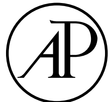
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Differentiation-dependent regulation of skeletal myogenesis by neuregulin-1

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Abstract

Neuregulins comprise a group of growth factor proteins that regulate the differentiation of skeletal muscle. Here, we report that neuregulins are regulators of myogenic differentiation and stimulate mitogenesis in L6 skeletal myoblasts. The mitogenic response to neuregulin-1 was differentiation-dependent and observed only in aligned, differentiating cells. Treatment of these cells with neuregulin-1 increased [^{3}H]thymidine incorporation and cell proliferation by 2- to 5-fold, while a minimal increase was seen in proliferating myoblasts. Neuregulin-1 did not induce DNA synthesis in fused, multinucleated myotubes. The increased DNA synthesis correlated with downregulation of myogenin and inhibition of myoblast fusion and myotube formation. These data suggest that neuregulins may regulate skeletal myogenesis in vivo and that this regulation is dependent on the state of differentiation of the myocytes.
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Skeletal myogenesis involves the proliferation and differentiation of somatic mesodermal precursors to form multinucleated myotubes [1,3,26]. The morphological events in muscle differentiation are associated with the expression of muscle specific transcription factors and structural genes. In early skeletal myogenesis, peptide growth factors, through interaction with cell surface receptors on myoblasts, inhibit myogenic differentiation and promote continued growth and proliferation [2,7,12]. This results from repression of expression of the myogenic basic helix loop helix (bHLH) genes or reduced activity of the bHLH proteins. bHLH members of the myoD family of transcription factors include MyoD, myogenin, myf5, and MRF4/myf6/herculin, along with a related dominant negative HLH protein, known as Id [1,3,26,33]. However, in late myogenesis, growth factors stimulate muscle differentiation by inducing the expression of muscle specific genes such as nicotinic acetylcholine receptors and muscle specific isoforms of actin, myosin, sodium channels, and creatine kinase [6,9,17,30]. The local expression of growth factors is critical in determining whether cells are maintained as proliferating myoblasts or acquire the differentiated phenotype. Various peptide growth factors, including fibroblast growth factor (FGF), transforming growth factor β (TGFβ), insulin-like growth factors, and hepatocyte growth factor (HGF), have been shown to regulate muscle differentiation using in vitro cultured muscle cells [2,7,12].

Neuregulins are a family of growth and differentiation factors known to be involved in the functional maturation of the neuromuscular junction [10]. Neuregulin-1 (NRG-1) family members include acetylcholine receptor inducing activity (ARIA), glial growth factor (GGF), heregulin, and neu differentiation factor (NDF) [9,21,27,34]. Neuregulins have been shown to stimulate the synthesis of acetylcholine receptors and sodium channels at the neuromuscular junction [6,9]. Neuregulins have also been shown to stimulate differentiation of L6 rat myotubes in culture. In these studies,

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neuregulins caused an increase in myotube formation and elevated creatine kinase and myosin heavy chain levels which are generally associated with terminal myogenic differentiation [13,18].

Neuregulins are expressed in motor neurons and are concentrated at neuromuscular junctions in both rat and chick [15,29]. Recently, NRG-1 expression has been demonstrated in dermamyotome of chick and *Xenopus*, suggesting that neuregulins play a role in early myogenesis [20,35]. Here, we report that neuregulins are potent inhibitors of differentiation and inducers of mitogenesis in skeletal myoblasts. Inhibition of differentiation by NRG-1 and the induction of mitogenesis were dependent on the differentiation state of the myoblast and correlated with an inhibition of myogenin expression. These results suggest a novel role for neuregulins in myogenesis.

Experimental procedures

Cell culture, proliferation, and differentiation. L6 myoblasts were plated in DMEM containing 10% fetal calf serum (FCS) for one or more days. The cultures were treated with the EGF-like domain of neuregulin β1 (residues 177–244; EGF β1; gift from Amgen, Thousand Oaks, CA) in DMEM containing either 10% FCS, 0.1% FCS or 0.1% bovine serum albumin. To measure DNA synthesis, L6 cells were treated with the indicated concentrations of EGF β1 for 18–24 h at 37 °C. The solutions were removed and [^{3}H]thymidine (1 μCi/ml) in DMEM was added for 4 h at 37 °C. At the end of the incubation, cells were fixed with methanol at −20 °C for 10 min. Cells were then washed three times with 10% trichloroacetic acid (TCA) and lysed with 0.1 M NaOH/0.1% sodium deoxycholate. Lysates were transferred to vials containing scintillant and vigorously vortexed. Radioactivity was measured by liquid scintillation counting. To quantitate cell proliferation, cells were trypsinized and counted using a hemacytometer 24–48 h after the addition of growth factors. To determine the fusion index, cells were fixed with 4% paraformaldehyde following treatment with EGF β1. The extent of myoblast fusion was determined as the number of multinucleated cells as a percentage of the total number of cells in randomly chosen fields under a microscope. Cells containing three or more nuclei were regarded as fused cells. Percent change from control reflects values obtained after NRG-1 treatment compared to cells from the same plate treated with vehicle alone under similar conditions.

Immunoblotting. L6 cells were harvested by adding hot SDS loading buffer directly into the 48-well culture plate. Samples were resolved in SDS/5% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Myogenin was detected using conventional chemiluminescence methods. Primary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and the peroxidase conjugated secondary antibody was from DAKO (Carpenteria, CA).

Results

Neuregulin-1 stimulates proliferation of skeletal myoblasts

To assess the mitogenic potential of neuregulins in skeletal myoblasts, we examined [^{3}H]thymidine incorporation in early passage L6 myoblast cultures following treatment with NRG-1. EGF β1 (1 nM) had little or no effect on [^{3}H]thymidine incorporation in myoblasts during the first 1–2 days of culture compared to control cultures (Fig. 1A). These cells were primarily undifferentiated, proliferating myoblasts (Fig. 1B). However, treatment of myoblasts with EGF β1 resulted in a 2- to 5-fold increase in [^{3}H]thymidine incorporation between days 3 and 7 in culture compared to untreated cultures (Fig. 1A). These cultures were characterized by elongated cells which were aligned and preparing to fuse (Fig. 1C). Experiments performed with the full length extracellular domain of neuregulin β1 (residues 1–244) similarly increased [^{3}H]thymidine incorporation in the cultures (data not shown). In contrast, [^{3}H]thymidine incorporation values after EGF β1 treatment of myoblast cultures >7 days old (myotubes, Fig. 1D) were not significantly different from untreated cultures (Fig. 1A). Thus, EGF β1 had no effect on [^{3}H]thymidine incorporation when cells had fused to form myotubes. Interestingly, EGF β1 did not induce DNA synthesis in late passage cells that were incapable of fusion, suggesting that the mitogenic response of myoblasts to neuregulins is dependent on the ability of the cells to differentiate (Fig. 1A). EGF β1 stimulated a concentration-dependent increase in [^{3}H]thymidine incorporation in differentiating myoblast cultures with a maximal response at ~1 nM (Fig. 2A). The EGF-like domain of neuregulin α (EGF α) was ineffective in stimulating [^{3}H]thymidine incorporation (Fig 2B). Glial growth factor (GGF) was previously reported to induce minimal mitogenic activity in myoblasts but only in the presence of a non-mitogenic concentration of serum [13]. However, the increased [^{3}H]thymidine incorporation in this study was seen both in the absence and presence of serum (data not shown).

The EGF β1 stimulated increase in [^{3}H]thymidine incorporation was paralleled by an increase in myoblast proliferation (Fig. 3A). After treatment of myoblast cultures with EGF β1, a 2–3-fold increase in cell numbers was observed which closely paralleled the increased [^{3}H]thymidine incorporation. The effect of EGF β1 was also associated with a decrease in the rate of cellular fusion in the myoblast cultures. Approximately 35% of cells in control cultures were fused cells (cells containing three or more nuclei), whereas only ~15% of cell were fused following treatment with EGF β1 (Fig. 3B). These results indicate that: (1) there is an increase in the number of cells in the cultures and this increase is accounted for by an increase in the number of unfused myoblasts in the cultures and (2) there is a baseline level of fusion at this time point (day 3), however, the remaining cells are either induced to proliferate by NRG-1 or they are held in an undifferentiated state such that they retain proliferative capacity.

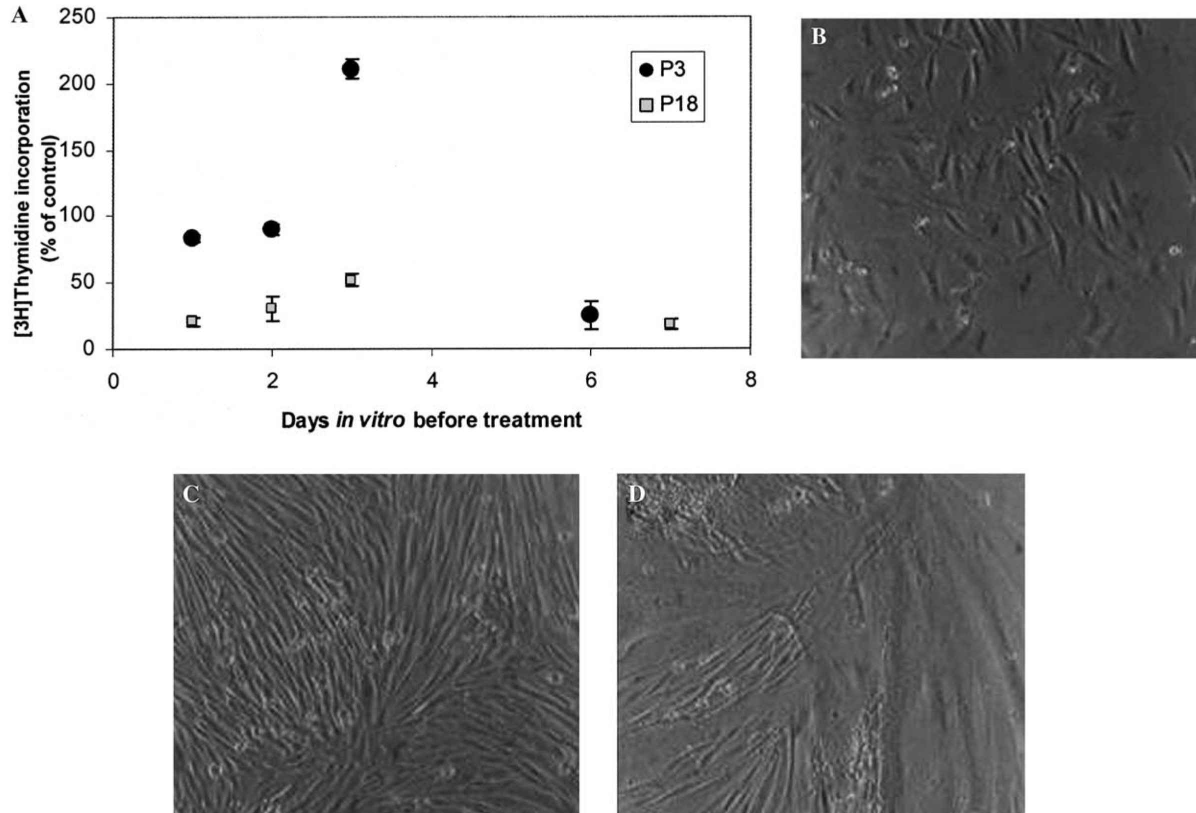

Fig. 1. Neuregulin stimulates DNA synthesis in differentiating L6 myoblasts. Early (P3) and late (P18) passage L6 cells were plated and treated with DMEM containing 0.1% bovine serum albumin ± 1 nM EGF β1 (NRG) after 1–7 days in culture (A). All results are presented as means ± SEM for triplicate experiments. (A) A representative experiment of three similar ones, each performed in triplicate. Mononucleated, proliferating myoblasts (B) were predominant after the initial plating of L6 cells (1–3 days in vitro). After 3–6 days in culture, cells were aligned and elongated (C) but later (<6 days in vitro) formed multinucleated myotubes (D).

Myogenesis in vivo and in vitro is characterized by sequential expression of myogenic transcription factors of the myoD family [1,3,26,33]. A negative regulation of both gene expression and functional activity of MyoD has been reported for a number of growth factors, including FGF and TGFβ [32], which is associated with the inhibition of myoblast differentiation. L6 cells express myogenin during the process of myogenic differentiation but do not express myoD [11]. Myogenin protein is not detectable in undifferentiated myoblasts (Fig. 4). During differentiation, myogenin was observed in differentiating myoblasts and differentiated myotubes. EGF β1 completely blocked myogenin expression in differentiating myoblast cultures. These results, taken together, demonstrate that neuregulins stimulate myoblast proliferation either as a result of or in addition to the inhibition of myogenic differentiation in the culture.

Discussion

Neuregulins have been shown to play a prominent role in skeletal myogenesis. Neuregulins stimulate differentiation of L6 rat myotubes in culture as demonstrated by increased myotube formation and elevated creatine kinase and myosin heavy chain levels which is generally associated with terminal myogenic differentiation [13,18]. NRG-1 expression has been demonstrated in dermamyotome of chick and *Xenopus*, suggesting that neuregulins play a role in early myogenesis [20,35]. Here, we reveal a novel role for NRG-1 in inhibiting early myoblast differentiation and promoting the proliferation of myoblasts.

GGF, an isoform of NRG-1, was previously reported to induce minimal mitogenic activity in L6 myoblasts but only in the presence of sub-mitogenic concentrations of serum [13]. In our studies, the increased [^{3}H]thymidine incorporation was seen in the absence or presence of serum. However, careful interpretation of our finding suggests that our results are consistent with those of Florini et al. [13] and support the idea that the effects of neuregulins depend on the differentiation state of the cells. Specifically, we are in total agreement with previous reports that neuregulins induce differentiation in myotube cultures and mature muscle cells. Previous reports from our laboratory clearly support these findings [6,9,29]. However, in this manuscript, we show that neuregulins block differentiation and induce (or allow)

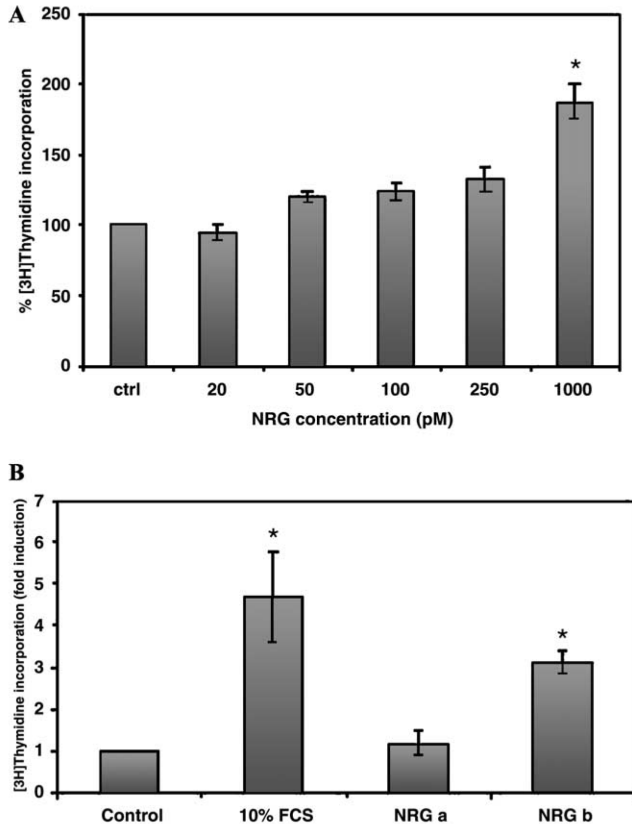

Fig. 2. Concentration dependence of stimulation of mitogenesis in L6 cells by neuregulin. L6 cells were plated as above and then treated with the indicated concentrations of EGF β1 (A). L6 cells from 3 day cultures were incubated for 24 h with medium containing 10% FCS, 1 nM EGF β1 (NRG β) or 100 nM EGF α (NRG α) (B). All results are presented as means ± SEM for triplicate experiments; * denotes $p < 0.05$.

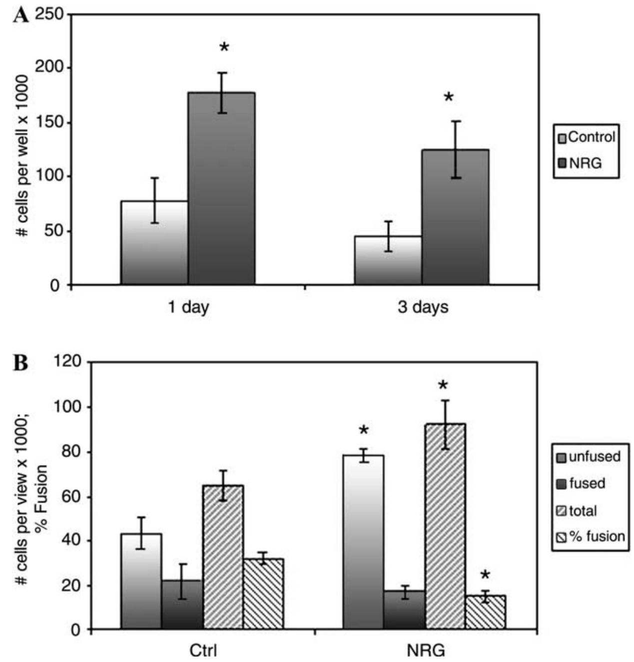

Fig. 3. Neuregulin blocks fusion and stimulates proliferation in differentiating L6 myoblast cultures. (A) L6 cells were plated as described and treated with EGF β1 (NRG) for 1 day or 3 days. Cells were trypsinized and counted with a hemacytometer as described in Experimental procedures. (B) To determine the fusion index, cells were treated with NRG and the extent of myoblast fusion was determined as described above. * denote significantly different compared to same parameter in untreated cultures, $p < 0.05$.

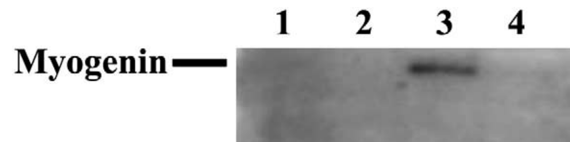

Fig. 4. Neuregulin inhibits the expression of myogenin in L6 myoblasts. Cell lysates were prepared and subjected to immunoblot analysis using an anti-myogenin antibody. Lanes are: (1) control proliferation myoblasts from 1 day culture; (2) neuregulin-treated proliferating myoblasts from 1 day culture; (3) control aligned myoblasts from 3 day culture; and (4) neuregulin-treated aligned myoblasts from 3 day culture.

proliferation in "differentiating" cells but not in myotube cultures. The experiments by Florini et al. [13] were performed on cells cultured for ~6 days in 10% horse serum, which accelerates myoblast differentiation in culture [16,19,28]. These cultures were likely composed of differentiated myotubes which explains the lack of a mitogenic response to neuregulin. The observation that neuregulins induce modest myogenic activity in the presence of "serum components" suggests to us that these "serum components" may delay differentiation in some myoblasts (more "differentiating" cells in the culture) thus allowing these cells to exhibit proliferative properties. In addition, the concentration of neuregulin used in that study was ~20 pM. The mitogenic effect of EGF β1 seen here occurred at concentrations greater than 250 pM. The concentrations of EGF β1 effective in stimulating myoblast mitogenesis are similar to those shown to induce proliferation of glia, keratinocytes, mammary epithelial cells, and embryonic chick heart cells [4,8,14,22–25]. In contrast, the effects of neuregulins in myotube differentiation occur at substantially lower concentrations with an optimal concentration of approximately 25 pM [13,18], similar to the effective concentrations of neuregulins used to induce the expression of AChRs, sodium channels, and creatine kinase activity [6,9,17]. Interestingly, EGF β1 did not induce DNA synthesis in late passage cells that were incapable of fusion. This suggests that the ability of neuregulins to stimulate [^{3}H]thymidine incorporation in skeletal myoblast cultures is dependent upon the competence of cells to fuse but is not possible following cell fusion.

Myogenesis is characterized by sequential expression of myogenic transcription factors and muscle specific genes [1,3,26,33]. NRG-1 blocked myogenin expression in differentiating myoblasts cultures, suggesting that neuregulins inhibit myogenesis. Myogenin is an early inducer of myogenic differentiation; therefore cells preparing to

fuse will express it. We propose that neuregulin inhibits myogenin expression which keeps myoblasts from moving to the next stage prior to fusion and retains the cells in a proliferative stage. However in myotube cultures, neuregulin is associated with an increase in skeletal myosin heavy chain and in other molecules related to terminal differentiation in muscle such as acetylcholine receptors and creatine kinase [9,13,17,18].

A number of studies suggest that differential expression of erbB receptors in skeletal muscle cells may explain the differentiation-dependent effects of neuroregulins on skeletal muscle cells. Alterations in erbB receptor expression patterns appear to correspond with the level of differentiation of skeletal myoblast cultures. Jo et al. [17] found that there is detectable erbB-2 and erbB-3 mRNA in skeletal muscle and in the C2 myoblast cell line, with substantially increased erbB-3 mRNA upon myotube formation. Kim et al. [18] demonstrated that there was detectable erbB-3 in L6 cells, with substantially increased erbB-3 mRNA in myotubes compared to myoblasts. A recent report similarly demonstrated an increase in erbB-3 expression in differentiated myotubes that was associated with the ability of neuroregulins to regulate glucose transport [31]. The endogenous expression of growth factors, as well as that of their receptors, is developmentally regulated during myogenesis. The induction or repression of growth factor receptors with the onset of differentiation is vital in ensuring an irreversible withdrawal from cell cycle and, consequently, a stable expression of muscle specific phenotype. Alternatively, other factors, such as developmentally regulated transcription factors [1,3,26,33] or adhesion molecules [5], may determine the outcome of neuregulin signaling during myogenesis.

Taken together, the results of our studies and others suggest that neuregulins play multiple and distinct roles during different stages of muscle development. Prior to myogenic differentiation, neuregulins likely serve to inhibit myogenic differentiation and promote continued growth and proliferation. Following differentiation in the embryo and in adults, neuregulins are associated with the induction and maintenance of muscle specific genes. In view of these findings, it is possible that during muscle regeneration in vivo, neuregulins, which are concentrated at neuromuscular junctions, could also control muscle growth and regeneration following damage. Studies are currently underway to determine the roles for neuregulins in muscle development and pathophysiology.

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