Stimulation of Myogenic Differentiation by a Neuregulin, Glial Growth Factor 2

ARE NEUREGULINS THE LONG-SOUGHT MUSCLE TROPHIC FACTORS SECRETED BY NERVES?*

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It has long been known that nerves stimulate growth and maintenance of skeletal muscles in ways not dependent on physical contacts, but numerous attempts to identify and characterize the myotrophic agent(s) secreted by nerves have been unsuccessful. We here suggest that products of the neuregulin gene may be these agents. The neuregulins are a family of proteins made by alternative splicing of a single transcript to give as many as 15 protein products. One member of this family, glial growth factor 2 (rhGGF2) is a very potent stimulator of myogenesis in L6A1 myoblasts, giving a maximal stimulation of cell fusion and creatine kinase elevation at a concentration of 1 ng/ml (18 pM). The stimulation of myogenesis is not rapid, but it is prolonged, continuing over a period of at least 6 days. The effects of rhGGF2 are additive with those of insulin-like growth factor I (IGF-I) or its analog R3-IGF-I, suggesting that the actions of these two myotropic agents differ in at least one rate-limiting step. We have observed one possible difference; unlike the IGFs, rhGGF2 does not induce elevation of the steady state level of myogenin mRNA.

The myotrophic actions of nerves have been known for a long time, and it has been widely recognized that nerves stimulate muscle formation and maintenance in ways not entirely dependent on direct physical contact. A review by Gutman (1) 20 years ago discusses a large body of early evidence for the existence of “long term maintenance regulations not mediated by nerve impulses” and states that “motor, sensory, or central neurons can supply the . . . agent.” Subsequent searches for such myotropic agents have not been successful (at least twice transferrin was isolated when a “muscle trophic factor” or “scatrin” was sought (2, 3)), but we have recently uncovered a strong candidate for the role of the myotrophic agent from

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1 The abbreviations used are: GGF2, glial growth factor 2; rhGGF2, recombinant human glial growth factor 2; IGF-I, insulin-like growth factor I; ARIA, acetylcholine receptor inducing activity; DMEM, Dulbecco’s minimal essential medium; CK, creatine kinase; CHO, Chinese hamster ovary.
Concentration of approximately 18 pM, the lowest effective concentration of rhGGF2 in DMEM-bovine serum albumin, was used to stimulate myogenesis (quantitated by guest on April 27, 2019http://www.jbc.org/Downloaded from)

FIG. 1. Concentration dependence of stimulation of myogenesis by rhGGF2. Myoblasts were plated under our standard conditions, washed with DMEM once after 24 h, and then treated with the indicated concentrations of rhGGF2 in DMEM-bovine serum albumin. Cultures were observed microscopically and frozen for CK and DNA determinations 72 h later. The results presented are means ± S.E. for triplicate incubations, as is true in all figures presented here.

Another aliquot of the same cell suspension was measured fluorometrically as described by Ewton et al. (17).

RESULTS

Concentration Dependence of the Stimulation of L6A1 Myoblast Differentiation by rhGGF2—Fig. 1 illustrates the results of an experiment (typical of four similar ones, each including triplicate incubations) in which an extended series of rhGGF2 concentrations was used to stimulate myogenesis (quantitated as the elevation of creatine kinase levels). It demonstrates very clearly that a biphasic curve characterizes the myogenic action of rhGGF2. (It should be understood that fusion to form myotubes closely parallels elevation of creatine kinase levels in all of these experiments.) The optimal concentration was 1 ng/ml; for a molecular weight of 55,000, this represents a molar concentration of approximately 18 pM, the lowest effective concentration of any growth factor having a major effect on muscle cells that has been reported. By contrast, IGF-1 (M = 7500) gives optimal stimulation of myogenesis at about 40 ng/ml, or about 6 nM, a full 333-fold lower potency. To be sure, much of the lower potency of IGF-1 can be attributed to secretion of inhibitory binding proteins by myoblasts; analogs with substantially reduced affinity for the IGF binding proteins (such as R3-IGF-I, in which an arginine has been substituted for glutamate as the third amino acid) exhibit potencies as much as 100 times as great as that of native IGF-1 (18), but that still leaves them only one-third as potent as rhGGF2 in stimulating myogenesis.

It should be noted that rhGGF2 in the serum-free medium used here had little or no effect on myoblast proliferation whether measured as Coulter-counted cells at 24 or 48 h or DNA/dish at 72 h (data not shown). (The flat cell number and DNA concentration dependence curves for both rhGGF2 and DMEM treatments, as well as our earlier experiments with IGFs, show that rhGGF2 does not simply enhance survival of the cells.) In this way, it differs dramatically from IGF-I, which is an active mitogen for skeletal myoblasts in serum-free medium. However, in the presence of serum components, rhGGF2 does exhibit a modest mitogenic activity, with approximately a doubling in cell numbers after a 48-h incubation; under these conditions, R3-IGF-I gave a 5-fold increase in cell numbers (data not shown).

Time Course of CK Elevation by rhGGF2 and IGF-I—In our “standard” 72-h incubations such as that presented in Fig. 1, rhGGF2 caused substantially less elevation of creatine kinase and increase in cell fusion than did IGF-I or its R3 analog. However, when the incubation was extended to longer periods, the amount of fusion observed microscopically and the levels of CK attained were nearly as great in rhGGF2-treated cells as in those incubated with IGF-I at 80 ng/ml, as shown in Fig. 2. (L6 myotubes formed in the presence of IGF-I tend to lift from the culture dish after 4 or 5 days, so extended incubations with IGF-I are not feasible.) It is not clear why the response to rhGGF2 is so much slower than that to IGF-I, but this has been a consistent result in our experiments.

Additivity of Effects of rhGGF2 and R3-IGF-I—The stimulation of differentiation by rhGGF2 adds it to the handful of agents known to stimulate myogenesis (the others are IGFs, thyroid hormones, and retinoic acid, see Ref. 20). Fig. 3 presents the results obtained when we examined the effects of two of these stimulatory agents, IGF-I and rhGGF2, in combination. As shown in the figure, whether IGF or R3-IGF-I was varied in the presence of optimal rhGGF2 (Fig. 3, A and B) or rhGGF2 was varied in the presence of optimal R3-IGF-I (Fig. 3C), the levels of CK attained with the two agents were clearly greater in combination than with either alone. Such observations are usually interpreted to mean that the agents operate by different mechanisms (i.e. different processes become rate-limiting at optimal concentrations of each hormone), but in this case interpretation is slightly clouded by the biphasic nature of the response curves of both hormones. This biphasic effect may be attributable to a requirement for interaction of a single ligand with two receptors (19) or induction of myogenesis-inhibiting oncoprogens such as Fos and Jun (20).

Differences in Actions of rhGGF2 and IGF-I—We (21) demonstrated some time ago that the induction of differentiation by IGF-I involves increased expression of the myogenin gene, although later results have made us aware that a simple increase in myogenin mRNA does not completely account for the increased myogenesis. We have found that the R3 analog of IGF-I (at 1 ng/ml, a concentration that stimulates differentiation but has little effect on cell proliferation, see Ref. 18), gives a substantial increase in myogenin mRNA, but we found little or no elevation of myogenin mRNA levels in rhGGF2-treated cells (data not shown). Another difference between rhGGF2 and IGF-I was detected when we measured phosphorylation of the insulin receptor substrate 1, which we observed in IGF-I-treated myoblasts but not in cells incubated with rhGGF2 (data not shown).
ARia has been shown to act on muscle to induce expression of myogenesis or cell division in skeletal muscle cells, but studies have given results consonant with our observations. It is not possible to know from knockout animals whether or not neuregulin gene products are required for skeletal myogenesis. Although myogenin mRNA is detectable as early as day 9.5 (22), the protein is not detectable at day 10.5 (23), apparently because the mRNA is not processed (24); the latter authors cite reports that formation of skeletal muscle is not detectable until day 14. It is well established that myogenin protein is essential for skeletal muscle formation (25, 26). ErbB-3 and ErbB-4. No results of knockouts of the ErbB-3 gene have been reported, so it is not yet possible to evaluate its possible role. Because the most likely receptor binding sequence in the neuregulins is identical in all members of the family (i.e. amino acids 177-226 of heregulin β, see Ref. 33), it seems likely that all neuregulins share the myotropic activities we have found for rhGGF2, although it is possible that other parts of the molecule may modulate the relative potencies of the members of the family.

It is not obvious how rhGGF2 could be effective in stimulating myogenesis in the absence of any apparent effect on expression of the myogenin gene, as myogenin knockouts are postnatal lethal because of the failure to develop functioning diaphragm muscle (25, 26). Possibilities currently under investigation by us include increased levels of myogenin protein resulting from increased translation of the basal levels of myogenin mRNA found in growing myoblasts and increased half-life of myogenin protein, as well as elevated levels of MEF2, which has been shown by Olson's group (34) to enhance myogenic responses to the MyoD family.

Are we correct in suggesting that the neuregulins are the long-sought myotropic factors secreted by nerves? As mentioned above, the timing of developmental events makes it difficult if not impossible to evaluate this point with knockout experiments, but there are several points which require that this possibility be considered seriously. They include the following.

1) The neuregulins have been shown to be produced by at least some cells of the nervous system (7, 14). 2) Within nerves, at least some neuregulins are localized at or near the neuro-muscular junction (28, 30). 3) The actions of neuregulins on skeletal muscle cells are associated with growth or differentiation, i.e. they are myotropic effects. (a) We found that rhGGF2 causes an increase in myotube formation and elevated CK levels (Fig. 1). (b) Induction of acetylcholine receptors (i.e. ARIA activity) is generally associated with terminal differentiation, and it is often used (as we use CK levels) to quantitate myogenic differentiation. (c) rhGGF2 has at least some mito-
long-sought myotrophic agents secreted by nerves. For identification of products of the neuregulin gene as the skeletal muscle differentiation (35).

We believe that these considerations make a substantial case for identification of products of the neuregulin gene as the long-sought myotrophic agents secreted by nerves.

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