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Neuregulin Stimulates Myogenic Differentiation in an Autocrine Manner*

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During myogenesis, mononucleated myoblasts form multinucleated myotubes by membrane fusion. Efficiency of this intercellular process can be maximized by a simultaneous progress, with a time window, of other neighboring myoblasts in the differentiation program. This phenomenon has been described as the community effect. It proposes the existence of a molecule that acts as a differentiation-inducing signal to a group of identical cells. Here ,we show that neuregulin is a strong candidate for this molecule in myoblast differentiation. The expression of neuregulin increased rapidly but transiently at early stage of differentiation of rat L6 cells. Neuregulin showed a potent differentiation-promoting activity in membrane fusion and expression of myosin heavy chain. The antibodies raised against neuregulin and its cognate receptor ErbB3, which were capable of neutralizing the signal pathway, inhibited myotube formation and expression of myosin heavy chain in both L6 cells and primary rat myoblasts. The progress of differentiation was mostly halted after the expression of myogenin and cell cycle arrest. These results suggest that the activation of an autocrine signaling of neuregulin may provide a basic mechanism for the community effect observed in the differentiation of the embryonic muscle cells.

Embryonic induction, a process in which a signal from one group of cells regulates the development of an adjacent group of cells, provides a major paradigm for understanding of cellular basis of development. For certain differentiation processes, however, interactions within a group of cells in an autocrine manner become a great consideration (1–3). One of these phenomena, the community effect, has been described to explain the requirement of identical neighbors for their completion of differentiation program as observed in muscle development of amphibian embryos (Refs. 4–9; for review, see Ref. 7). The formation of skeletal muscle involves an intercellular process, in which mononucleated myoblasts form multinucleated myotubes by fusion of cellular membrane. The fusion involves specific cell-cell adhesion molecules that mediate recognition between myoblasts (10, 11). This process inevitably proposes the existence of a time window for fusion to occur, and spatiotemporal coordination becomes an important factor to accomplish the homogeneity and uniformity of differentiation. The community effect, presumably mediated by a local signal molecule made from the differentiating myoblasts, may establish this spatiotemporal coordination. This model assumes a checkpoint that can be passed by only when the concentration of this molecule exceeds a certain threshold level (12, 13).

The NRGs[1] (also known as acetylcholine receptor inducing activity, GGF, heregulin, or neu differentiation factor) are a group of polypeptide growth factors that play roles in the developing heart and nervous system. ErbB2, ErbB3, and ErbB4 are members of a subfamily of receptor tyrosine kinases that are activated by NRG through direct or indirect interaction (14, 15). NRG is essential in the developing heart; $NRG^{-/-}$ embryos fail to form ventricular trabeculae and die in mid-gestation (16). In the developing nerves, NRG, ErbB2, and ErbB4 are required for the formation of the sympathetic nervous system, such as neural crest precursor cells (17). NRGs, secreted from motor neuron terminals, also regulate the process of neuromuscular synapse formation (18, 19).

Glial growth factor 2 (GGF2), one member of NRG family secreted from motor neuron terminals, has recently been shown to stimulate myoblast differentiation in immortalized cell lines (20). The exogenous addition of GGF2 to the cultured myoblasts stimulates cell fusion and accumulation of creatine kinase. Based on these observations, Florini *et al.* (20) suggested GGF2 as a candidate for myotrophic agents secreted by nerves. Here, we provide evidence for the existence of an NRG-ErbB3 autocrine signaling pathway in an early stage of myoblast differentiation. Disruption of the NRG-ErbB3 signaling by the antibodies raised against NRG and ErbB3 receptor markedly suppressed both myotube formation and muscle-specific gene expression in L6 cell line and rat primary myoblasts. The neutralizing antibodies appeared to influence mostly at the differentiation stage(s) after the expression of myogenin and cell cycle arrest. These results are consistent with a model that NRG plays a role as a local signal molecule that mediates the community effect observed in the differentiation of the embryonic muscle cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibody Treatment, and Immunocytochemistry —The L6 rat myoblast cell line was obtained from the American Type Culture Collection. Cultures were plated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (proliferation medium

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[1] The abbreviations used are: NRG, neuregulin; GGF, glial growth factor; PM, proliferation medium; DM, differentiation medium; MHC, myosin heavy chain; EGF, epidermal growth factor; RT, reverse transcription; PCR, polymerase chain reaction; Rb, retinoblastoma protein; CK, creatine kinase; DMEM, Dulbecco's modified Eagle's medium; Ab, antibody.

(PM)) at 1.5×10^5 cells per 100-mm dish. The cell differentiation was induced by switching the medium to DMEM containing 5% horse serum (differentiation medium (DM)) at 3 days after plating. In the antibody inhibition experiments, the anti-ErbB3 antibody (Ab5) or the anti-NRG antibody (Ab2) was included in DM. To investigate the effects of NRGα1 or NRGβ1 on myogenesis, cells were cultured for 3 days in PM, washed with DMEM, and further cultured in DMEM containing 0.05% bovine serum albumin in the presence or absence of NRG. Ab5, Ab2, NRGα1, and NRGβ1 were purchased from NeoMarkers.

Primary rat muscle cell cultures were prepared essentially as described (21). Because the differentiation of primary rat muscle cells does not require the switching the culture medium to DM, Ab5 or Ab2 was directly added to the cultures at 4 h after plating for the antibody inhibition experiments. Fusion indices were measured as described previously (22). For immunocytochemistry, cells were fixed with 3.7% paraformaldehyde and incubated for 1 h with MF20, a monoclonal antibody specific to the skeletal myosin heavy chain (MHC). MHC was visualized using a horseradish peroxidase-linked system, which employs diaminebenzoate as a substrate (Vectastain ABC kit, Vector Laboratories Inc.).

Preparation of Antibodies—RNA from C2C12 myogenic cell line was subjected to RT-PCR using oligonucleotide primers corresponding to the epidermal growth factor (EGF)-like domain of rat NRG (5′-TCTGGAGAGTATATGTGCAAAGTGATCAGC-3′, 3′-GCAGTAGGCCACCACACACATGATGCC-5′). A 480-base pair amplified fragment was subcloned into pGEM-T (Promega) and sequenced. Its sequence showed 90% identity with that of the rat neu differentiation factor cDNA. The 480-base pair fragment of NRG cDNA was subcloned into pGEX-4T (Amersham Pharmacia Biotech). The resulting glutathione *S*-transferase fusion protein containing the NRG sequence (amino acids 107–265) was produced in *Escherichia coli* and purified using glutathione-Sepharose (Amersham Pharmacia Biotech). After separation in SDS-polyacrylamide gel electrophoresis, the band corresponding to a molecular mass of 42 kDa was cut out, minced, and injected three times into albino rabbit at 4-week intervals. Upon immunoblot analysis, the resulting antiserum, but not the preimmune serum, was found to specifically interact with the 42-kDa glutathione *S*-transferase-NRG fusion protein in the *E. coli* lysates. The antiserum also recognized the extracellular domain of NRG α1 and β1 expressed in *E. coli* as a 30-kDa protein.

Mouse monoclonal antibodies were used to detect ErbB2 (Ab-3; Oncogene), β-tubulin (T4026; Sigma), and myogenin (F5D; Pharmingen). p21 was detected with a mixed monoclonal antibody (05-345; Upstate Biotechnology). The antibodies against Rb (C-15) and p27 (C-19) were purchased from Santa Cruz Biotechnology. Creatine kinase (CK) was detected using a rabbit polyclonal antibody, prepared as described previously (23).

RNA Analysis by RT-PCR—RNA was isolated from L6 cells using the TRIzol (Life Technologies, Inc.) method at the proper time intervals. First-strand synthesis was done on 1 μg of RNA in a final volume of 20 μl using random primers and Superscript reverse transcriptase (Life Technologies, Inc.). One microliter of the sample was subjected to PCR using the primers corresponding to the EGF-like domain of rat NRG as above. The level of NRG RNA was measured by hybridizing with ^{32}P-labeled internal oligonucleotide probes (5′-ACATCAACATCCACGACTGGGACCAGCCATCT-3′). To ensure that the assay was in the linear range, the cycle number and amounts of RNA were varied.

Immunoprecipitation Analysis—Whole cell extracts were prepared with radioimmune precipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 1 μg/ml leupeptin) and precleared by centrifugation. The precleared lysates were incubated with 2 μg of an anti-ErbB3 antibody (C-17; Santa Cruz Biotechnology) for 2 h at 4 °C, followed by addition of 50 μl of protein-A Sepharose beads (10% (v/v) suspension). After incubation for 1 h at 4 °C, the beads were washed three times with radioimmune precipitation buffer. Bound ErbB3 proteins were subjected to immunoblot analysis using an anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). The ErbB3 proteins were then detected by an enhanced chemiluminescence method (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Expression of ErbB3 Receptor and Neuregulin in L6 Cells—Recently, GGF2 has been suggested to act as a myotrophic agent secreted from nerve cells (20). If the differentiation of L6 myogenic cell is under the influence of NRG, L6 myoblasts should have receptors for NRG. To determine whether the functional ErbB3, the cognate receptor of NRG, is indeed expressed during the myogenesis of L6 cells, immunoprecipitation experiments were performed using an antibody raised against ErbB3 (Fig. 1*a*). Consistent with the previous report (18), the terminally differentiated myotubes expressed ErbB3. ErbB3 was expressed also in the undifferentiated myoblasts, although to a lower level than in myotubes. Moreover, the ErbB3 receptor in the myoblasts was tyrosine-phosphorylated upon treatment with NRGα1 or NRGβ1, indicating its functional responsiveness.

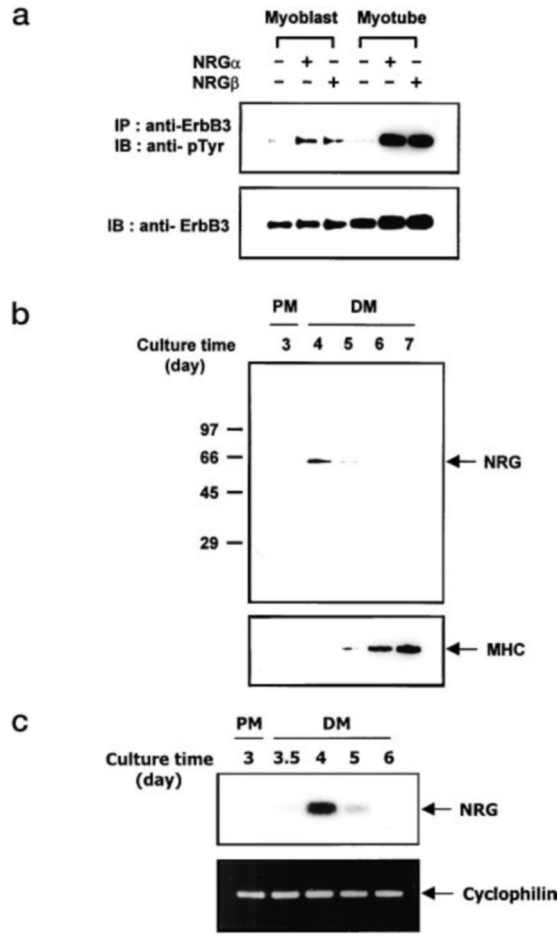

FIG. 1. **Expression of ErbB3 receptor and neuregulin in L6 cells.** *a,* activation of ErbB3 receptors expressed in myoblast and myotube. ErbB3 in the extracts of L6 cells was immunoprecipitated with an anti-ErbB3 antibody. Phosphotyrosine levels of ErbB3 were then determined by immunoblot with an anti-phosphotyrosine antibody 4G10. *b,* a transient expression of neuregulin during myogenesis. The cell extracts were analyzed by immunoblot with an anti-NRG antibody. Expression of MHC is shown as a differentiation marker. *c,* a transient increase of neuregulin mRNA during myogenesis. The total RNAs of L6 cells were subjected to RT-PCR as described under "Experimental Procedures." The RT-PCR products were hybridized with a ^{32}P-labeled internal oligonucleotide probe. Cyclophilin served as an internal control.

Interestingly, we observed a low level of tyrosine phosphorylation of ErbB3 in the control L6 cells that were not treated with NRGs (Fig. 1*a*). This observation led us to speculate that ErbB3 might be activated by endogenously expressed NRG. In fact, previous reports have shown the expression of NRGs in muscle cells. However, most attention has been focused on the action of these ligands in the neuromuscular junction, and their role at the early stage of myogenic differentiation has not been well documented (24, 25). We therefore examined in detail the expression pattern of NRGs in the course of L6 myogenesis. When the differentiation was induced by replacing the culture medium with a low mitogen-containing medium, the expres-

sion of NRG with a size of about 65 kDa was dramatically induced 1 day after the medium change (Fig. 1*b*). It is striking that the expression of NRG maintained only for 1 day and rapidly declined thereafter. As shown in Fig. 1*c,* the changes in the level of NRG mRNA closely correlated with the alterations in the NRG protein level. Thus, the expression of NRG is most likely to be under transcriptional control, although an increase in NRG mRNA stability may give rise to a similar result. These results demonstrate that NRG is transiently induced at the early stage of myogenesis, whereas ErbB3 receptor is constitutively expressed throughout the differentiation process. Because the anti-NRG antibody used in this immunoblotting analysis was raised against the EGF-like domain, a region common to most isoforms of NRG, it remains uncertain which isotype of NRG is expressed in the L6 myoblasts.

NRGα1 Stimulates the Differentiation of L6 Myoblasts—In order to determine whether NRG may play a role as a local signaling molecule for myogenesis, L6 myoblasts were treated with the recombinant α1-NRG and β1-NRG isoforms, which contain EGF-like domain. Because NRG have been reported to show a modest mitogenic activity in the presence of serum components, the cells were treated with NRGs after the medium change with a serum-free medium (20). As shown in Fig. 2*a,* NRGα1 stimulated membrane fusion in a dose-dependent manner. This stimulatory effect could be seen at its concentrations as low as 0.1 nM (Fig. 2*a*). At 0.5 nM, the onset of myoblast fusion in the NRGα1-treated cells was observed about 1 day earlier than that in the control cells (Fig. 2*c, lower panel*). NRGα1 at the same concentration also stimulated the accumulation of muscle-specific proteins, such as MHC, as assessed by immunostaining (Fig. 2*b*) and immunoblot analysis using an anti-MHC antibody (Fig. 2*c, upper panel*). Similar results were obtained when the same experiments were performed with NRGβ1, although its effect was slightly less potent than that of NRGα1. These results indicate that NRG facilitates both the morphological and biochemical differentiation of L6 cells at its concentrations comparable to those used previously for determining its effect on proliferation and differentiation of other cells. For example, some cellular responses, such as proliferation and differentiation of mammary epithelial cells, are known to require 0.3 nM of NRG to be effective (26–28). In addition, recombinant proteins containing only the EGF-like domain have been reported to stimulate the NRG receptors at subnanomolar concentrations *in vitro* (29, 30).

Inhibition of NRG Receptor Activation Suppresses the Differentiation of L6 Cells—If there is a NRG-ErbB3 autocrine signaling necessary for myogenesis, disruption of this signaling must suppress the differentiation process. To test this possibility, we took advantage of the availability of antibodies that neutralize the NRG-ErbB3 signal. As shown in Fig. 3*a,* the activation of ErbB3 receptor by exogenous NRGα1 was dramatically suppressed by treatment of the cells with 10 μg/ml of the anti-ErbB3 monoclonal antibody (Ab5) or the anti-NRG polyclonal antibody (Ab2). These results indicate that Ab5 and Ab2 were capable of neutralizing the NRG-ErbB3 signal pathway effectively. We then examined the effect of Ab5 and Ab2 on myogenesis. The differentiation of L6 cells was induced by changing the medium with DM at 3 days after plating, followed by immediate treatment with either Ab5 or Ab2. Both the formation of myotube and the expression of MHC were markedly inhibited by the antibodies, whereas the differentiation was not influenced by the treatment with normal mouse or rabbit immunoglobulin (Fig. 3, *b–d*). These results demonstrate the existence of an autocrine signaling pathway for NRG in L6 cells, which is necessary to initiate the process of muscle-specific gene expression and myotube formation.

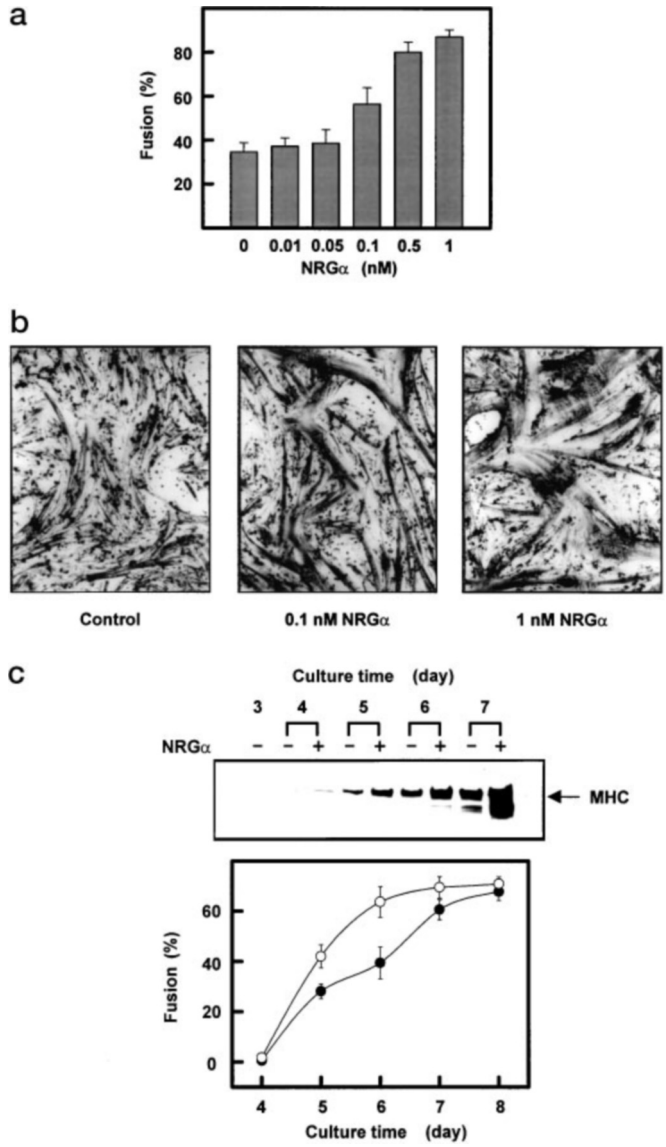

FIG. 2. **NRGα1 stimulates the differentiation of L6 myoblasts.** L6 cells were cultured for 3 days in PM and then incubated with or without NRGα1 in DMEM containing 0.05% bovine serum albumin. *a* and *b,* concentration dependence of membrane fusion to NRGα1. Cells were fixed at 6 days after plating. Fusion index was determined (*a*), and the cells were stained with the anti-MHC antibody, MF20 (*b*), as described under "Experimental Procedures." *Bars* show mean ± S.E. for three independent experiments. *c,* time course of the myogenic induction by NRGα1. Cells were treated with (○) or without (●) 0.5 nM NRG-α1. Extent of fusion was determined at the indicated time points. The results presented are means ± S.E. for triplicate determinants. Cell extracts were prepared at the indicated time points and subjected to an immunoblot analysis with the anti-MHC antibody, MF20.

Moreover, the extent of inhibition appeared to be precisely correlated with the potency of neutralizing antibodies. An extensive exposure visualized a low level of tyrosine phosphorylation of ErbB3, when Ab2, the ligand-blocking antibody, was incubated with NRGα1, whereas tyrosine phosphorylation of ErbB3 was not detected with Ab5, the receptor-blocking antibody (Fig. 3*a*). These results demonstrate that Ab5 is more potent than Ab2 in neutralizing the NRG-ErbB3 signal pathway. In parallel, Ab5 inhibited the expression of MHC and membrane fusion of L6 cells more effectively than Ab2 (Fig. 3, *b* and *c*). A low level of MHC was detected upon a prolonged exposure when the myogenesis of L6 cells was inhibited with Ab2 but not with Ab5 (Fig. 3*b*).

Inhibition of NRG Receptor Activation Suppresses the Differ-

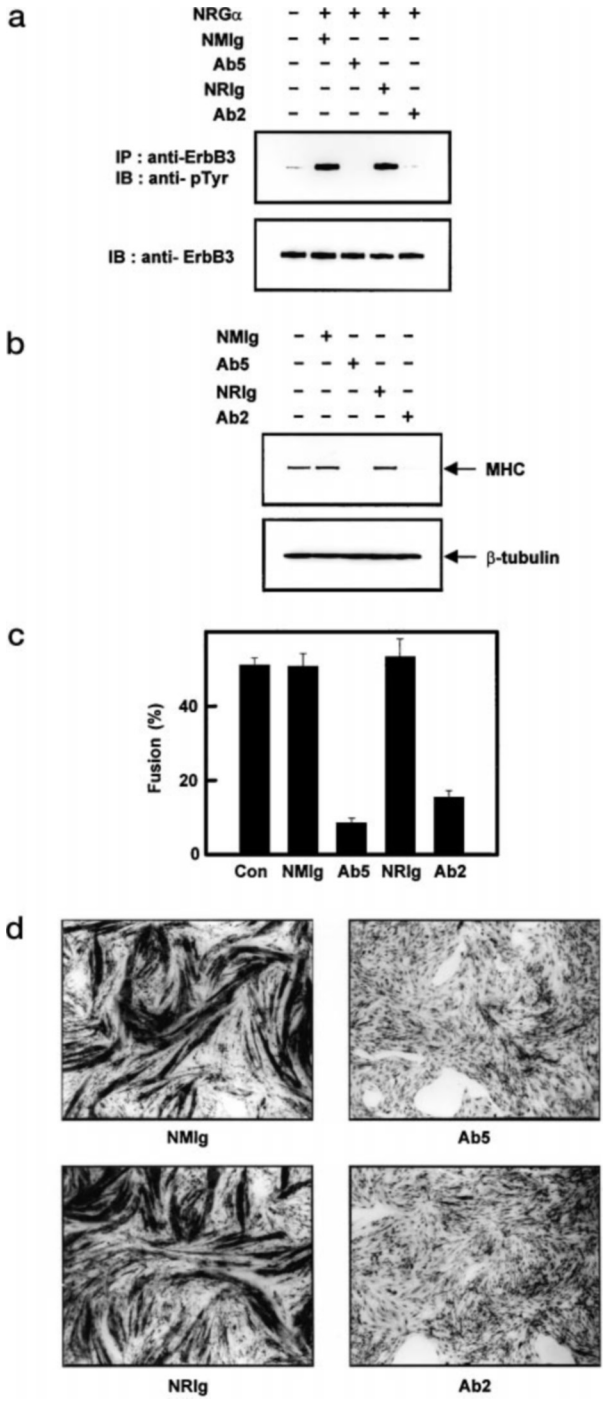

FIG. 3. **Inhibition of NRG receptor activation suppresses the differentiation of L6 cells.** *a,* inhibition of the NRGα1-mediated tyrosine phosphorylation of ErbB3 by Ab5 and Ab2 antibodies. L6 myoblasts were incubated with Ab5 or Ab2 antibody (10 μg/ml) for 1 h at 37 °C and then treated with 0.5 nM NRGα1. After incubation for 5 min, tyrosine phosphorylation of ErbB3 receptor was monitored as described in Fig. 1*a*. *NMIg* and *NRIg* indicate normal mouse and rabbit Ig, respectively. *b–d,* inhibition of the differentiation of L6 myoblast by Ab5 and Ab2 antibodies. L6 cells were cultured for 3 days in PM and switched to DM. At the time of the medium change, the indicated antibodies were added at 10 μg/ml. Expression of MHC (*b* and *d*) and membrane fusion (*c*) were determined with the cells 5 days after plating. *NMIg,* normal mouse Ig; *Ab5,* anti-ErbB3 monoclonal antibody; *NRIg,* normal rabbit Ig; *Ab2,* anti-NRG polyclonal antibody.

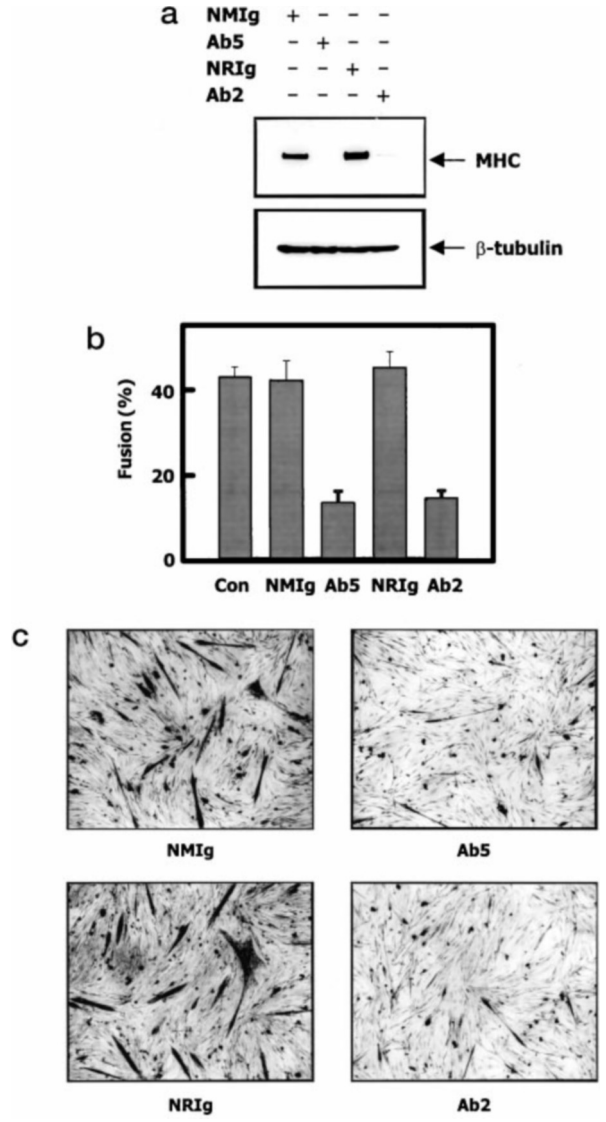

FIG. 4. **Inhibition of NRG receptor activation suppresses the differentiation of rat primary myoblasts.** Rat primary myoblasts were prepared as described under "Experimental Procedures." Four hours after the cell plating, they were treated with Ab5 or Ab2 antibody. Expression of MHC (*a* and c) and membrane fusion (*b*) were determined with the cells 3 days after plating. *NMIg,* normal mouse Ig (30 μg/ml); *Ab5,* anti-ErbB3 monoclonal antibody (30 μg/ml); *NRIg,* normal rabbit Ig (50 μg/ml); *Ab2,* anti-NRG polyclonal antibody (50 μg/ml).

entiation of Rat Primary Myoblasts—L6 cell is an established cell line, and it may not truly represent *in vivo* situation. In an attempt to investigate whether the autocrine signaling of NRG also works in the process of muscle differentiation *in vivo*, we used primary culture of rat myoblasts. Treatment of rat primary myoblasts with Ab5 (30 μg/ml) or Ab2 (50 μg/ml) caused a severe inhibition in expression of MHC and membrane fusion (Fig. 4). On the other hand, normal mouse or rabbit immunoglobulin failed to inhibit the differentiation of myoblasts into myotubes at the same concentrations. Compared with L6 cells, it is noticeable that rat primary myoblasts required higher concentrations of neutralizing antibodies for inhibition of the differentiation processes. Moreover, the inhibitory effect on primary culture was less prominent than that on L6 cell line. It may be explained by the observation that the primary cultures of rat myoblasts were significantly contaminated by fibroblasts (less than 30% of total cell number at the time of plating and more than 60% 2 days after plating), which are known to secrete NRGs (31, 32). Another explanation could be the heterogeneity in the population of rat primary myoblasts, a part of which might have already committed to differentiation program at the time of the preparation of the primary cultures. Nevertheless, the significant suppression of MHC expression

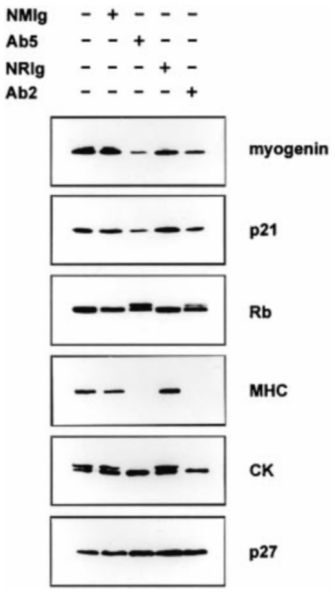

FIG. 5. **Effect of inhibition of NRG receptor activation on the expression of various proteins related with myogenesis.** L6 cell extracts were prepared as described in Fig. 3*b*. Expression of myogenin, p21, Rb, MHC, and CK was assessed by immunoblot. The cell cycle inhibitor, p27, was also analyzed as above for an internal control.

and membrane fusion by the neutralizing antibodies supports our notion that the activation of autocrine signaling of NRG is required for muscle differentiation *in vivo*.

Contribution of NRG Signal Pathway to the Myogenic Program—The formation of skeletal muscle proceeds through sequential developmental events following commitment of mesodermal precursor cells, withdrawal from cell cycle, and terminal differentiation into multinucleated myotubes. The commitment to the differentiation pathway is characterized by the expression of myogenin (33, 34). Induction of p21 and dephosphorylation of Rb are closely related with the exit from cell cycle (35, 36). The induction of myogenin and the exit from cell cycle are proposed to be prerequisite for terminal differentiation indicated by myotube formation and muscle-specific gene expression, such as MHC and CK. To examine whether the neutralizing antibodies selectively suppressed specific stage(s) of the myogenic differentiation, immunoblot analysis was performed using antibodies against myogenin, p21, Rb, MHC, and CK in the presence and absence of Ab5 or Ab2. Both Ab5 and Ab2 dramatically suppressed the expression of MHC and CK (Fig. 5). These antibodies also suppressed the expression of myogenin and p21, but rather moderately. The phosphorylation of Rb reflects its activity in cell cycle. Rb in the absence of the neutralizing antibodies migrated as a single band of 100 kDa, indicating that most of Rb is hypophosphorylated when myoblasts exit from cell cycle (Fig. 5). In contrast, a part of Rb from Ab5- and Ab2-treated cells showed a retarded mobility, indicating that cell cycle was not completely arrested. These results demonstrate that the commitment to the differentiation pathway and the irreversible cell cycle arrest were partially blocked by the neutralizing antibodies. On the other hand, the neutralizing antibodies more effectively suppressed the terminal differentiation characterized by cell fusion and expression of MHC and CK.

The expression of myogenin is induced by another myogenic factor, myf5, expressed at the early stage of myogenic differentiation. Myogenin also promotes its own gene expression by itself. Therefore, the partial blockage of myogenin expression by the neutralizing antibodies can be explained by a possibility that the NRG-ErbB3 signaling be involved in autoregulation of myogenin expression. Concurrently, the NRG-ErbB3 signaling appears to be also required for the ability of myogenin to induce the terminal differentiation as evidenced by the finding of complete inhibition of MHC expression and myotube formation by the neutralizing antibodies. The partial blockage of cell cycle arrest is also explained by this hypothesis because myogenin is partly responsible for the completion of cell cycle arrest (34, 37). It is noticeable that the extents of suppression were closely correlated with the potencies of neutralizing antibodies. Ab5, the stronger neutralizing antibody, more effectively suppressed the hypophosphorylation of Rb and the induction of myogenin and p21.

NRGs play a role as an inducing signal in various vertebrate development, such as trabeculae formation in heart, growth and differentiation of Schwann cells, morphogenesis of mammary gland, and formation of neuromuscular synapse (25, 38, 39). These developmental processes are presumably mediated by local actions of NRGs in paracrine manner, in which NRG secreted from one group of cells induces the development of neighboring target cells. Here, we provide evidence for activation of an NRG autocrine signaling during the differentiation of embryonic muscle cells. We have shown that the expression of NRG was transiently but dramatically up-regulated after the cells were switched to the differentiation state. The receptors for NRG, ErbB2, and ErbB3 were also found to exist and be active in L6 myoblasts. Furthermore, a substantial inhibition of myogenesis by the antibodies that neutralize the NRG signal pathway is consistent with the model that locally synthesized NRGs may regulate the differentiation of embryonic muscle cells through an autocrine mechanism. NRG has been shown to act in an autocrine manner in other cases. Schwann cells express NRG mRNA, and the production of NRG is required for the proliferation of Schwann cell in response to transforming growth factor-β, basic fibroblast growth factor, or hepatocyte growth factor (31). Mature muscle cells also synthesize NRG and ErbBs, and both ligands and receptors are localized to synaptic site (24). Although NRG is also secreted from nerve terminals, NRG-ErbB signaling at the synapse may play a role, at least in part, as an autocrine mechanism (25).

The neutralizing antibodies against NRG and ErbB3 showed the differential effect on each event of myogenic progression. They partially suppressed the dephosphorylation of Rb and the expression of myogenin and p21 but completely inhibited myotube formation and MHC expression. These results indicate that NRG has a differentiation-promoting activity, although it is not absolutely required for the commitment of myoblasts to differentiate. More importantly, these results also indicate the existence of a checkpoint between the expression of myogenin and the terminal differentiation. In *Xenopus* and mammal, muscle precursor cells of mid-gastrulae contain transcripts of muscle-specific transcription factors, and the level of these factors first becomes detectable at this stage (40–43). But the expression of muscle-specific transcription factors has proven to be insufficient for autonomous differentiation of muscle precursor cells (41). Our study proposes that NRG, produced by the myoblasts at early stage of differentiation, may be required to proceed through a checkpoint presumably for the cells located after the expression of myogenin and the exit from cell cycle. When NRG reaches a threshold concentration in an autocrine manner, it may precisely coordinate the asynchronously committed myoblasts for the spatiotemporal progress of differentiation. It will be interesting to determine the nature of checkpoint controlled by NRGs.

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