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Original article

Growth factor and cytokine interactions in myogenesis. Part I. The effect of TNF-α and IFN-γ on IGF-I-dependent differentiation in mouse C2C12 myogenic cells

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Abstract

The aim of this study was to examine the potential interactions of IGF-I with TNF-α and IFN-γ with regard to regulation of the myogenesis and proliferative potential of mouse C2C12 myoblasts. The stimulation of myogenesis by IGF-I (30 nmol/l) was manifested by an enhanced myoblast fusion and expression of myosin heavy chain (MHC) during the first 3 days of differentiation. IGF-I-dependent fusion and MHC expression was reduced by TNF-α and IFN-γ. Both cytokines prevented the stimulatory effect of IGF-I on MyoD expression with minor modification of the myogenin level. Both TNF-α and IFN-γ activated the expression of cyclin A in myoblasts restimulated to proliferation; however, when used in combination with IGF-I these cytokines prevented the rise in cyclin A induced by growth factor. In conclusion: i) TNF-α and IFN-γ reduce IGF-I-dependent myogenesis which was manifested by the reduction of myoblast fusion and MHC cellular levels, ii) Molecular mechanisms of inhibitory action of TNF-α and IFN-γ on IGF-I-mediated differentiation involve a decrease in MyoD whereas myogenin level plays a minor role, iii) TNF-α and IFN-γ increase the proliferative potential of myoblasts; however, they reduced the mitogenic effect of IGF-I, manifested by a decrease of IGF-I-stimulated cyclin A expression in myoblasts reinduced to proliferation. Interactions among IGF-I and proinflammatory cytokines are therefore important to establish a number of myoblasts and the onset of myogenesis during muscle regeneration.

Key words: Cyclin A, IFN-γ, IGF-I, myogenesis, myogenic regulatory factors, TNF-α

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Introduction

Skeletal muscle is terminally differentiated tissue; however, it may adapt to physiological demands such as growth, training and injury. Muscle regeneration after injury seems to follow the same procedure as muscle development during embryogenesis (Karalaki et al. 2009). Myogenesis is controlled by members of a family of muscle-specific basic helix-loop-helix (bHLH) proteins that, in association with members of the ubiquitous E2A and myocyte enhancer factor-2 (MEF2) families, activate the differentiation program i.e. transcription of regulatory and structural muscle-specific genes (Ishido et al. 2004). One of these factors, MyoD plays a specific role in muscle cell activation and in the transition from proliferation to differentiation, via cell cycle arrest and activation of the expression of multiple additional transcription factors and the muscle-specific genes in the differentiation program. Myogenin is crucial for the progression of normal fusion and more particularly at the onset of fusion (Cao et al. 2006).

Muscle damage with a lack of regeneration manifests itself in several diseases, including cancer cachexia (Tisdale et al. 2008), AIDS (Abad et al. 2002) and sepsis (Saini et al. 2006) and proinflammatory cytokines, in particular TNF-α, are implicated in the loss of lean body mass that occurs under these conditions. Interferon-γ (IFN-γ), a cytokine with pleiotropic effects on immune and nonimmune cells, is also thought to play a role in muscle wasting and hypercatabolism; however, the evidence supporting this claim is primarily indirect (Madihally et al. 2002). On the other hand, the role of inflammatory cytokines in skeletal muscle regeneration recently became evident and TNF-α appears to be important both in muscle repair and myogenesis (Chen et al. 2007).

Insulin-like growth factor-I (IGF-I) is considered a major anabolic factor in skeletal muscle, since it is critical in promoting growth, and in the maintenance and regeneration of this tissue. IGF-I is unique among growth factors as it can stimulate both proliferation and differentiation of muscle precursor cells, which are usually considered to be mutually exclusive events (Paul and Rosenthal 2002). In our recent study a marked impairment of IGF-I signalling and acute metabolic response in C2C12 myotubes exposed to proinflammatory cytokines has been found (Grzelkowska-Kowalczyk and Wieteska-Skrzeczyńska 2010). The aim of the present study was to examine the potential modifications of mechanisms of IGF-I-dependent proliferation and differentiation of myogenic cells, exposed to TNF-α and IFN-γ. We investigated myoblast fusion and the expression of myosin heavy chain in murine C2C12 myoblasts during differentiation, as well as the cellular content of molecular markers of cell cycle and differentiation: cyclin A, MyoD and myogenin.

Materials and Methods

Cell culture

C2C12 mouse myoblast cell line (satellite cells from thigh muscle) purchased from the European Collection of Animal Cell Culture (ECACC) was used for the study. The cells were free of contamination and were maintained in an exponential phase of growth in 10% (v/v) FBS/DMEM, together with antibiotic-antimycotic mixture, in controlled humidified air supplemented with 5% CO_2, at 37°C. The growing medium was changed every other day until cells reached 90% confluence. The cells were then subjected to 5-day differentiation (switch to differentiation medium – 2% (v/v) horse serum HS/DMEM) in the presence of IGF-I (30 nmol/l), TNF-α (1 ng/ml), IFN-γ (1 ng/ml), or a combination of growth factor with each cytokine. The concentration of TNF-α and IFN-γ used in the present experiments was chosen on the basis of a previous study (Grzelkowska-Kowalczyk and Wieteska-Skrzeczyńska 2006 and unpublished data) showing that this dose of cytokines is effective in inhibiting the metabolic effects of insulin in C2C12 myogenic cells. Control cultures were mantained in 2% HS/DMEM. To preserve the characteristics of the C2C12 cell line, the splitting of cells was done up to a maximum of 7 times.

Myoblast fusion

In order to visualize the range of morphological changes in C2C12 cultures on the 3rd and on the 5th day of myogenesis monolayers were washed twice with ice-cold PBS and fixed with 75% methanol (v/v) for 20 minutes. Two ml of Giemsa stain (0.04% w/v in methanol) (Sigma-Aldrich) was added to the dishes and incubated for 2 minutes. An equal volume of distilled water was added for 1 min. Nuclei were counted using a phase-contrast microscope (IX 70, Olympus). To determine myotube formation the average number of nuclei in ten random fields was recorded for each dish. The results were presented as: fusion index (%) = (number of nuclei in myotubes)/(total number of nuclei in myoblasts and myotubes) x 100, as described by Dedieu et al. (2002).

Reinduction of proliferation

Quiescent myoblasts were obtained as described previously (Kitzmann et al. 1998). Briefly, confluent C2C12 cells were allowed to differentiate for 3 days in the presence of the experimental factors. During this period a majority of myoblasts differentiate and fuse into myotubes, whereas some myoblasts stop prolif-

erating but do not differentiate. Myotubes are more susceptible to trypsinization than residual myoblasts, thus a limited trypsinization (0.15% trypsin-EDTA, 30 seconds) of differentiating cell cultures gave rise to 2 populations of cells: detached myotubes and quiescent undifferentiated myoblasts still adherent to the culture dishes. These quiescent cells were then allowed to reenter the cell cycle by addition of fresh growth medium to the culture. Control cultures were mantained in 10% FBS/DMEM.

Immunoblotting

Aliquots of whole cell lysates corresponding to 50 μg of total protein were subjected to SDS-PAGE. After electrotransfer the membranes were probed with an appropriate primary antibody (Santa Cruz Biotechnology), and were then exposed to the appropriate secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Membranes were also reprobed with goat polyclonal anti-actin antibody to ensure that all lanes contained equal amounts of total protein. The blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's directions. The bands were scanned and analyses of optical density were performed using Kodak EDAS 290/Kodak 1D 3.5 system (Eastman Kodak Company, Rochester, NY, USA). Optical density of the band of each studied protein was presented in arbitrary units in relation to the optical density of actin.

Statistics

The data were expressed as means ± S.E. and were representative of three separate experiments performed in triplicate. Student's t-test was used for the comparison of two means (treatment effect). The results were also evaluated using ANOVA, with actin data included as a covariate. For each assay individual values were used in statistical analysis with n = 9/treatment condition. Statistical significance was set at $P < 0.05$.

Results

In our experiments C2C12 myogenic cells placed in differentiation medium began to fuse after 3 days of culturing, and the fusion index on the 5th day amounted to 39.6%, (Fig. 1). TNF-α caused a 3.5 fold increase in myoblast fusion on the 3rd day of myogenesis. IFN-γ did not modify the myoblast fusion on the 3rd day; however, it significantly decreased the fusion index on the 5th day of differentiation (by 30% in comparison to the value obtained in control culture on the same day, $P < 0.05$). Supplementation of differentiation medium with IGF-I resulted in a significant rise in the fusion index on the 3rd day of differentiation (26.5% vs 2.33% of fusion in IGF-I-treated and control cultures, respectively, $P < 0.05$). Both TNF-α and IFN-γ affected IGF-I-mediated myoblast fusion. This was manifested by a decrease in the fusion index on the 3rd day by 33.5% and 58%, respectively ($P < 0.05$). The inhibition of IGF-I-dependent myoblast fusion by TNF-α and IFN-γ on the 5th day was similar and the fusion index amounted to appr. 80% of the value detected in cultures exposed to IGF-I.

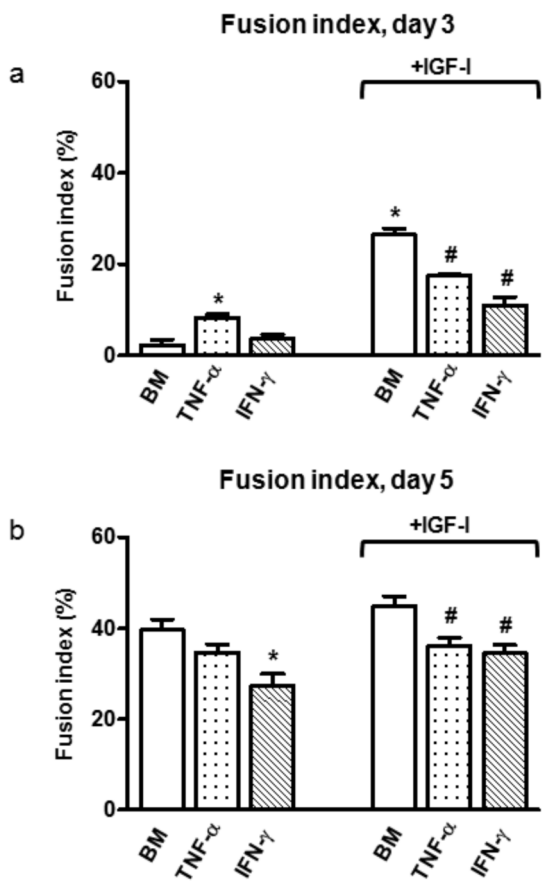

Fig. 1. Fusion index in C2C12 myogenic cell cultures differentiated in 2% HS/DMEM (Basal medium, BM), supplemented with TNF-α (1 ng/ml) or IFN-γ (1 ng/ml), in the absence or presence of 30 nmol/l IGF-I, assessed on the 3rd day (a) and on the 5th day of differentiation (b). The results represent the mean ± SE, with n = 9/treatment condition. * – significantly different *versus* control value on the same day, # – significantly different *versus* IGF-I treatment on the same day.

A gradual increase in expression of the heavy chain subunit of myosin on the 3rd and the 5th day of differentiation occurred in control cultures (Fig. 2).

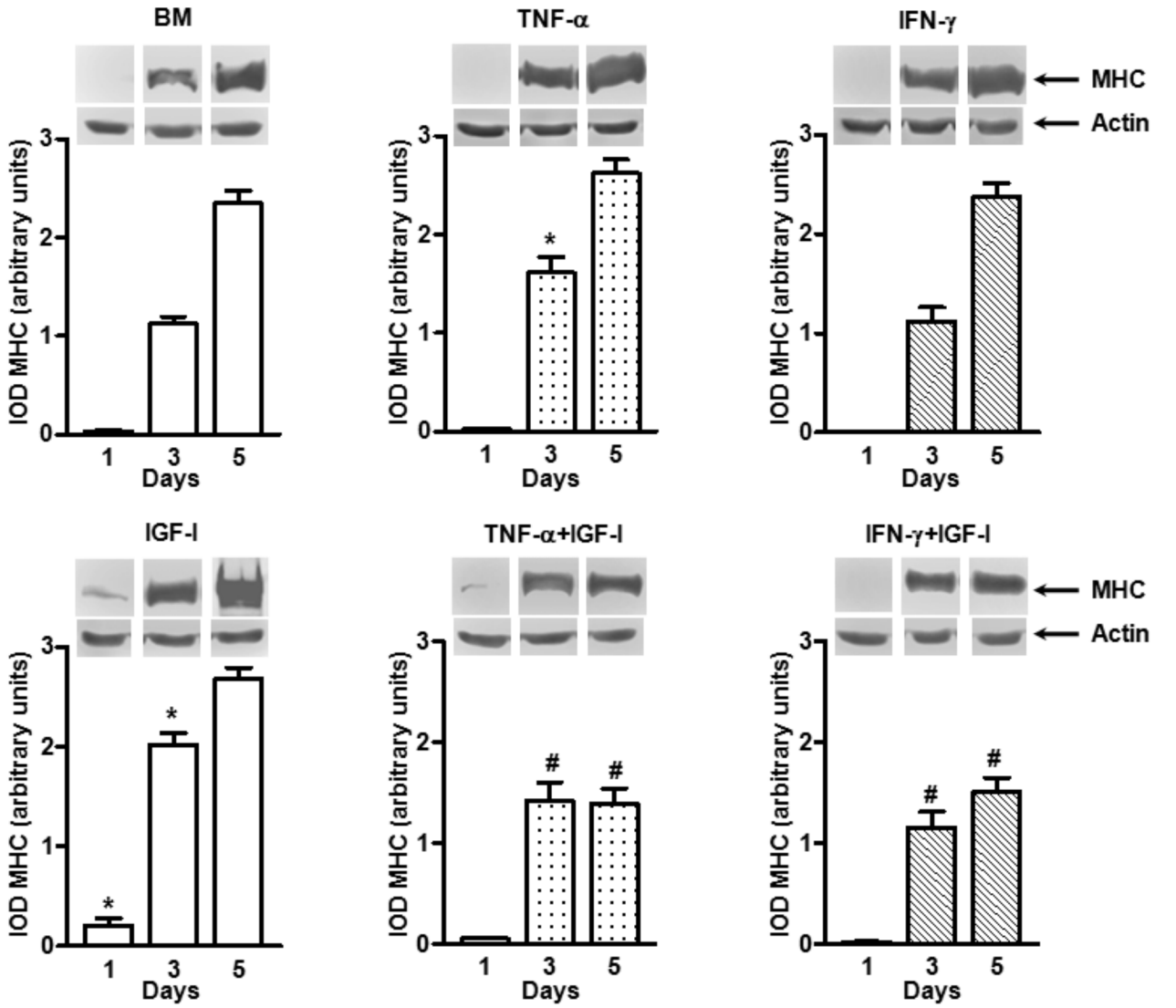

Fig. 2. Cellular content of myosin heavy chain (MHC) in C2C12 myogenic cell cultures subjected to differentiation in 2% HS/DMEM (Basal medium, BM), supplemented with TNF-α (1 ng/ml) or IFN-γ (1 ng/ml), in the absence or presence of 30 nmol/l IGF-I. Total actin protein at subsequent time points of the experiments is also presented. Blots are representative of three separate experiments. The results represent the mean ± SE, with n = 9/treatment condition. * – significantly different *versus* control value on the same day, # – significantly different *versus* IGF-I treatment on the same day.

Addition of TNF-α to differentiation medium caused a significant rise in the MHC expression on the 3rd day ($P < 0.05$), whereas IFN-γ had no effect. IGF-I stimulated the MHC expression on the 1st and the 3rd day in comparison to the values obtained in control cultures ($P < 0.05$). TNF-α and IFN-γ markedly reduced IGF-I-dependent MHC expression on the 3rd and the 5th day of differentiation ($P < 0.05$).

In the control myoblasts the expression of MyoD did not change during the first three days of differentiation, and then a decrease was observed on the 5th day (Fig. 3). TNF-α caused a drop in MyoD cellular content on the 3rd day, whereas IFN-γ inhibited MyoD expression on the 3rd and 5th day of differentiation. IGF-I significantly stimulated MyoD expression in C2C12 myoblasts during 5 days of differentiation. In C2C12 cultures exposed to TNF-α or IFN-γ and IGF-I the MyoD level was significantly lower in the 5-day period of the experiment.

IGF-I clearly stimulated myogenin expression with the maximum level on the 3rd day (Fig. 4). TNF-α and IFN-γ exerted no significant effect on myogenin expression in myoblasts differentiated under control conditions and slightly reduced the myogenin protein level on the 1st day and on the 3rd day, respectively, in the presence of IGF-I.

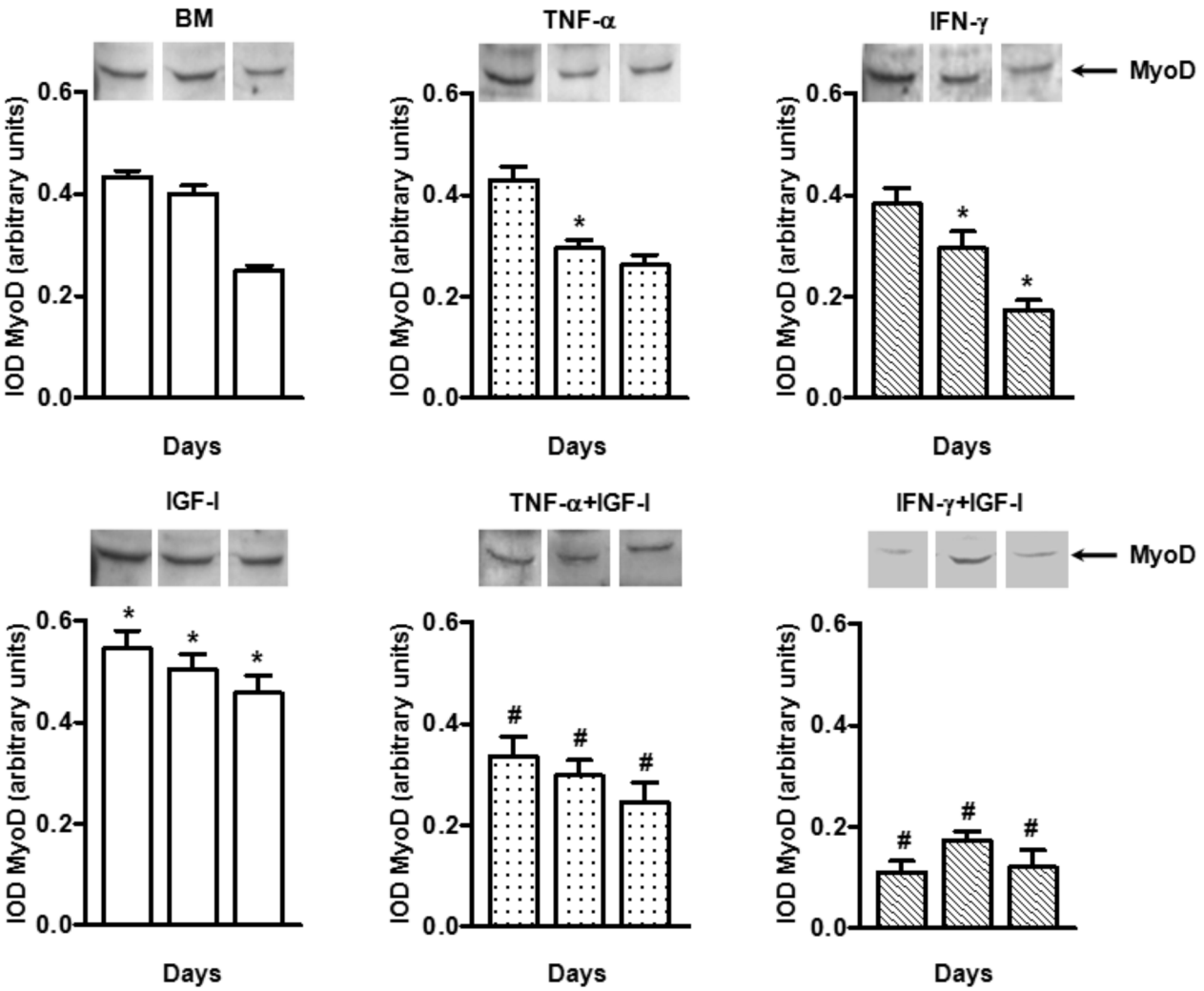

Fig. 3. Cellular content of MyoD in C2C12 myogenic cell cultures subjected to differentiation in 2% HS/DMEM (Basal medium, BM), supplemented with TNF-α (1 ng/ml) or IFN-γ (1 ng/ml), in the absence or presence of 30 nmol/l IGF-I. Blots are representative of three separate experiments. The results represent the mean ± SE, with n = 9/treatment condition. * – significantly different *versus* control value on the same day, # – significantly different *versus* IGF-I treatment on the same day.

Addition of growth medium to residual myoblasts obtained from differentiating culture resulted in a reinduction of proliferation, manifested by cyclin A expression (Fig. 5). Exposing of C2C12 myoblasts to TNF-α or IFN-γ for 3 days caused a significant increase in cyclin A level in cells reinduced to proliferation by 24-hour incubation under standard growth conditions ($P < 0.05$). Supplementing of growth medium with IGF-I led to a marked rise in the cyclin A level in myoblasts restimulated to proliferation ($P < 0.05$). Preincubation with each cytokine caused a 2-fold decrease in IGF-I-stimulated cyclin A expression in myoblasts restimulated to proliferation.

Discussion

Cytokines released by the cells of the immune system influence muscle development, growth and metabolism. A direct effect of the cytokines on the mechanisms controlling muscle regeneration is, however, still controversial, because of their potential importance in satellite cell activation (Chen et al. 2007) and, simultaneously, of the involvement in muscle wasting in several pathological conditions (Yeh et al. 2008).

Data of the present study indicate that TNF-α and IFN-γ can modify the process of differentiation with a clear inhibitory effect on IGF-I-induced myogenesis (assessed on the basis of the fusion index, Fig. 1) and

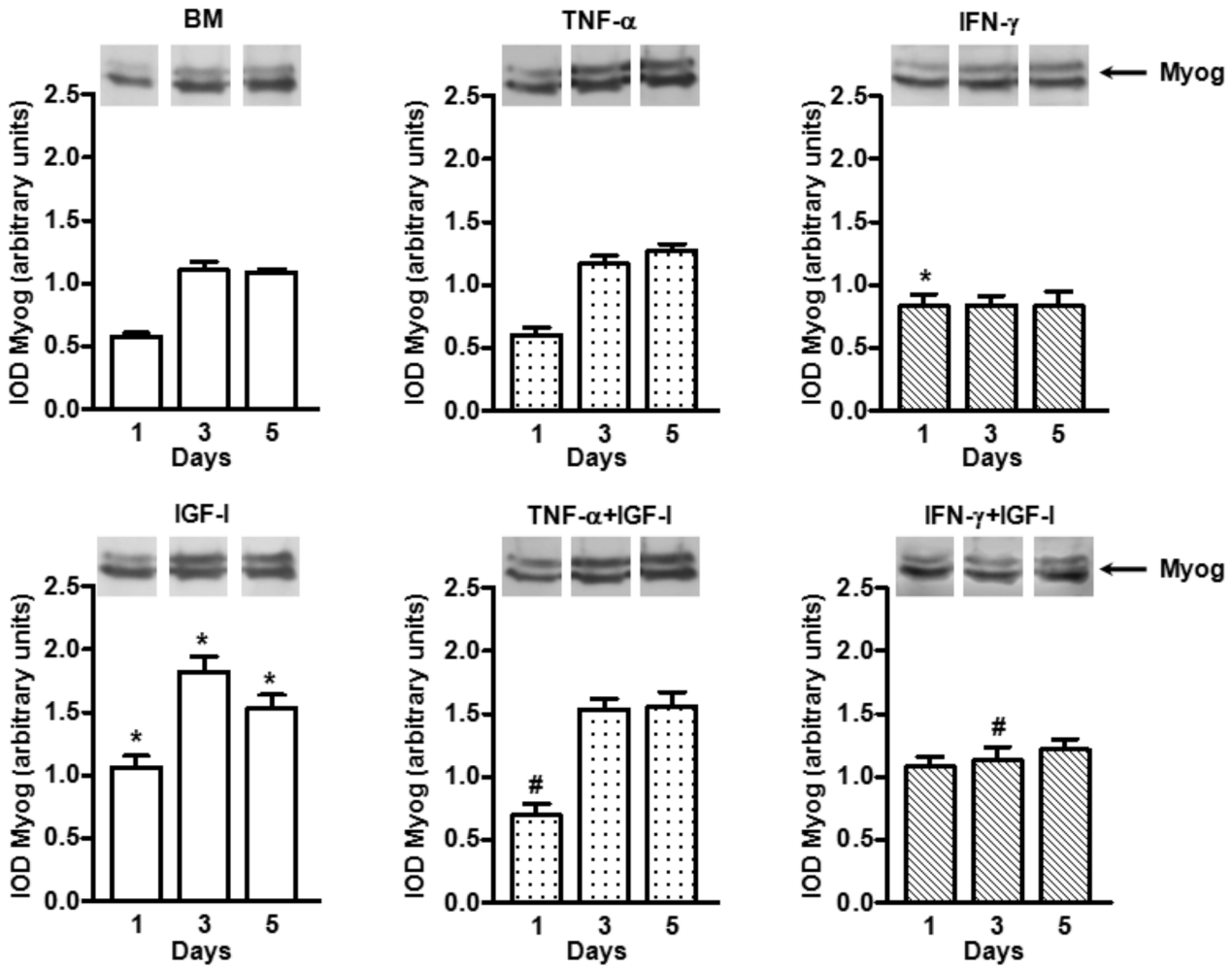

Fig. 4. Cellular content of myogenin (Myog) in C2C12 myogenic cell cultures subjected to differentiation in 2% HS/DMEM (Basal medium, BM), supplemented with TNF-α (1 ng/ml) or IFN-γ (1 ng/ml), in the absence or presence of 30 nmol/l IGF-I. Blots are representative of three separate experiments. The results represent the mean ± SE, with n = 9/treatment condition. * – significantly different *versus* control value on the same day, # – significantly different *versus* IGF-I treatment on the same day.

myotube growth (manifested by MHC level, Fig. 2). Interestingly, the addition of TNF-α alone to the differentiation medium increased myoblast fusion on the 3rd day of observation, which was compatible with augmented MHC level and suggests that this cytokine accelerates the early steps of myogenesis. This conclusion is in accordance with the previous report by Li and Schwartz (2001) showing that TNF-α stimulates MHC expression during the early stages of differentiation but attenuates MHC accumulation at late stages of differentiation. IFN-γ did not exert a stimulatory effect on myoblast fusion and, conversely, inhibited formation of myotubes on the 5th day of observation. Thus, according to our data, TNF-α and IFN-γ have direct and differential effects on early myogenesis.

In the present study the proliferative potential of myoblasts pre-exposed to TNF-α or IFN-γ was assessed by the level of cyclin A, which controls the G1/S and G2/M transitions, two major cell cycle points, and is an established marker of regenerative tissue potential (Cheng et al. 2007). Three-day-exposition of C2C12 myoblast cultures to TNF-α or IFN-γ resulted in a marked increase in cyclin A level in cells reinduced to proliferation (Fig. 5), which resembles the activation of satellite cells *in vivo* by molecules released in skeletal muscle by rapidly infiltrating inflammatory cells following muscle injury (Goetsch et al. 2003). IFN-γ and its receptor are expressed in the C2C12 muscle cell line and this cytokine has recently been found to be required for proliferation and fusion of C2C12 cells (Cheng et al. 2008). It has been demonstrated that the IFN-γ receptor blocking antibody reduced proliferation and fusion of these myogenic cells, pointing to the role of this cytokine in the formation of new muscle fiber and muscle healing, which is in apparent contrast to our study indicating disturbances in myoblast differentiation. However, Cheng et al. (2008) eliminated the effect of endogenous IFN-γ on

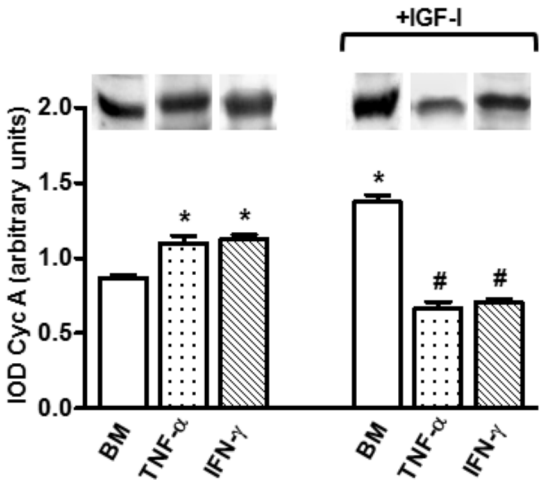

Fig. 5. Cellular content of cyclin A (Cyc A) in C2C12 myoblasts derived from cultures subjected to differentiation in 2% HS/DMEM (Basal medium, BM), supplemented with TNF-α (1 ng/ml) or IFN-γ (1 ng/ml), in the absence or presence of 30 nmol/l IGF-I. Blots are representative of three separate experiments. The results represent the mean ± SE, with n = 9/treatment condition. * – significantly different *versus* value obtained in cultures treated with basal medium (BM), # – significantly different *versus* value obtained in cultures treated with IGF-I (IGF-I).

proliferation and fusion of C2C12 cells by blocking the specific receptor, whereas in our study the effect of IFN-γ added to the differentiation medium was explored. Even thought IFN-γ seems to be required for efficient cell proliferation and fusion, an excess of this cytokine could impair the formation of muscle fibers. The effect of cytokines on several differentiation markers has already been reported. For example, exposition of differentiated C2C12 myotubes (Di Marco et al. 2005) or myoblasts (Dehoux et al. 2007) to TNF-α in combination with IFN-γ caused a significant reduction in MHC level with no effect on the fusion index or creatine kinase release. One may expect that prolongation of the present experiment could result in a reduction of MHC in cytokine-treated myogenic cells as a consequence of: i) decreased level of MyoD, a myogenic transcription factor driving the transcription of MHC, ii) activation of proteolytic pathways, mainly the ubiquitin-proteasome system in the presence of cytokines (Langen et al. 2004).

In agreement with the myoblast fusion pattern, both TNF-α and IFN-γ significantly reduced IGF-I-stimulated MyoD expression (Fig. 3). These results are consistent with the recent report (Langen et al. 2004), showing that in C2C12 myoblast cultures subjected to differentiation in the presence of TNF-α (10 ng/ml) a striking reduction in MyoD level, assessed by immunoblotting, was observed after 72 h of differentiation. TNF-α treatment increased MyoD degradation through the ubiquitin-proteasome pathway (Tintignac et al. 2005), which resulted in a sustained myoblast proliferation and inhibition of myogenic differentiation.

Proinflammatory cytokines and IGFs are released during muscle repair and guide muscle regeneration (Karalaki et al. 2009). TNF-α has already been shown to inhibit IGF-I-stimulated hypertrophy of C2C12 cells (assessed by MHC levels) and expression of two critical myogenic regulatory factors – MyoD and myogenin in C2C12 myoblasts (O'Connor et al. 2008). The lack of evident changes in cellular content of myogenin under cytokine treatment presented in our study (Fig. 4) is therefore in contrast with previous observations. A possible explanation of such discrepancy could be a relatively minor contribution of myogenin to myoblast differentiation under experimental conditions used in the study. In these experiments MyoD appeared to be a critical myogenic factor in determining myogenesis progression and, simultaneously, its level, even if decreased in the presence of TNF-α and IFN-γ, was sufficient to activate the expression of myogenin.

The impairment of differentiation of C2C12 myoblasts by TNF-α and IFN-γ only in the presence of IGF-I indicates that in target cells the integration of numerous signals occurs via an intracellular cross-talk among distinct ligand-activated systems and an activation of one pathway may interfere with signal transduction of others, leading to the modulation or inhibition of original biological effects. An intringuing example of such an interaction i.e. the pro-apoptotic effect of a very low dose of IGF-I used in combination with TNF-α in myoblast cultures has recently been described (Saini et al. 2008). In our experiments we did not observe any symptoms of apoptosis; moreover, the assessment of protein synthesis in all experimental treatments gave a similar pattern of results (Wieteska-Skrzeczyńska et al. submitted). This indicates that decreased formation of myotubes in cultures exposed to TNF-α or IFN-γ and IGF-I resulted from the modification of myogenesis and not from increased cell mortality. According to our results, treatment of C2C12 myoblasts with TNF-α or IFN-γ decreased IGF-I-stimulated cyclin A expression in myoblasts restimulated to proliferation, indicating that cytokine-IGF-I interactions are not limited to myogenesis regulation.

Our results permit the following conclusions: i) TNF-α and IFN-γ reduce IGF-I-dependent myogenesis, which was manifested by the reduction of myoblast fusion and MHC cellular levels, ii) Molecular mechanisms of inhibitory action of TNF-α and IFN-γ on IGF-I-mediated differentiation involve a decrease in MyoD, whereas myogenin level plays a minor role, iii) TNF-α and IFN-γ increase the proliferative potential of myoblasts; however, they reduced

the mitogenic effect of IGF-I, which was manifested by a decrease in IGF-I-stimulated cyclin A expression in myoblasts reinduced to proliferation. The interactions among IGF-I and proinflammatory cytokines are therefore important in establishing the number of myoblasts and the onset of myogenesis during muscle regeneration.

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