Polish Journal of Veterinary Sciences Vol. 16, No. 2 (2013), 255–264

DOI 10.2478/pjvs-2013-0036

Original article

Interleukin-1β stimulates early myogenesis of mouse C2C12 myoblasts: the impact on myogenic regulatory factors, extracellular matrix components, IGF binding proteins and protein kinases

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Abstract

The purpose of the study was to examine the mechanisms important for early myogenesis in mouse C2C12 myogenic cells exposed to interleukin-1β. Cyclin A and cyclin B1 were increased by interleukin-1β (1 ng/ml), but the level of cyclin D1 and total DNA content was unaffected. Fusion index and the rate of protein synthesis was increased in the presence of IL-1β, but these effects were limited to 3-day-treatment. IL-1β increased the level of MyoD, myogenin and MHC on the 3rd day of differentiation, without altering the content of the active form of myostatin, as well as it augmented the level of fibronectin, integrin β1 and full length 100 kDa form of ADAM12. IL-1β caused a decrease in IGFBP-4 and IGFBP-6 levels and a marked increase in IGFBP-5. The phosphorylation of PKB and ERK1/2 and the cellular content of p38 were elevated by IL-1β. We conclude that the myogenic effect of IL-1β was limited to the onset of myoblast fusion and was associated with: i) increase in the level of myogenic transcription factors i.e. MyoD and myogenin expression, ii) modification of extracellular matrix assembly and signaling, manifested by an increase in fibronectin, integrin-β1 and ADAM12 content, iii) drop in IGFBP-4 and IGFBP-6, and an increase in IGFBP-5, that could alter the local IGF-1 bioavailability, and iv) increase in phosphorylation of PKB and ERK1/2, and the expression of p38 kinase, leading to activation of intracellular pathways essential for myogenic differentiation.

Key words: Cyclins, ECM, IGFBPs, IL-1β, myogenic regulatory factors, signaling

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Introduction

Proinflammatory cytokines are usually considered as catabolic factors that mediate the development of skeletal muscle wasting. TNF-α, a very potent cytokine, is the archetype in this category, however it exerts many effects probably via the stimulation of IL-1β release (Zoico and Roubenoff 2002). Several reports suggest direct or indirect actions of IL-1β on skeletal muscle. Both plasma IL-1β and hindlimb muscle ubiquitin-proteasome pathway were elevated in tumor-bearing and cachectic mice (Cannon et al. 2008). According to in vivo (Cai et al. 2004) and in vitro studies (Luo et al. 2003, Chevrel et al. 2005) IL-1β activated p38 MAPK and NF-kB: the signaling elements that promote the proteolysis in muscle and the reduction of myofibrillar proteins (Li et al. 2009).

On the other hand, the view that cytokines may contribute in a positive way to skeletal muscle growth and metabolism was supported by several other studies. IL-6, considered also as a physical activity-associated myokine (Pedersen 2011), TNF-α (Chen et al. 2007) and IFN-γ (Cheng et al. 2008) have been shown to play a physiological role in myogenesis and muscle repair, however the importance of other proinflammatory cytokines is less understood.

Skeletal muscle has a remarkable ability to regenerate itself. Muscle regeneration after injury seems to follow the same procedure as muscle development during embryogenesis (Karalaki et al. 2009). This is a complex process comprising highly coordinated events in a sequence of satellite cell activation, proliferation, and differentiation leading to injury repairing or formation of new myofibers. Sequential expression of myogenic regulatory factors, i.e. Myf 5, MyoD, myogenin and MRF4 is required for the formation of myofibers and muscle-specific gene expression (Charge and Rudnicki 2004), whereas myostatin is a potent negative regulator of myoblast proliferation and differentiation (Langley et al. 2004).

The importance of extracellular matrix (ECM) molecules as part of the myogenesis signaling mechanism has been demonstrated. Osses and Brandan (2002) have reported that neither the expression of myogenin alone nor its localization to myoblast nuclei was sufficient to drive skeletal muscle differentiation. Moreover, the shift of the myogenic pathway of C2C12 myoblasts into the osteogenic lineage by inhibitors of proteoglycan sulfation has been described (Osses et al. 2009).

IGF-I is a central anabolic factor in skeletal muscle, critical in promoting both proliferation and differentiation of skeletal muscle cells, muscle hypertrophy and muscle regeneration following injury (Nystrom et al. 2009) and several cytokines have long been considered to induce IGF-I resistance. For example, in intact animals IL-1α elevated circulating IGFBP-1, effectively reducing IGF bioavailability (Benbassat et al. 1999) pointing to the systemic mechanisms of cytokine catabolic effects. Regarding in vitro models, several studies reported the inhibition of myogenic effect of IGF-I (O'Connor et al. 2008, Wieteska--Skrzeczyńska et al. 2011), whereas others provided evidence for the contrary, i.e. synergistic interactions of cytokines with IGF-I system in controlling myoblast number and differentiation (Al-Shanti et al. 2008).

The major purpose of the present study was the examination of mechanisms controlling proliferation, differentiation, fusion and growth of myogenic cells, exposed in vitro to proinflammatory cytokine, IL-1β. We investigated the cellular content and localization of cyclins: A, B1, and D1 in proliferating murine C2C12 myoblasts, and the fusion index, protein synthesis, and expression of MyoD, myogenin, an active form of myostatin, and myosin heavy chain (MHC) after induction of differentiation. The cellular levels of some extracellular matrix elements, i.e. fibronectin, integrin α5, β1 subunits, and disintegrin metalloprotease ADAM12, main binding proteins controlling IGF-I bioavailability and playing a key role in myogenic differentiation, i.e. IGFBP-4, -5, -6 as well as expression and phosphorylation of intracellular proteins essential for the mitogenic and myogenic effects, i.e. protein kinase B (PKB), extracellular signal-regulated kinase ERK1/2 and p38 in differentiated C2C12 myoblasts were investigated.

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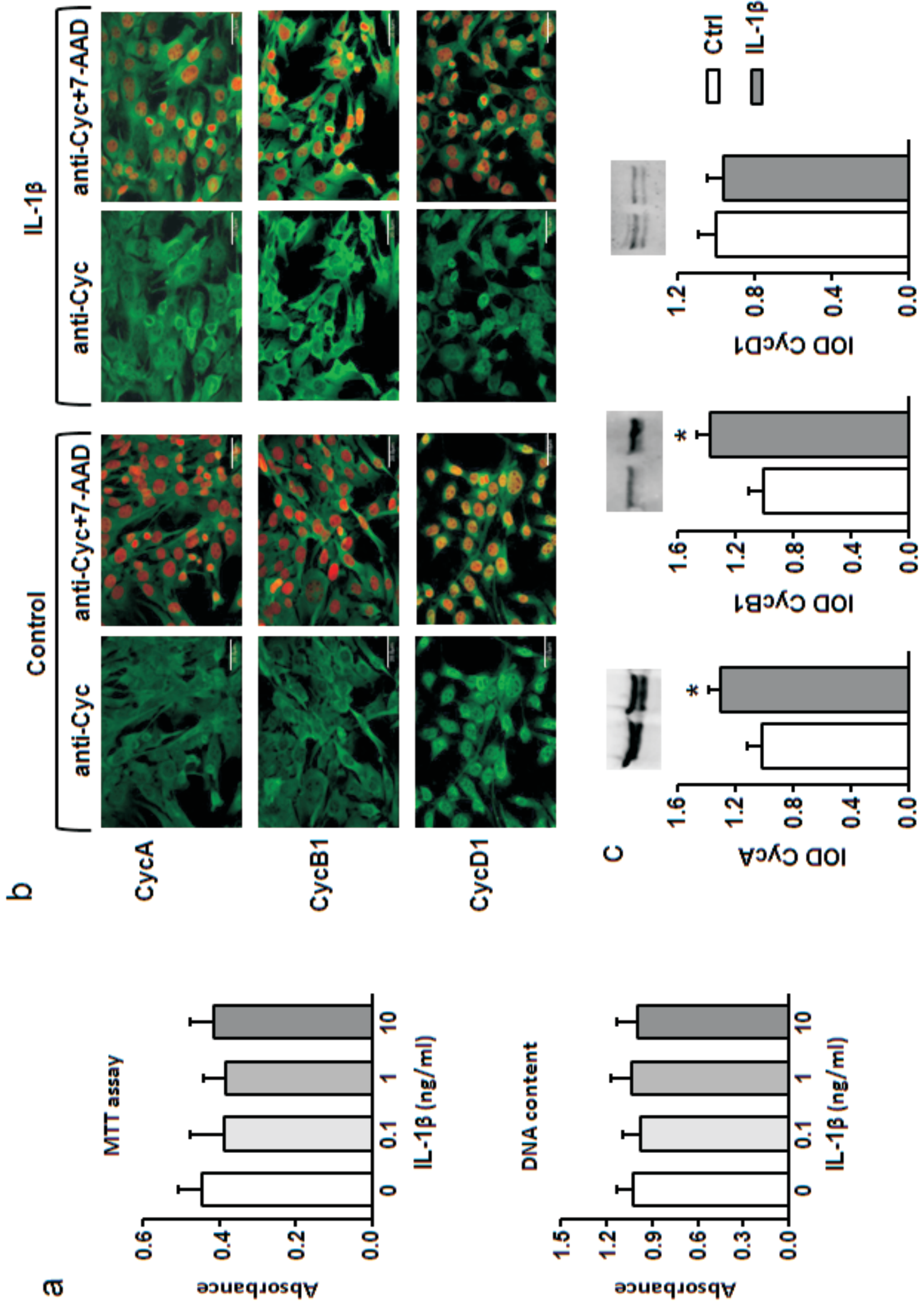
Fig. 1. **a.** Cell respiration (MTT assay) and DNA content (crystal violet test) in mouse C2C12 myoblasts exposed to different concentration of interleukin-1f (IL-1β) for 24 hours. The results represent the mean ± SD, with n = 12/treatment condition. **b.** Cellular content and localization of cyclin A (CycA), cyclin B1 (CycB1) and cyclin D1 (CycD1) in mouse C2C12 myoblasts proliferating for 24 hours in 10% FBS/DMEM (Control) or in the presence of IL-1β (1 ng/ml). Cell cultures were stained with antibodies against appropriate cyclins (green) and simultaneous nuclear staining with 7-AAD (red) was performed. Images are representative of 10 independent fields in three separate experiments. Bar, 20 μm. **c.** The level of CycA, CycB1 and CycD1 in whole cell lysate assessed by immunoblotting. Blots are representative of three separate experiments performed in triplicate. The densitometric quantitation of the specific bands is presented in arbitrary units, with the value obtained in the control group (Ctrl) set as 1. The results represent the mean ± SD, with n = 9/treatment condition. * – significantly different *versus* control value.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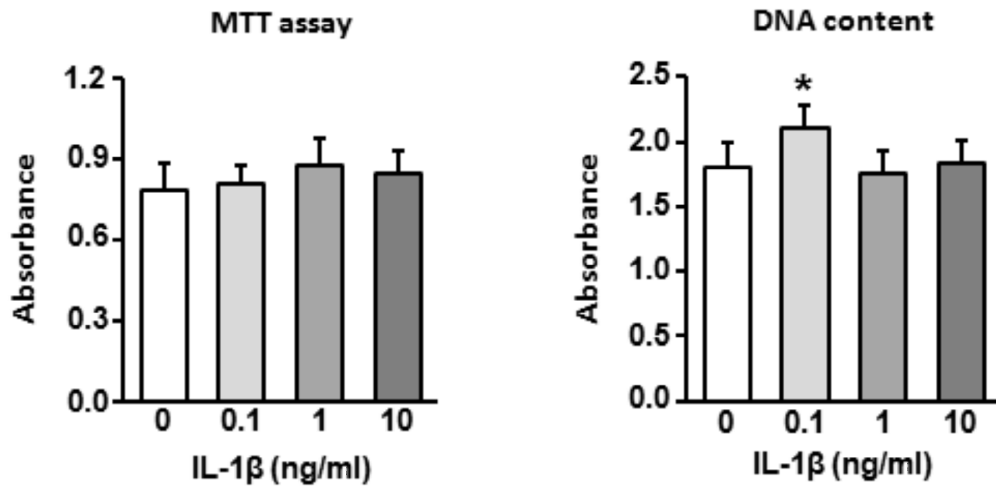

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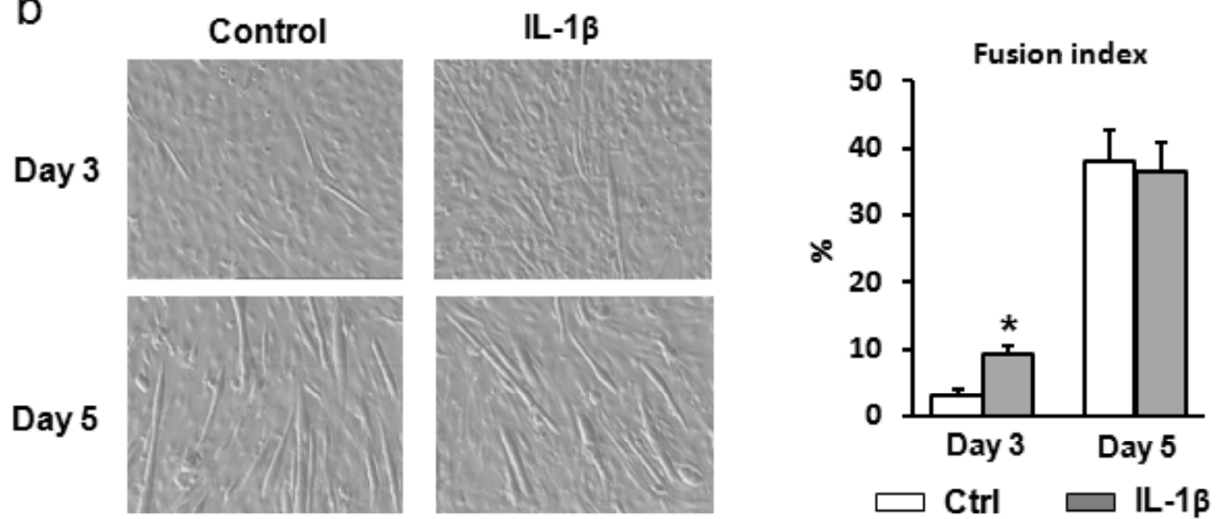

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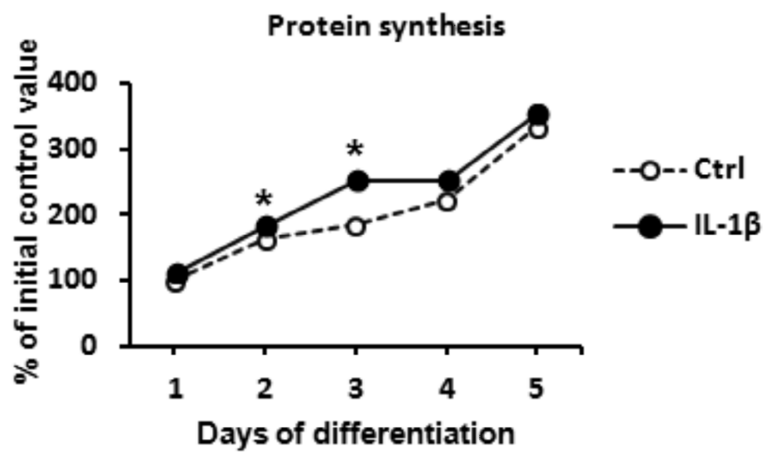

Materials and Methods

Cell culture

C2C12 mouse myoblast cell line (satellite cells from thigh muscle) purchased from the European Collection of Animal Cell Culture (ECACC), free of contamination, were maintained in an exponential phase of growth in 10% (v/v) FBS/DMEM added with antibiotic-antimycotic mixture, in controlled humidified air supplemented with 5% CO_2, at 37°C. The growing medium was changed every 48 hrs. Cell cultures at approximate 40% confluence were treated with interleukin-1β at concentration range of 0.1-10 ng/ml for 24 hrs. In another set of experiments, cells at approximately 90% confluence were subjected to differentiation (switch to differentiation medium – 2% (v/v) horse serum HS/DMEM) in the presence of IL-1β. For experiments focused on cellular levels of proteins involved in mechanisms controlling cell cycle and differentiation the IL-1β concentration of 1 ng/ml was chosen. To preserve the characteristics of the C2C12 cell line, the splitting of cells was done up to a maximum of 7 times.

Assessment of cell viability and proliferation

Viability of proliferating and differentiating cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in 96-well plates and after different incubation time, 180 µL of MTT solution (0.5 mg/ml) in PBS was added to each well. The plates were then incubated for 4 h at 37°C. The reaction product i.e. the formazan precipitate was then solubilized in 100% DMSO (100 µl/well).

The crystal violet assay was performed to determine the total amount of nuclear DNA corresponding with cell proliferation. The cells cultured in 96-well plates were fixed with 75% and 100% methanol for 20 min and then monolayers were stained using crystal violet solution (2 mg/ml) for 5 min. The excess of unbound dye was removed by washing the plates with water. The bound crystal violet was released by adding 1% SDS for 30 min.

In both assays the absorbance was measured on a multidetection microplate reader Infinite 200 PRO Tecan™ (TECAN, Mannedorf, Switzerland) at a wavelength of 570 nm.

Assessment of protein synthesis

To determine the changes in protein synthesis, 4-hour-labeling with [^{3}H]leucine (ICN Polfa Rzeszów, final activity 1 µCi/ml) was used. The results in d.p.m. were expressed as a % of the value obtained in control conditions.

Myoblast fusion

In order to visualize the morphological changes in C2C12 cultures on the 3rd day of myogenesis cell monolayers were washed twice with ice-cold PBS, fixed with 75% methanol (v/v) and then the Giemsa staining was performed. Nuclei were counted using a phase-contrast microscope (IX 70, Olympus), and 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).

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

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Fig. 2. Cell respiration (MTT assay) and DNA content (crystal violet test) in differentiating mouse C2C12 myocytes exposed to different concentration of interleukin-1β (IL-1β) for 3 days. The results represent the mean ± SD, with n = 12/treatment condition. * – significantly different *versus* control value. **b.** Representative phase-contrast images (magnification x100) of C2C12 myogenic cells on the 3rd and on the 5th day of differentiation in the absence (Control) and presence of IL-1β (1 ng/ml). The fusion index values represent the mean ± SD, with n = 10/treatment condition. **c.** The rate of protein synthesis during 5-day differentiation of C2C12 myogenic cells. The data are presented in arbitrary units, with the initial control value set as 100%. The results represent the mean ± SD, with n = 7/treatment condition. * – significantly different *versus* control value for the same day.

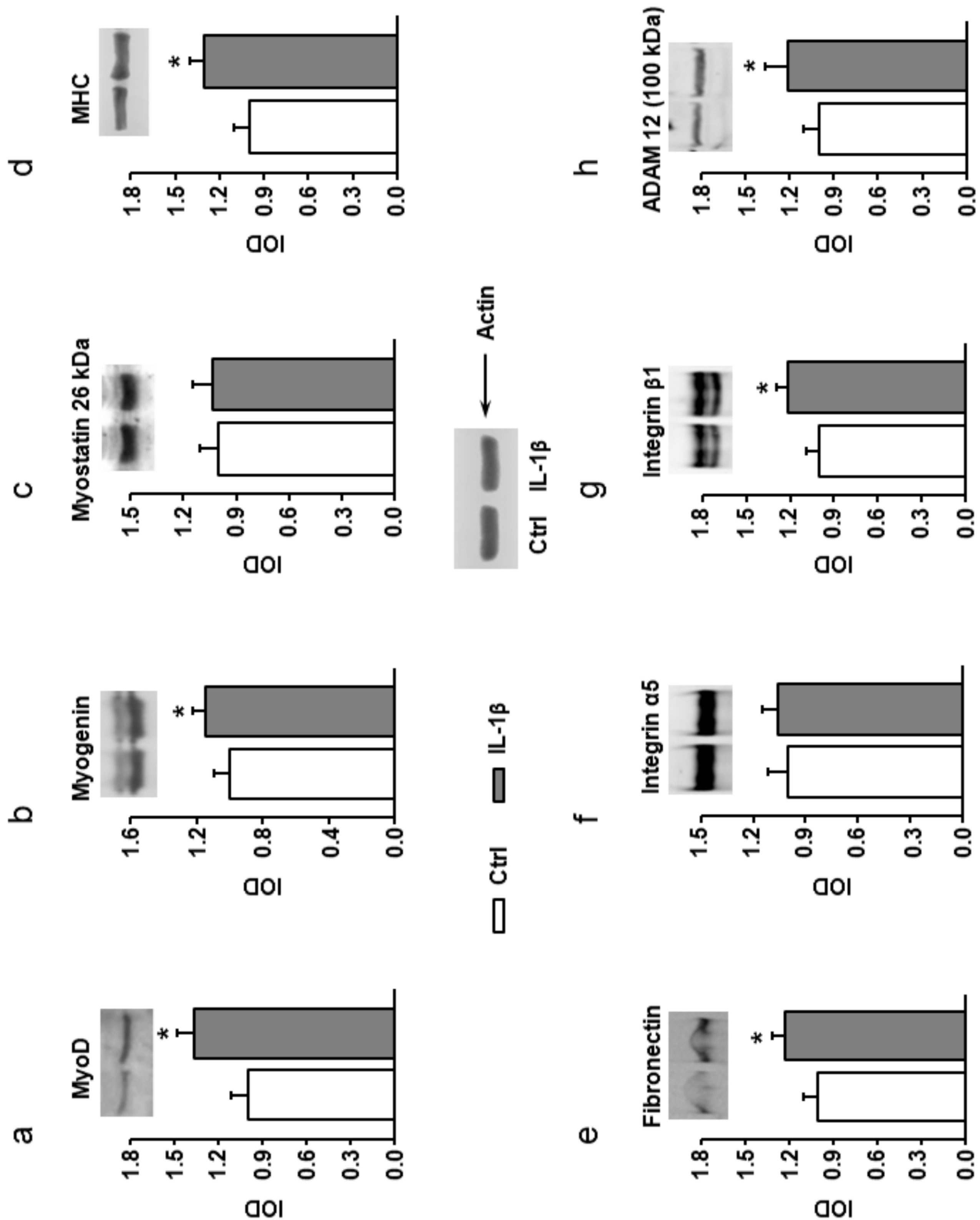

Fig. 3. Cellular content of MyoD (**a**), myogenin (**b**), active form of myostatin (**c**), myosin heavy chain (MHC, **d**), fibronectin (**e**), integrin-α5 (**f**), integrin-β1 (**g**) and long chain form of ADAM12 (**h**) in mouse C2C12 myogenic cells subjected to 3-day differentiation in 2% HS/DMEM (Control, Ctrl), or in the presence of IL-1β (1 ng/ml). Total actin protein isolated from control and experimental cultures is also presented. Blots are representative of three separate experiments performed in triplicate. The results represent the mean ± SD, with n = 9/treatment condition. * – significantly different *versus* control value.

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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).

Immunofluorescence staining and confocal microscopy

Cell cultures were carried out directly on glass Lab-tec coverslips (Nunc Inc., USA). The cells were fixed with 3.7% paraformaldehyde for 20 min at room temperature, permeabilized with 0.05% Triton X-100 in PBS and then incubated overnight in darkness at 4°C with primary antibody anti-cyclin A, anti-cyclin B1, or anti-cyclin D1 (all purchased from Santa Cruz Biotechnology) diluted 1:100 in PBS. The slides were rinsed three times with PBS and incubated for 1 hr with Alexa Fluor 488 secondary antibodies (Eugene, USA) diluted 1:500 in PBS. For nuclear visualization cells were stained with 7-aminoactinomycin D (7-AAD, 5 μg/ml) in PBS for 15 min at room temperature. After rinsing, the coverslips were mounted on glass slides using Fluorview mounting medium (Sigma-Aldrich) and the cells were visualized by confocal laser scanning microscope FV-500 system (Olympus Optical Co, Hamburg, Germany). The combination of excitation/emission were: Argon 488 nm laser with 505-525 nm filter for Alexa Fluor 488 and HeNe 543 nm laser with 610 nm filter for 7AAD nucleus staining. Stack of cross-sections were gathered separately for each fluorescence channel.

Statistics

The data were expressed as means ± S.D. Differences were assessed using one-way ANOVA for comparison of multiple means. When ANOVA detected a significant overall difference, Tukey's post hoc test for multiple comparison was performed. Student's t-test was used for the comparison of two means, and the statistical differences were marked when $P<0.05$.

Results

Interleukin-1β at concentration ranging between 0.1-10 ng/ml did not affect mouse C2C12 myogenic cells viability as was demonstrated by MTT assay and by DNA content in proliferating myoblasts (Fig. 1a). The cytokine markedly increased the level of cyclin A, assessed by immunoblotting, which was confirmed by higher cyclin A immunofluorescence seen in confocal microscopy. IL-1β also augmented the level of cyclin B1, associated with mitosis, both in the cytoplasm and in the myoblast nuclei. Cyclin D1 was localized mainly in the nucleus, and its level was not affected by IL-1β (Fig. 1b-c).

IL-1β in either concentration used did not affect cell viability during 3 days of differentiation (Fig. 2a). The DNA content in differentiating cultures was slightly but significantly increased by IL-1β at 0.1 ng/ml (by 16% in comparison to control group, $p<0.01$). The fusion index was significantly elevated under IL-1β treatment (1 ng/ml) on the 3rd day of differentiation, but was similar to control value on the 5th day (Fig. 2b). Exposure of C2C12 myocytes to IL-1β significantly increased the rate of protein synthesis on the 2nd and on the 3rd day of observation (by 12% and 36% in comparison to appropriate control value, respectively, $p<0.05$, Fig. 2c).

Interleukin-1β did not alter the cellular level of actin in differentiating myocytes (Fig. 3). The cytokine markedly increased the level of differentiation markers assessed on the 3rd day after induction of myogenesis, i.e. MyoD ($P<0.0001$), myogenin ($P=0.0038$) and myosin heavy chain ($P<0.0001$), however it did not modify the cellular content of the active form of myostatin 26 kDa ($P=0.48$). IL-1β elevated the level of fibronectin in C2C12 myogenic cells ($P=0.0005$). The expression of the integrin α5 (a component of the adhesion receptor for fibronectin) was not modified ($P=0.23$), whereas the integrin β1 (forming functional heterodimers with integrin a subunits) was significantly increased in the presence of IL-1β ($P<0.0001$). The cytokine also caused an increase in cellular content of full length 100 kDa form of ADAM12 ($P=0.0025$).

IL-1β led to the slight but significant decrease in IGFBP-4 ($P=0.0048$) and IGFBP-6 ($P=0.003$) levels as well as to a marked increase in IGFBP-5 ($P<0.0001$) on the 3rd day of differentiation (Fig. 4a-c). The phosphorylation of PKB was slightly but significantly augmented in the presence of IL-1β ($P=0.026$). The cytokine also increased the phosphorylation of ERK1/2 ($P=0.0002$) without changing the total ERK1/2 protein, as well as it augmented the cellular content of p38 ($P=0.0002$, Fig. 4d-f).

Discussion

The present study revealed the effect of proinflammatory cytokine, IL-1β, on the processes leading to muscle growth and development. Exposition of C2C12 myoblasts on this cytokine resulted in an increase in cellular level of cyclin A, which controls the G1/S and G2/M transitions, two major cell cycle

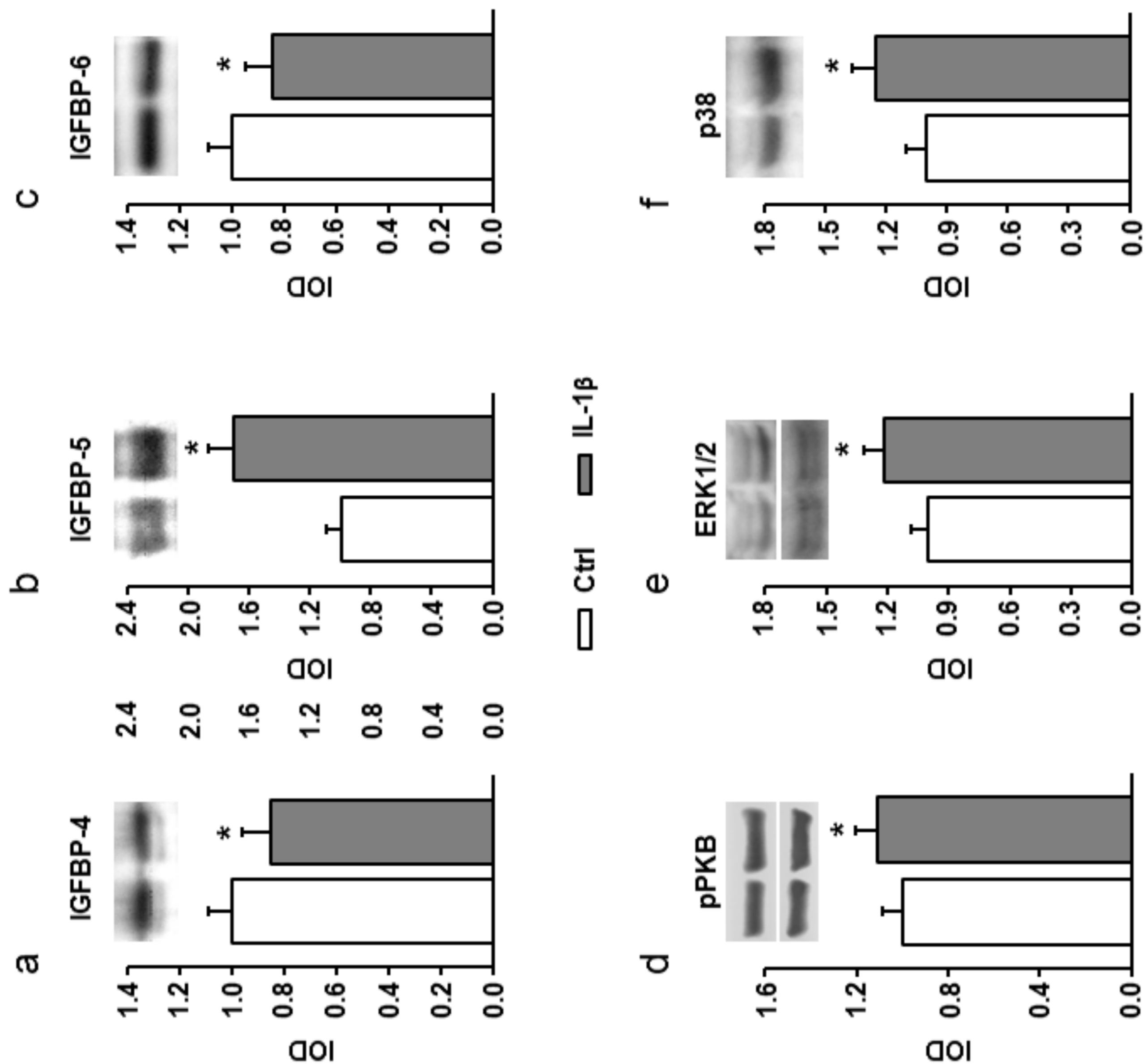

Fig. 4. Cellular content of IGFBP-4 (**a**), IGFBP-5 (**b**), IGFBP-6 (**c**), phosphorylated and total form of protein kinase B (PKB, **d**), phosphorylated and total form of extracellular signal-regulated kinase 1/2 (ERK1/2, **e**) and p38 (**f**) in mouse C2C12 myogenic cells subjected to 3-day differentiation in 2% HS/DMEM (Control, Ctrl), or in the presence of IL-1β (1 ng/ml). Blots are representative of three separate experiments performed in triplicate. The densitometric quantitation of the phosphorylated PKB and ERK1/2 is presented in arbitrary units as a ratio: the density of the bands identified by the phosphospecific antibody/the density of the bands identified by an antibody which recognizes total kinase protein, with the value obtained in the control group set as 1. The results represent the mean ± SD, with n = 9/treatment condition. * – significantly different *versus* control value.

points, and is established marker of regenerative tissue potential (Cheng et al. 2007), as well as the cyclin B1, associated with the mitosis (Fig. 1). The stimulatory effect of IL-1β does not concern all the cyclins essential for cell cycle progression since cyclin D1, important for transition through the G1 phase, although localized in the nuclei, was not augmented in cytokine-treated myoblast, and probably for this reason we did not observe activation of cell proliferation under these conditions. Exposure to IL-1β resulted in a marked stimulation of early myogenesis, manifested by an increase in fusion index and total protein synthesis assessed on the 3rd day after induction of differentiation (Fig. 2). These effects were in agreement with the augmented myosin heavy chain level and resulted from the increase of key myogenic regulatory factors, i.e. MyoD and myogenin expression, but appeared to be independent of myostatin regulation (Fig. 3). It should be noticed, however, that the stimulatory effect of IL-1β did not persist till the 5th day of

myogenesis, as assessed by the fusion index and rate of protein synthesis. Thus, the influence of IL-1β on cell cycle regulatory proteins and the early myogenesis resembles the activation of satellite cells by factors released after injury in skeletal muscle in vivo, due to infiltration of inflammatory cells (Goetsch et al. 2003).

IL-1β has long been considered a putative mediator of inflammation-associated muscle atrophy (Cai et al. 2004, Cannon et al. 2008). Recent studies have shown that chronic exposure to IL-1β reduced myofibrillar proteins and myotube diameter in C2C12 culture (Li et al. 2009), which is in apparent contrast to our observation indicating the promotion of myoblast growth and differentiation under IL-1β treatment. In potential explanation of this discrepancy the differences in experimental protocols should be taken into consideration, as Li et al. (2009) used mature myotubes subjected to cytokine treatment, whereas in our study the effects of IL-1β on cell cycle regulatory mechanisms and the onset of myogenesis were investigated, and appeared to be limited in time to the period preceding myoblast fusion.

We also examined the expression of some extracellular matrix components, keeping in mind the role ECM assembly and remodeling in differentiation (Osses et al. 2009) and growth factor signaling response (Li et al. 2006). According to our results in C2C12 myogenic cells IL-1β elevated the level of fibronectin, integrin β1 and full length 100 kDa form of ADAM12, without the alteration of the integrin α5 subunit expression (Fig. 3). Such modifications could create an environment promoting myogenesis, where increased level of integrin β1 allow its redistribution in functional heterodimers with integrin subunits other than α5, that appear during progressing differentiation. In agreement with our study, a rise in cellular content of metalloprotease ADAM12 has already been reported in myoblasts undergoing fusion (Lafuste et al. 2005).

The promotion of myogenesis by IL-1β could be, at least, partly explain by the alterations in IGFBPs levels. IL-1β decreased the expression of IGFBPs, which attenuate IGF-I activity at the level of skeletal muscle tissue (i.e. IGFBP-4 and -6), and, simultaneously, it increased the level of IGFBP-5 (Fig. 4a-c), which positively regulate IGF-I action (Yin et al. 2004). Such modifications could affect the balance between several BPs by shifting the system toward increased IGF-I bioavailability and activity in muscle cells. In fact, the positive interaction of other cytokines (TNF-α combined with IL-6) with IGF system (i.e. IGFBP-5, IGF-I and IGF-II receptors) to achieve a benefitial effect on skeletal myoblast growth has already been described (Al-Shanti et al. 2008).

Treatment of myoblasts induced to differentiation with IL-1β resulted in increased phosphorylation and/or cellular content of protein kinases playing an essential role in growth factor-regulated myogenesis (De Alvaro et al. 2008, Jo et al. 2009), i.e. PKB, ERK $^{1}/_{2}$ and p38 (Fig. 4d-f). This observation is compatible with myogenic effect of IL-1β proved by morphological data (Fig. 2) and supports the hypothesis about the intracellular cross-talk among distinct ligand-activated system in order to integrate numerous signals for optimal tissue growth and development.

In summary, our data show that IL-1β does not decrease myoblast proliferation and exerts beneficial effects on early myogenesis, that was manifested by the increase in the fusion index, MHC and total protein synthesis in mouse C2C12 myogenic cells. In view of well-documented catabolic action of IL-1β in mature myotubes and skeletal muscle such observations indicate that the effects of cytokines are cell dependent and could be mediated by various mechanisms. The myogenic effect of IL-1β was limited to the onset of myoblast fusion and was associated with: i) increase in the level of myogenic transcription factors i.e. MyoD and myogenin expression, ii) modification of extracellular matrix assembly and signaling, manifested by an increase in fibronectin, integrin-β1 and ADAM12 content, iii) drop in IGFBP-4 and IGFBP-6, and an increase in IGFBP-5, that could alter the local IGF-1 bioavailability, and iv) increase in phosphorylation of PKB and ERK1/2, and the expression of p38 kinase, leading to activation of intracellular pathways essential for myogenic differentiation.

Acknowledgements

This study was supported by grant (No: N N308 006236) from the Polish Ministry of Science and Higher Education in 2009-2012.

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