factor I; MHC, myosin heavy chain; ROS, Reactive Oxygen Species; TNF-α, Tumor Necrosis Factor-α.

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