Tumor necrosis factor-α alters integrins and metalloprotease ADAM12 levels and signaling in differentiating myoblasts

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Abstract

The extracellular matrix (ECM) is important in the regulation of myogenesis. We hypothesized that tumor necrosis factor-α (TNF-α) modifies ECM during differentiation of mouse C2C12 myoblasts. Exogenous TNF-α (1 ng/ml) stimulated myoblast fusion on the 3rd day (by 160% vs control) but not on the 5th day of myogenesis. The level of integrin α5 was significantly augmented by TNF-α during 5 day-differentiation; however, integrin β1 was higher than control only on the 3rd day of cytokine treatment. Both the abundance of integrin α5 bound to actin and the level of integrin β1 complexed with integrin α5 increased in the presence of TNF-α, especially on the 3rd day of differentiation. Similarly, the stimulatory effects of TNF-α on integrin α3, metalloprotease ADAM12 and kinases related to integrins, FAK and ILK, were limited to the 3rd day of differentiation. We concluded that TNF-α-induced changes in ECM components in differentiating myogenic cells, i.e. i) increased expression of integrin α5, β1, α3, and metalloprotease ADAM12, ii) enhanced formation of α5β1 integrin receptors and interaction of integrin α5-cytoskeleton, and iii) increased expression of kinases associated with integrin signaling, FAK and ILK, were temporarily associated with the onset of myocyte fusion.

Key words: ADAM12, FAK, ILK, integrins, myogenesis, TNF-α

Introduction

The proinflammatory cytokine, tumor necrosis factor-α (TNF-α) influences muscle development, growth and metabolism through numerous mechanisms. It has been shown to promote muscle wasting in several pathological conditions (Yeh et al. 2008) and to impair anabolic hormone/factor signaling in mature myotubes in vitro (Grzelkowska-Kowalczyk and Wieteska, 2006, O’Connor et al. 2008, Grzelkowska-Kowalczyk and Wieteska-Skrzeczyńska 2010). TNF-α is important in satellite cell activation (Chen et al. 2007) and, simultaneously, it inhibited myoblast differentiation by alteration of IGF-I bioavailability (Wieteska-Skrzeczyńska et al. 2011 b).

Extracellular matrix (ECM) is involved in the regulation of myoblast proliferation, migration, adhesion and differentiation (Krauss et al. 2005, Bellayr...
et al. 2013), and is required for skeletal muscle cells to respond to growth factors (Li and Velleman 2009, Miura et al. 2010, Yasaka et al. 2013). It has been shown that neither the expression of myogenin, one of muscle regulatory transcription factors (Zammit et al. 2006), nor its localization to myoblast nuclei were sufficient to drive skeletal muscle differentiation, if interactions cell-ECM were inhibited (Osses et al. 2009). Integrins, heterodimeric transmembrane receptors, transmit extracellular signals into the cell by mechanisms that involve cytoskeletal proteins and additional signal transduction molecules (Askari et al. 2009), such as integrin-linked kinase ILK (Honda et al. 2009) and focal adhesion kinase FAK (Michael et al. 2009). Matrix metalloproteinases play important roles during myogenesis by remodeling and degradation of ECM components (Nishimura et al. 2008). Among the matrix enzymes, a disintegrin and metalloprotease ADAM12 is required for fusion of myoblasts into multinucleated myotubes (Lafuste et al. 2005).

Whether and how TNF-α affects components of the extracellular matrix during myogenic differentiation is not known; however, several lines of evidence suggest a link between proinflammatory cytokines and ECM function during regenerative myogenesis. ECM proteins, together with the vasculature system, muscle-resident cells and muscle fibers, create a niche for muscle stem cells, important in controlling proliferation and differentiation to maintain muscle tissue (Wilschut et al. 2010). The levels of matrix proteinase inhibitors were high in quiescent satellite cells (Pallafacchina et al. 2010), and satellite cell activation was associated with matrix proteinase synthesis, which facilitate cell migration to the injured site (Nishimura et al. 2008).

In the present work the interest was focused on alterations of the expression of ECM-associated molecules in mouse C2C12 myogenic cells subjected to 5-day differentiation in the presence of TNF-α. The cellular contents of integrin α3, α5, β1 subunits, and disintegrin metalloprotease ADAM12 were assessed. The formation of integrin α5β1, integrin α5-actin, ADAM12-α-actin complexes, and the levels of integrin-dependent kinases FAK and ILK were also examined. We found that TNF-α affected the levels and interactions of cellular proteins related to ECM in differentiating C2C12 myogenic cells, and the majority of cytokine effects were temporarily associated with the onset of myocyte fusion.

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 with added antibiotic-antimycotic mixture, in controlled humidified air supplemented with 5% CO2, at 37°C. The cell cultures were subjected to 5-day differentiation (switch to differentiation medium – 2% (v/v) horse serum HS/DMEM) in the presence of TNF-α (1 ng/ml). According to our previous study this cytokine concentration affects myogenesis, IGF-I bioavailability and signaling in C2C12 myoblasts (Wieteska-Skrzeczyńska et al. 2011a,b).

Assessment of cell viability and DNA content

Crystal violet assay was performed to determine the total amount of nuclear DNA corresponding to the cell number. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Grabiec et al. 2013). 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.

Myoblast fusion

The morphological changes in C2C12 cultures during myogenesis were visualized using Giemsa staining. Nuclei were counted using a phase-contrast microscope (IX 70, Olympus Optical Co, Hamburg, Germany), 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 previously (Wieteska-Skrzeczyńska et al. 2011a).

Immunoblotting

Cell lysates comprising cytosolic and membrane-enriched fractions were obtained using RIPA buffer supplemented with protease and phosphatase inhibitor cocktail and octyl-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration in the lysates was determined using a BCA kit according to the manufacturer’s instructions and aliquots corresponding to 100 μg of total protein were subjected to SDS-PAGE. The membranes were probed with appropriate primary antibody (all purchased from Santa Cruz Biotechnology). The secondary antibody was conjugated with appropriate IR fluorophores: IRDye® 680 or IRDye® 800 CW
Table 1. Effect of TNF-α (1 ng/ml) on DNA content, cell viability and fusion of differentiating mouse C2C12 myogenic cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
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<tbody>
<tr>
<td>DNA content (absorbance)</td>
<td>Ctrl 0.92 ± 0.03</td>
<td>TNF-α 0.92 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ctrl 1.04 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α 1.13 ± 0.04</td>
</tr>
<tr>
<td>Cell viability (absorbance)</td>
<td>Ctrl 0.77 ± 0.02</td>
<td>TNF-α 0.89 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ctrl 0.94 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α 1.11 ± 0.03*</td>
</tr>
<tr>
<td>Fusion index (%)</td>
<td>Ctrl 5.0 ± 0.47</td>
<td>TNF-α 8.0 ± 0.47*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ctrl 36.2 ± 1.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α 37.2 ± 2.05</td>
</tr>
</tbody>
</table>

Data represent means ± SE, n=12
* – significantly different vs control value (Ctrl) for the same day.

Fig. 1. Effect of TNF-α (1 ng/ml) on integrin-α5 (a) and integrin-β1 (b) in differentiating C2C12 myogenic cells. Integrated optical density (IOD) of the bands is presented in arbitrary units (a.u.), with the value obtained in control on the 3rd day set as 100%. Representative blots and means ± SE (n = 9), are shown. The abundance of integrin-α5 and integrin-β1 in complexes was assessed by immunoprecipitation (IP). Control probing with the antibody used for immunoprecipitation was performed (IB). * – significantly different vs control (Ctrl) for the same day.

Immunoprecipitation

To assess potential protein interactions and protein distribution in complexes, a stage of immunoprecipitation prior to immunoblotting was used. Cell lysates containing approximately 300 μg of total protein were subjected to 12-hour incubation with primary antibody previously adsorbed on Protein A/G Plus agarose (Santa Cruz Biotechnology). Antigen-antibody complexes adsorbed on agarose
beds were recovered by centrifugation, and subjected to SDS-PAGE, electrotransfer and blotting with appropriate antibody, as described above. Interactions of examined proteins were assessed on the basis of: i) the abundance of integrin β1 co-precipitated with integrin α5, ii) the abundance of integrin α5 co-precipitated with actin, and iii) the abundance of α-actinin co-precipitated with ADAM12. Control probing with the antibody used for immunoprecipitation was also performed, to ensure that equal amounts of protein were precipitated and recovered from cell lysates.

Statistics

The results of CV, MTT tests and fusion index are representative of four separate experiments, whereas the data obtained by immunoblotting and immunoprecipitation represent three separate experiments, performed in triplicate. The data were expressed as means ± S.E. Student t-test was used for the comparison of two means. The statistical differences were marked when p<0.05.

Results

Exogenous TNF-α (1 ng/ml) did not significantly altered the cell number; however, it slightly augmented cell respiration assessed on the 3rd day and on the 5th day of myogenesis (by 16%, p=0.0005 and by 17%, P<0.0001 vs appropriate control, respectively). The fusion index was significantly elevated by TNF-α on the 3rd day of differentiation (by 160%, p=0.0003), but was similar to control value on the 5th day (Table 1).

The level of integrin α5, a subunit of fibronectin surface receptor, was significantly augmented by TNF-α and this effect was visible during 5 days of observation (Fig. 1a). Moreover, the abundance of integrin α5 bound to actin was markedly higher in cells treated with TNF-α, especially on the 3rd day of differentiation. The cellular content of integrin β1 was markedly higher under 3-day cytokine treatment in comparison to the control group (Fig. 1b). In cell cul-
Fig. 4. Effect of TNF-α (1 ng/ml) on the expression of the focal adhesion kinase, FAK (a) and the kinase dependent on integrin signaling, ILK (b) in differentiating C2C12 myogenic cells. Integrated optical density (IOD) of the bands is presented in arbitrary units (a.u.), with the value obtained in control on the 3rd day set as 100%. Representative blots and means ± SE, with n = 9 are shown. * – significantly different vs control (Ctrl) for the same day.

Apart from well-documented catabolic effects of TNF-α on mature myofibers (O'Connor et al. 2008), this cytokine appears to be secreted by myoblasts (Borge et al. 2009), plays a physiological role in myogenesis (Chen et al. 2007), stimulates protein synthesis (Plaisance et al. 2008) and promotes myoblast fusion (Wieteska-Skrzeczyńska et al. 2011a). In the present study we report that TNF-α also affects proteins related to extracellular matrix, which provide an appropriate and permissive environment for myogenic differentiation (Osses et al. 2009).

Myogenic cells change their integrin expression pattern in the course of differentiation (Cachaco et al. 2005). Proliferating and migrating myoblasts express high amounts of the fibronectin-binding α5β1 integrin, and during myotube formation they switch to the laminin-binding integrin subunits (Brakebusch & Farsler 2005). Our present results indicate that α5β1 integrin complexes are essential in myoblast fusion. The integrin α5 and integrin β1 subunits, as well as the levels of integrin β1 complexed with integrin α5 and integrin α5 bound to actin were markedly higher in cells treated with TNF-α for 3 days (Fig. 1), suggesting the stimulation of functional adhesive receptor formation and the activation of an integrin-cytoskeleton connection. We have also shown that integrin α3 expression increased markedly in myogenic cells treated with TNF-α. During the 5-day myogenic differentiation the highest level of integrin α3 was noticed on the 3rd day, corresponding with the onset of fusion and indicating that integrin α3 is important in this stage of myogenesis. Our results are in agreement with an earlier study performed on rat primary myoblasts, showing that overexpression of the full-length integrin α3 subunit induced myoblast fusion, whereas the inhibition of integrin α3 extracellular domain impaired this process (Brzóska et al. 2006).

Skeletal myoblasts induced in vitro to differentiate either form multinucleated myotubes or give rise to quiescent undifferentiated „reserve cells” which share several characteristics with muscle satellite cells. Cao et al. (2003) reported very high express-
ion of metalloprotease ADAM12 in proliferating C2C12 myoblasts and in reserve cells in comparison to mature myotubes. Remarkably, overexpression of ADAM12 induced a quiescent-like phenotype and did not stimulate differentiation. In the present study, treatment with TNF-α increased the expression of ADAM12 in myocytes (Fig. 3). Such an observation suggests that TNF-α could play a role in determination of the reserve cell pool during myogenic differentiation acting via stimulation of ADAM12 expression. TNF-α decreased the binding of ADAM12 to α-actinin (Fig. 3), suggesting impairment of the mechanism controlling distribution of metalloprotease to the cell membrane required for its cleavage (Wewer et al. 2005). This indicates that ADAM12 is linked to the cytoskeleton and that the cytoskeleton may regulate distribution of ADAM12 on the cell surface, where localized proteolysis and/or cell-cell contacts occur (Wewer et al. 2005). Apparently, ADAM12-cytoskeleton interactions, and, in turn, ADAM12 breakdown are diminished in the presence of TNF-α, that is in line with increased cellular level of the metalloprotease under cytokine treatment. In our previous study a long 100 kDa isoform of ADAM12 was increased in myoblasts differentiating for 3 days in the presence of IL-1β (Grabiec et al. 2013) and IGF-I (Grzelkowska-Kowalczyk et al. 2015), suggesting similar effects of proinflammatory cytokines and anabolic growth factors on early stages of myogenesis.

Apart from their role in the adhesion of the muscle fiber with ECM (Postel et al. 2008) and the regulation of muscle cell spreading (Goel and Dey, 2002), integrin related kinases, FAK and ILK, have been established as positive regulators of the insulin signaling pathway (Bisht et al. 2007, Gupta et al. 2009, Zong et al. 2009). In this regard, our present data demonstrating an increase in FAK and ILK expression under TNF-α treatment have some discrepancy with cytokine-induced insulin resistance described in fully differentiated myotubes and mature muscle fibers (summarized in O’Connor et al. 2008). Our results are, however, compatible with previous studies describing the positive effects of factors released after muscle injury on satellite cells (Goetsch et al. 2003) and the transient stimulatory effects of proinflammatory cytokines on myoblasts in culture (Grabiec et al. 2013), and support the diversity of proinflammatory cytokine effects in proliferating and fully differentiated cells.

In conclusion, TNF-α affects the level and, probably, the function of proteins related to extracellular matrix in differentiating myogenic cells. The majority of cytokine effects, i.e. i) increased expression of integrin α5, β1, α3, and metalloprotease ADAM12, ii) enhanced formation of α5β1 integrin receptors and interaction of integrin α5-cytoskeleton, and iii) increased expression of kinases associated with integrin signaling, FAK and ILK were temporarily associated with the onset of myocyte fusion.

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References


