FEBS 19514 FEBS Letters 418 (1997) 275–281

NG108-15 cells express neuregulin that induces AChR α-subunit synthesis in cultured myotubes

San Pun, Jie F. Yang, Yu P. Ng, Karl W.K. Tsim*

Department of Biology and Biotechnology Research Institute, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, China

Received 25 September 1997

Abstract A cholinergic neuroblastoma×glioma hybrid cell line NG108-15 is able to form functional synapses, and contains both AChR-aggregating and AChR-inducing activities when co-cultured with myotubes. Several lines of evidence indicate that the AChR-inducing activity of NG108-15 cells is derived from neuregulin. The conditioned medium of cultured NG108-15 cells induced the expression of AChR α-subunit as well as the tyrosine phosphorylation of erbB-3 receptor. NG108-15 cells expressed neuregulin with a protein of ~100 kDa in size and transcripts of ~6.8 kbp, ~2.6 kbp and ~1.8 kbp; mRNAs encoding β1 and α2 isoforms of neuregulin were revealed. NG108-15 cells were induced to differentiate by chemicals, and the chemical-induced differentiation of NG108-15 cells increased the level of neuregulin mRNA expression ~3-fold while the expression of a housekeeping gene remained relatively unchanged. The activity of neuregulin in the conditioned medium of NG108-15 cells was reduced by treating the medium with heparin and anti-neuregulin antibody. In addition, NG108-15 cells were transfected with antisense neuregulin cDNA and its expression of neuregulin was reduced, while its neuregulin-induced tyrosine phosphorylation activity was markedly decreased. This is the first direct demonstration that the NG108-15 cell-induced AChR up-regulation on cultured myotubes is mediated by neuron-derived neuregulin.

Key words: Acetylcholine receptor; ARIA expression; Development; Neuromuscular junction; Synaptogenesis

1. Introduction

During the formation of vertebrate neuromuscular junctions, motor neurons make contact with muscle fibers and direct the formation of postsynaptic specializations [1]. These specializations include the aggregation of acetylcholine receptors (AChRs), acetylcholinesterase (AChE) and other synaptic proteins. The increase in postsynaptic AChR density, up to approximately 10000 receptors/μm^2, is primarily due to the aggregation of AChRs already present on the membrane at the time of nerve-muscle contact as well as an increase in local AChR synthesis [1]. The aggregation of AChRs is induced by agrin derived from the presynaptic terminus [1–4]. In the local synthesis of RNAs encoding AChR subunits, AChE and other synapse-specific proteins are highly concentrated in the synaptic regions [1,5,6]. The up-regulation of AChR synthesis at the neuromuscular junction is induced by innervated motor neurons. The motor nerve provides two distinct mechanisms to achieve this striking localization of AChRs: (i) it releases factors, such as calcitonin gene-related peptide [7], ascorbic acid [8] and acetylcholine receptor inducing activity (ARIA) [9], that stimulate the synaptic expression of AChR; and (ii) nerve-evoked electrical activity represses the synthesis of AChR in the extrasynaptic regions [10].

ARIA, first isolated from chick brain, is the best candidate for the nerve-derived signal for postsynaptic gene regulation. ARIA could mimic several effects of motor axons on the muscle target that include: inducing the synthesis of AChR in aneural myotubes [9,11], increasing the number of voltage-gated sodium channels [12] and the expression of the ε-subunit of AChR characteristic of the adult AChR [13–15]. The ARIA's signaling pathway in muscle is mediated by erbB-2 and/or erbB-3 receptors [15–17]. It is believed that ARIA released from the developing motor nerve terminals activates its receptor on the postsynaptic muscle membrane and induces the postsynaptic gene expression at the neuromuscular junction [9,18,19].

The chick ARIA cDNA encodes a considerably larger transmembrane precursor designated pro-ARIA with a predicted protein of 602 amino acids [11]. From N- to C-terminus, pro-ARIA has immunoglobulin (Ig)-like, epidermal growth factor (EGF)-like, hydrophobic and intracellular domains [11]. Mature ARIA of 42 kDa in size is believed to be produced by proteolytic cleavage of pro-ARIA at the K_{205} and R_{206} dibasic amino acid residues adjacent to the hydrophobic domain [11]. Sequence analysis shows that ARIA belongs to the neuregulin family, which has diverse functions in neural development [20,21]. Members of neuregulin include rat *neu* differentiation factor [22], human heregulin [23] and bovine glial growth factor [24]. Thus, the term neuregulin has been used to describe all splice variations observed for this family [21,24]. Through alternative RNA splicing, many isoforms are generated from this family; the most common splicing site is at the C-terminus of the EGF-like domain that determines two major classes of isoforms termed α and β. Within each class, there are subclasses that are classified according to the region downstream of the intracellular domain [22,23]. However, the full biological activity of neuregulin is restricted at the EGF-like domain [18,15,25].

A study model for in vitro synaptogenesis is the co-culture of myotube with a neuroblastoma×glioma hybrid cell line NG108-15 that was derived by somatic cell hybridization [26]. Apart from the formation of functional synapses with myotubes, several lines of evidence indicate that NG108-15 cells are very similar to motor neurons. The differentiated NG108-15 cells are neuronal in light microscope appearance, have membranes of high electrical activity and capable of

*Corresponding author. Fax: (852) 2358-1559.
E-mail: BOTSIM@USTHK.UST.HK

Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor; Bt_2-cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate; PBS, phosphate-buffered saline (pH 7.5); PCR, polymerase chain reaction; PGE1, prostaglandin E1; TTX, tetrodotoxin

PII S0014-5793(97)01369-0

generating acetylcholine-induced action potentials, have clusters of synaptic vesicles, have a high level of acetylcholine transferase activity, and have agrin responsible for the AChR aggregation when co-cultured with myotubes [3,26,27]. Although AChR-inducing activity has been reported in the cultured NG108-15 cells [28], the nature of that activity is not known. In order to establish the NG108-15 cell-myotube co-culture system, we used molecular genetic approaches to show that NG108-15 cells expressed neuregulin and its activity could be blocked by heparin, antibody and antisense cDNA transfection.

2. Materials and methods

2.1. Cell cultures

Neuroblastoma×glioma NG108-15 hybrid cells were cultured in 100 mm culture dishes as described by [28]. NG108-15 cells were induced to differentiate by treatment with one of the following media supplemented with 1 mM N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2-cAMP; Sigma, St. Louis, MO), 10 μM prostaglandin E1 (PGE1; Sigma) and 1 mM theophylline, 1.5% (v/v) dimethylsulfoxide (DMSO; Sigma), or serum starvation. After 2 days of treatment, cells were washed and harvested for RNA or protein extraction.

Primary chick myotube cultures were prepared from the hind-limb muscles dissected from embryonic day 11 chick embryos. The muscles were then dissociated according to the modified protocol previously described [29]. Muscle cells were cultured in MEM supplement with 10% heat inactivated horse serum, 2% (v/v) chick embryo extract, 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. In the co-cultures, NG108-15 cells were plated onto the 3-day-old chick myotubes in a 35 mm tissue culture plate for 2 days. The cells were rinsed with phosphate-buffered saline (PBS) pH 7.6, and used for immunohistochemical analysis. The C2C12 myoblasts were cultured and induced to fuse by reducing the serum to 1.5% [15]. All tissue culture chemicals were from Gibco-BRL (New York).

2.2. Induction of AChR α-subunit

In the AChR α-subunit induction assay, the chick myotubes were treated with conditioned medium of NG108-15 cells overnight, and total RNA was collected. Total RNA was electrophoresed in a 1% formaldehyde-agarose gel and transferred overnight onto a nylon membrane and hybridized with a [^{32}P]dCTP-labeled cDNA probe of ~1.2 kbp chick AChR α-subunit [30].

The pnlac Z plasmid containing 850 bp chick AChR α-subunit promoter tagged with β-galactosidase gene was described in [31] and provided by Dr. Joshua Sanes from Washington University School of Medicine. The cDNA was transfected into 2-day-old chick myotube cultures by using calcium phosphate precipitation [32]. Two days after transfection, the myotubes were cultured with NG108-15 cells for 36–48 h. In β-galactosidase staining, cell cultures were fixed for 5 min in PBS containing 2% paraformaldehyde and 0.2% glutaraldehyde at room temperature, and then rinsed with PBS. The staining reaction was developed for 16–24 h at 37°C in PBS, pH 7.6, containing 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$. After the color development, the culture was fixed with ethanol and observed under a Zeiss Axiophot microscope.

2.3. Immunochemical analysis

The cultured NG108-15 cell was fixed and stained by an anti-neuregulin antibody (α and β isoform-specific), then followed by fluorescent-conjugated secondary antiserum as described in [30]. For Western blot analysis, NG108-15 cells were lysed in 0.5% (w/v) SDS in 50 mM phosphate buffer (pH 8.0) and the protein concentrations were determined [33]. About 0.5 mg of protein was immunoprecipitated by the anti-neuregulin antibody (α and β isoform-specific), followed by protein G precipitation. Samples were electrophoresed on a 7.5% polyacrylamide gel and transferred onto nitrocellulose membrane (MSI, Westborough, MA). The membranes were blocked with 5% (w/v) dry milk in 20 mM Tris base pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween 20, for 1 h at 37°C, followed by incubation with anti-neuregulin antibody (α isoform-specific; 1:1000). Immunoreactivity was detected by ECL Western Blot System (Amersham, UK) following the instructions from the supplier.

In the phosphorylation studies, fused C2C12 myotubes were treated with the conditioned medium from NG108-15 cells for 30 min [30]. The treated cells were resuspended in RIPA buffer (PBS pH 7.4, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM aprotinin). The erbB-3 receptor was immunoprecipitated with an antibody against erbB-3 (C17; Santa Cruz Biotech., Santa Cruz, CA) at 1:1000 dilution. The immunoprecipitated proteins were collected on protein G agarose beads and fractionated by 7.5% SDS-PAGE [25,32]. Electrophoresed proteins were transferred onto nitrocellulose membrane. The membrane was blocked with buffer containing 2.5% BSA for 1 h at 37°C, followed by incubation with horseradish peroxidase-conjugated anti-tyrosine phosphorylation antibodies RC 20 (Transduction Lab., Lexington, KY) diluted 1 in 1000. For the blocking of neuregulin, the conditioned medium was treated with heparin (50 μg/ml) or anti-neuregulin antibody (1:500 dilution) for 30 min before it applied onto the cultured C2C12 myotubes. Anti-neuregulin α isoform specific antibody was purchased from Transduction Lab. (Lexington, KY) and anti-neuregulin α and β isoform-specific antibody was raised in our laboratory by using the recombinant EGF-like domain of neuregulin as antigen [30].

2.4. RNA isolation and RT-PCR analysis

Total RNA was isolated by using Micro RNA Isolation Kit (Stratagene, CA, USA). RNA concentration and purity were determined by absorbance at 260 nm. In RT-PCR analysis, 5 μg of total RNAs was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) by random oligonucleotide priming in a 20 μl reaction. One-fifth of the reverse transcription product was used as a template in PCR analysis with primers described below. PCR was carried out for 30 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 2 min in a 25 μl volume containing 0.8 mM dNTPs, 1×PCR buffer and 0.625 U of *Taq* polymerase (Gibco-BRL). The PCR products were analyzed in a 12% polyacrylamide gel. The amplified DNAs were directly cloned into pCR II vector (Invitrogen, CA). The identity of the cloned PCR products was confirmed by DNA sequencing using T7 Sequencing kit (Pharmacia Biotech, Sweden). The PCR primers were designed according to rat neuregulin cDNA sequence [22]. Sets of primers flanking the EGF-like domain are S-1: 5′-GAC CTG TCA AAC CCG TCA AG-3′ (sense; aa 937–956) and AS-1: 5′-AGC ACC CTC TTC TGG TAG AGT-3′ (antisense; aa 1067–1047).

2.5. Northern blot analysis

RNA samples were fractionated on a 1% formaldehyde gel. Ethidium bromide was used to assess the equal loading of different samples [32]. After the electrophoresis, samples were transferred to a

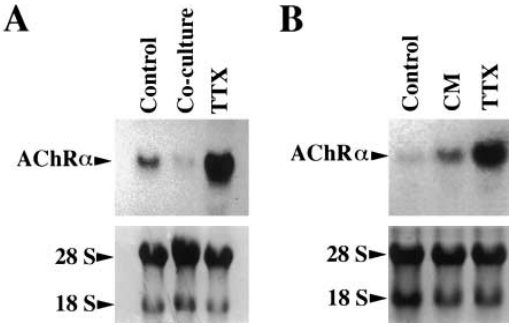

Fig. 1. The conditioned medium of NG108-15 cells induces the up-regulation of AChR α-subunit mRNA synthesis in cultured myotubes, but down-regulates in neuron-myotubes co-cultures. A: Four-day-old chick myotubes were co-cultured with NG108-15 cells (co-culture) for 1 day. Tetrodotoxin (TTX) at 1 μM was applied as a positive control, while the untreated myotube is the negative control. Total RNA was isolated from the cells and 10 μg of RNA was subjected to 1% formaldehyde-agarose gel. The membrane was probed with AChR α-subunit cDNA, and a transcript of ~3.2 kbp was detected. B: Like A except the myotubes were treated with the dialyzed NG108-15 cell conditioned medium (CM) for 1 day. Three independent experiments showed similar results. The lower panel shows the ribosomal RNA staining with 18S and 28S as markers.

charged nylon membrane (Hybond-N, Amersham, UK) and were UV cross-linked. Blots were hybridized with probes labeled with [α-^{32}P]dCTP (Amersham) by Oligolabelling Kit (Pharmacia Biotech). Hybridization was performed at 42°C overnight in 40% deionized formamide, 5×Denhart's solution, 0.5% SDS, 5×SSC, 10% dextran sulfate and 0.1 mg/ml denatured salmon sperm DNA. After hybridization, the filters were washed twice with 2×SSC with 0.1% SDS at room temperature for 30 min each, and then twice with 0.1×SSC with 0.1% SDS at 55°C for 30 min each. The washed filters were exposed to X-ray film with double intensifying screens at −80°C. The cDNAs probes were: rat neuregulin (~0.5 kbp from 525–1060 bp) [22], glyceraldehyde 3-phosphate dehydrogenase (~0.4 kbp) and chick AChR α-subunit (~1.2 kbp) [30].

2.6. cDNA construction and transfection

The partial cDNA encoding rat neuregulin was cloned by RT-PCR with specific primers according to published sequences [22]. The primers were: 5′-ATC TTC GGC GAG ATG TCT GAG CG-3′ (sense: 325–347) and 5′-TCT TCT GGT AGA GTT CCT CCG C-3′ (antisense: 1060–1039). The cloned product was confirmed by DNA sequencing. The pcDNA 3 plasmid (Invitrogen), containing a G418 resistant gene and under control of a cytomegaloviral promoter, was used as a mammalian expression vector throughout this study. The cDNAs encoding neuregulin$_{325-478}$ and neuregulin$_{525-1060}$ were created by RT-PCR with artificial cloning sites at both ends. The cDNA inserts were silica-gel-purified (Geneclean II, Bio-101, La Jolla, CA) and ligated at the corresponding restriction enzyme site of pcDNA 3 for subcloning. The identity of cDNA constructs were confirmed by DNA sequencing.

NG108-15 cells were transfected with calcium phosphate precipitation and the transfection efficiency was consistently over 60% [32]. For transient transfection, NG108-15 cells' conditioned medium was collected 3 days after transfection [3].

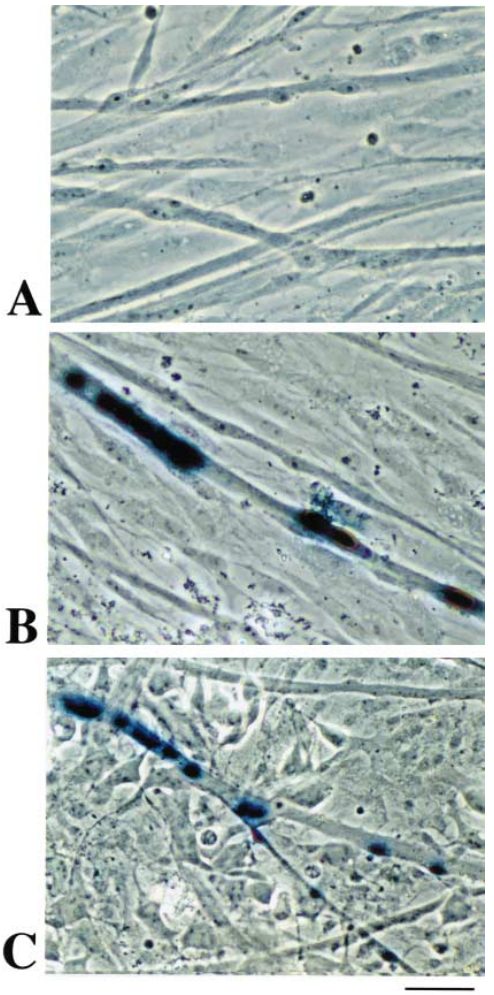

Fig. 2. The conditioned medium or the co-culture of NG108-15 cells induces the expression of AChR α-subunit promoter. The pnlac Z plasmid containing 850 bp chick AChR α-subunit promoter tagged with β-galactosidase gene was transfected into 2-day-old chick myotube cultures by using calcium phosphate precipitation. Two days after transfection, the myotubes were either treated with NG108-15 cell conditioned medium or co-cultured with NG108-15 cells for 2 days. The β-galactosidase staining was developed. Blue color indicates the induction of the promoter. A: pnlac Z transfected but without treatment. B: pnlac Z transfected and treated with the conditioned medium. C: pnlac Z transfected and co-cultured with NG108-15 cells. Scale bar, 100 μm.

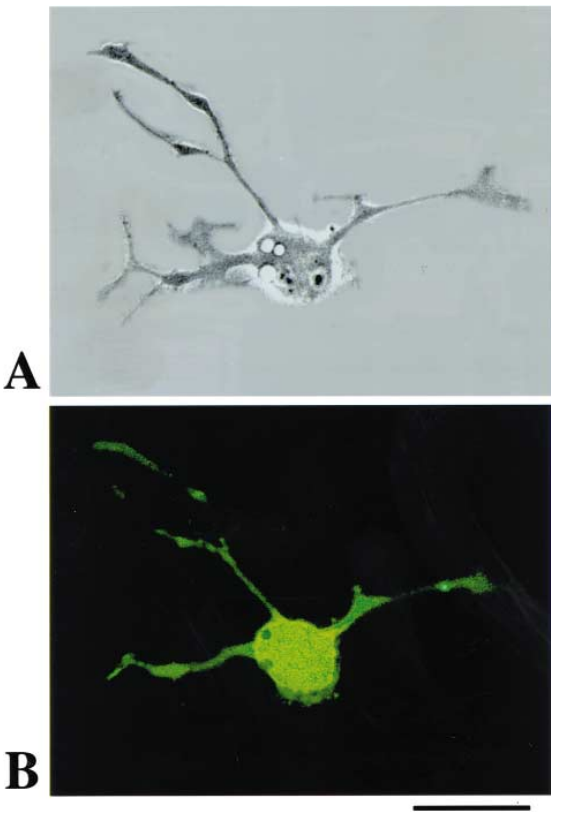

Fig. 3. Anti-neuregulin antibody recognized its antigen in differentiated NG108-15 cell. NG108-15 cells cultured for 3 days were fixed and then permeabilized with 0.5% Triton X-100. Cells were stained with anti-neuregulin antibody (α and β isoform-specific), then followed by FITC-conjugated secondary antibody. A: A phase of cultured NG108-15 cell. B: Same view as A but with fluorescence optic. A similar result was observed by using anti-neuregulin α isoform-specific antibody. Scale bar, 100 μm.

3. Results

3.1. AChR-inducing activity derived from NG108-15 cells

When NG108-15 cells were co-cultured with chick myotubes, the expression of AChR α-subunit mRNA was down-regulated (Fig. 1A). The regulation of AChR by NG108-15 cells in co-culturing with myotubes could be due to two factors: (i) the repression by secreted acetylcholine, (ii) the stimulation by an unknown AChR-inducing factor(s). In order to discriminate the two opposing factors, the conditioned medium of cultured NG108-15 cells was collected, dialyzed, and applied onto cultured chick myotubes. The AChR α-subunit mRNA at ~3.2 kbp in the treated myotubes was in-

creased ~5-fold (Fig. 1B). When the pnlac Z plasmid containing AChR α-subunit promoter was transfected into cultured myotubes, the conditioned medium or the co-culture of NG108-15 cells induced the expression of β-galactosidase staining on the transfected myotubes (Fig. 2). These results indicate the existence of a soluble AChR-inducing factor(s) that could be derived from the cultured NG108-15 cells.

3.2. Expression of neuregulin in NG108-15 cells

In cultured NG108-15 cells, antibody against neuregulin recognized an antigen in the differentiated neurons. The cell body and the neurites were labeled (Fig. 3). Using cDNA encoding rat neuregulin as a probe in Northern blot analysis, three transcripts (~6.8 kbp, ~2.6 kbp, ~1.8 kbp) were revealed; the transcript below ~1.8 kbp represented the degraded product because it was not consistent in all samples (Fig. 4A). In contrast, a single transcript of ~7.5 kbp and ~6.5 kbp was detected in avian [11,30] and amphibian (Yang et al., manuscript submitted) respectively. Differentiation of NG108-15 cells by either Bt_2-cAMP, PGE1 and theophylline, or DMSO increased the level of neuregulin mRNA expression

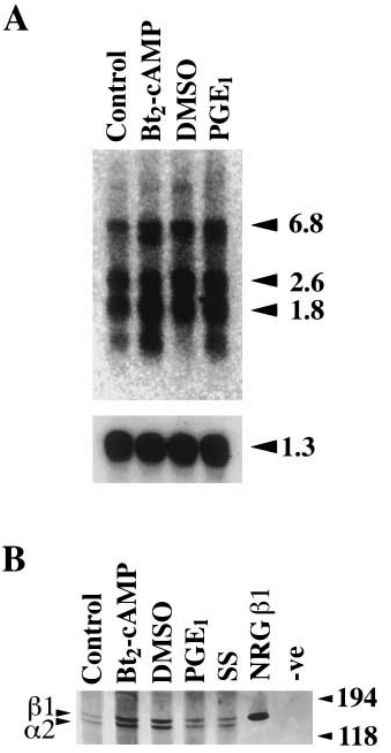

Fig. 4. mRNAs encoding neuregulin are increased during the differentiation of NG108-15 cells in culture. NG108-15 cells were induced to differentiate by different chemicals, such as Bt_2-cAMP, DMSO, PGE1+theophylline (PGE1) and serum starvation (SS) for 3 days, then poly(A)$^+$ RNAs were isolated from cultures. A: Neuregulin mRNAs were expressed at ~6.8 kbp, ~2.6 kbp and ~1.8 kbp as indicated. The expression of the transcripts were increased ~3- to ~4-fold after differentiation. The expression of glyceraldehyde 3-phosphate dehydrogenase (lower panel), with a transcript size of ~1.3 kbp as indicated, remained relatively unchanged. Control is the undifferentiated cells. B: RT-PCR was done with S-1 and AS-2 primers flanking the EGF-like domain of neuregulin. The PCR products were analyzed by a 12% acrylamide gel and stained by ethidium bromide. NG108-15 cells expressed predominantly β1 and α2 neuregulin isoforms. Neuregulin β1 cDNA (NRGβ1) serves as a positive control. Size markers in bp are shown.

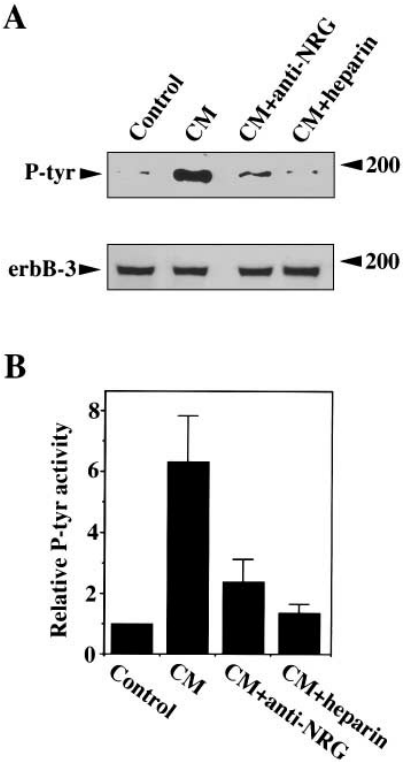

Fig. 5. Inhibition of NG108-15 cell-induced erbB-3 tyrosine phosphorylation by heparin and anti-neuregulin antibody. The conditioned medium of NG108-15 cells was treated with heparin (50 μg/ml; CM+heparin), or anti-neuregulin antiserum (1:500 dilution; CM+anti-NRG) for 30 min before applied onto C2C12 myotubes. Conditioned medium (CM) or unconditioned medium (Control) served as controls. The treated myotubes were lysed in RIPA buffer. The erbB-3 receptor was immunoprecipitated with a 1:1000 dilution of a rabbit antibody (C17) against erbB-3. The immunoprecipitated proteins were collected on protein G agarose beads and fractionated by 7.5% SDS-PAGE. A: Electrophoresed proteins were transferred onto nitrocellulose membrane, and detected by peroxidase-conjugated anti-tyrosine phosphorylation antibodies RC 20 (upper panel), or by anti-erbB-3 antibody (lower panel). 200 kDa marker is shown. B: The amounts of protein recognized by antibody on an immunoblot were determined by densitometry. Arbitrary units from the densitometer reading are used and the control phosphorylation is 1. Relative values are in mean ± S.E.M., $n=4$.

~3.0- to ~4.0-fold (Fig. 4A). All transcripts were increased in similar magnitude in the chemical-induced differentiated NG108-15 cells. The expression level of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase, with a transcript size of ~1.3 kbp, remained relatively unchanged in differentiated NG108-15 cells (Fig. 4A) as reported previously [3]. Transcripts encoding various isoforms of neuregulin were analyzed by RT-PCR on RNAs isolated from NG108-15 cells. Two PCR products of 144 bp and 129 bp were revealed; they corresponded to β1 and α2 isoforms of neuregulin (Fig. 4B). However, the expression profile of β1 and α2 isoforms of neuregulin was not affected in chemical-induced differentiated NG108-15 cells (Fig. 4B).

3.3. The blocking of neuregulin activity from NG108-15 cells

The conditioned medium of NG108-15 cells was applied onto cultured C2C12 myotubes; the tyrosine phosphorylation of erbB-3 receptor was increased (Fig. 5A). In order to determine the NG108-15 cell-induced tyrosine phosphorylation, in-

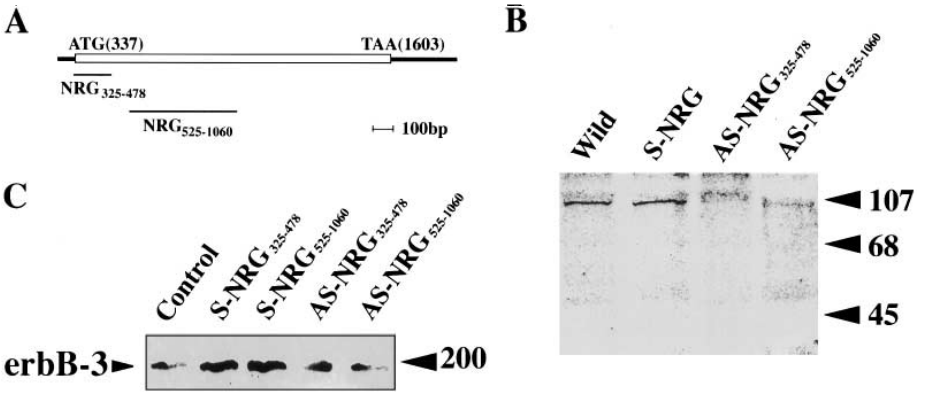

Fig. 6. Transient transfection of neuregulin antisense cDNA reduces the tyrosine phosphorylation activity of NG108-15 cells. A: A map shows the location of antisense neuregulin (NRG) cDNA constructs. Open box is the coding region, bold line shows the non-coding regions, and nucleotide numbers are in parentheses. The numbering is according to rat neuregulin cDNA as described in [22]. Two different antisense cDNA constructs were used: $NGR_{325-478}$ and $NRG_{525-1060}$. Bar, 100 bp. B: Transfection of antisense neuregulin cDNAs reduces the expression of neuregulin in NG108-15 cells. Protein (~0.5 mg) from sense transfected control (S-NGR, including either $S\text{-}NGR_{325-478}$ or $S\text{-}NRG_{525-1060}$), anti-sense transfected ($AS\text{-}NGR_{325-478}$ and $AS\text{-}NRG_{525-1060}$) and wild type NG108-15 cells were immunoprecipitated by anti-neuregulin antibody (α and β isoform-specific). The precipitated proteins were loaded onto a 7.5% SDS gel. Anti-neuregulin antibody (α isoform-specific) was used for the Western blot analysis. Two independent antibodies were used to confirm the specificity of the recognized 100 kDa as neuregulin. The antisense transfected stable cells show a reduction in ~100 kDa expression. Molecular weight markers are shown in kDa. C: Conditioned medium was collected from the transfected cells (sense control: $S\text{-}NGR_{325-478}$ and $S\text{-}NRG_{525-1060}$, and antisense transfected: $AS\text{-}NGR_{325-478}$ and $AS\text{-}NRG_{525-1060}$) or control cells. They were applied onto cultured C2C12 myotubes. Tyrosine phosphorylation assay on the erbB-3 receptor indicated that the neuregulin activity was decreased in the antisense transfected NG108-15 cells. The 200 kDa marker is indicated.

deed, is mediated by neuregulin derived from the cultured neurons. The conditioned medium of NG108-15 cells was treated with heparin and anti-neuregulin antibody. Both agents are able to bind neuregulin [18,30]. Fig. 5B shows the tyrosine phosphorylation activity of the conditioned medium is reduced by 60–70% by heparin and antibody respectively.

Whether neuregulin is the primary factor in NG108-15 cell-induced AChR synthesis and tyrosine phosphorylation is not established. We used the antisense cDNA transfection approach to demonstrate directly the pivotal role of neuregulin in NG108-15 cells. Two regions of rat neuregulin cDNA were chosen for anti-sense cDNA construction. Fig. 6A shows the localization of antisense neuregulin cDNA constructs: $NRG_{325-478}$ and $NRG_{525-1060}$. Because of the low expression level of neuregulin in NG108-15 cells, anti-neuregulin antibody was used to immunoprecipitate the antigen from the cell lysate for the Western blot analysis. Transfection of antisense neuregulin cDNAs ($AS\text{-}NRG_{325-478}$ and $AS\text{-}NRG_{525-1060}$) in NG108-15 cells reduced the expression of neuregulin, at ~100 kDa, in NG108-15 cells as compared to the wild type or the sense transfected control NG108-15 cells (Fig. 6B). The conditioned medium of the transfected NG108-15 cells was collected and tested for its erbB-3 receptor phosphorylation analysis. The activity of NG108-15 cell-induced tyrosine phosphorylation in antisense transfected cells was markedly reduced (Fig. 6C) indicating that neuregulin is the primary AChR-inducing factor in NG108-15 cells.

4. Discussion

Although AChR-inducing activity has been reported in the cholinergic neuroblastoma hybrid cell line NG108-15 [26,28], our results provide several lines of evidence to demonstrate that the AChR-inducing activity derived from NG108-15 cells is, indeed, neuregulin. First, the conditioned medium from NG108-15 cells increased the expression of AChR α-subunit mRNA in cultured chick myotubes, and it stimulated the tyrosine phosphorylation of erbB-3 receptor in cultured C2C12 myotubes. Second, the cultured NG108-15 cells expressed antigen recognized by anti-neuregulin antibody. It also expressed mRNAs encoding neuregulin (~6.8 kbp, ~2.6 kbp, ~1.8 kbp) and they were up-regulated by chemical-induced differentiation. Third, the activity of neuregulin in the conditioned medium of NG108-15 cells was blocked by treating the medium with heparin or anti-neuregulin antibody. Lastly, the antisense neuregulin cDNA transfection in NG108-15 cells reduced the expression of neuregulin, and that paralleled the decrease of tyrosine phosphorylation activity of the transfected neuronal cells. Although the neuregulin-induced erbB receptor phosphorylation was blocked in the antisense cDNA transfection, other AChR-inducing activities derived from NG108-15 cell could be eliminated.

In the antisense cDNA transfection, several attempts to create stable neuregulin-deficient NG108-15 cells have failed. The failure to obtain the stable transfectant may be due to vital, but not known, functional roles played by neuregulin in neuronal cells. Neuregulin has been reported to be mitogenic for a variety of cell types, such as Schwann cells and fibroblasts [24]. Recent studies have shown that antibodies against either erbB-2 or neuregulin reduced the proliferation of Schwann cells [34]. Moreover, neuregulin has been shown to promote the proliferation of blastema [35], and the differentiation of various neuronal cells [34,36]. In molecular genetic analysis, knock-out mutations in mice have also shown that neuregulin [20], erbB-2 [37] and erbB-4 [38] are all lethal and they are essential for normal vertebrate development.

Although NG108-15 cells in culture express β1 and α2 isoforms of neuregulin, both isoforms contain the full biological activity of neuregulin. Recent studies have demonstrated that the AChR-inducing activity of neuregulin requires only the EGF-like domain regardless of whether it is the α or β isoform [25,30]. In addition, mRNAs encoding neuregulin in NG108-15 cells were increased by chemical-induced differen-

tiation. All transcripts showed an equal magnitude of response. The up-regulation of neuregulin in differentiated NG108-15 cells could reflect a neuronal character of the cultured cells. Indeed, the differentiated NG108-15 cells also showed an up-regulation of agrin mRNA and AChR-aggregating activity when they were co-cultured with myotubes [3]. This distinct character of NG108-15 cells in up-regulating the expression of neuregulin and agrin could be a good model system to study the regulation mechanism of these synapse-inducing molecules in neurons, in particular, information on this aspect is still lacking.

Fischbach and his colleagues proposed that ARIA is released from the developing motor nerve terminals, activates its receptors on the postsynaptic muscle membrane and thus induces postsynaptic gene expression at the neuromuscular junctions [18]. In vertebrate neuromuscular junctions, three different cell types including neuron, muscle and Schwann cell could express neuregulin in vivo [6,13,30]. Although neuregulin is expressed in muscle and is regulated by innervation during development [30], it is not known whether the postsynaptic muscle fibers release their neuregulin to the synaptic cleft per se. Furthermore, whether muscle ARIA could play a role in the formation of postsynaptic specializations remains to be explained. In order to find evidence to support the 'neuregulin release hypothesis' as proposed by Fischbach et al. [18], the antisense cDNA transfection could provide a useful tool in elucidating the role of muscle-derived neuregulin in the formation of neuromuscular junctions. Although neuregulin knock-out mutant mice were created, most of the originally attributed functions of neuregulin during the formation of neuromuscular junctions cannot be studied in these neuregulin-deficient mice because of their early embryonic lethality. Thus, we are now creating neuregulin-deficient myotubes by antisense cDNA transfection in C2C12 cells and testing their response to the neuregulin-induced AChR up-regulation and tyrosine phosphorylation of erbB-3 receptor. This genetic approach has been shown to be successful in determining the roles of neuron-derived and muscle-derived agrin in inducing the AChR aggregation in the NG108-15 cell-myotube co-cultures [3,4].

Functional synapses could be formed in NG108-15 cell-myotube co-cultures. Many aspects of NG108-15 cells are very similar to motor neurons in vitro: (i) they are both cholinergic in nature and form functional neuromuscular synapses when co-cultured with muscle cells [26,28]; (ii) both of them contain agrin as the primary AChR-aggregating factor that induces AChR aggregation in the neuron-muscle co-cultures [3]; (iii) both of them express neuregulin as the primary AChR-inducing factor; and (iv) the AChE-inducing activity has also been reported in NG108-15 cells (Choi et al., manuscript submitted). However, NG108-15 cells provide a better cell type as compared to motor neurons. The cultured NG108-15 cells are homogeneous, easy to culture, capable of being transfected with DNA to a high percentage of efficiency. Thus, the cholinergic neuronal nature of NG108-15 cells could replace motor neurons in neuron-muscle co-culture, and that could serve as an in vitro model system for the study of the formation of neuromuscular junctions.

Acknowledgements: We are grateful to Tina Dong, Lisa Yung, H.Y. Choi and Annie Fong from our laboratory and to William Chau from the University Animal Care Facility for their expert technical assistance. We thank Dr. Joshua Sanes from Washington University School of Medicine for providing pnlac Z plasmid. The research was supported by grants from the Research Grants Council of Hong Kong and the Biotechnology Research Institute at The Hong Kong University of Science and Technology.

References

[1] Hall, Z.W. and Sanes, J.R. (1993) Cell/Neuron 72 (Suppl.), 99–121.
[2] McMahan, U.J. (1990) Cold Spring Harbor Symp. Quant. Biol. 55, 407–418.
[3] Pun, S. and Tsim, K.W.K. (1997) Mol. Cell. Neurosci. (in press).
[4] Pun, S., Ng, Y.P., Yang, J.F., Ip, N.Y. and Tsim, K.W.K. (1997) J. Neurochem. (in press).
[5] Merlie, J.P. and Sanes, J.R. (1985) Nature 317, 66–68.
[6] Moscoso, L.M., Chu, G.C., Gautam, M., Noakes, P.G., Merlie, J.P. and Sanes, J.R. (1995) Dev. Biol. 172, 158–169.
[7] Fontaine, B., Klarsfeld, A. and Changeux, J.P. (1987) J. Cell Biol. 105, 1337–1342.
[8] Horovitz, O., Knaack, D., Podleski, T.R. and Salpeter, M.M. (1989) J. Cell Biol. 108, 1823–1832.
[9] Falls, D.L., Harris, D.A., Johnson, F.A., Morgan, M.M., Corfas, G. and Fischbach, G.D. (1990) Cold Spring Harbor Symp. Quant. Biol. 55, 397–406.
[10] Laufer, R. and Changeux, J.P. (1989) Mol. Neurobiol. 3, 1–52.
[11] Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S. and Fischbach, G.D. (1993) Cell 72, 801–815.
[12] Corfas, G. and Fischbach, G.D. (1993) J. Neurosci. 13, 2118–2125.
[13] Jo, S.A., Zhu, X., Marchionni, M.A. and Burden, S.J. (1995) Nature 373, 158–161.
[14] Martinou, J.C., Falls, D.L., Fischbach, G.D. and Merlie, J.P. (1991) Proc. Natl. Acad. Sci. USA 88, 7669–7673.
[15] Si, J., Luo, Z. and Mei, L. (1996) J. Biol. Chem. 271, 19752–19759.
[16] Tansey, M.G., Chu, G.C. and Merlie, J.P. (1996) J. Cell Biol. 134, 465–476.
[17] Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D. and Yarden, Y. (1994) Biol. Chem. 269, 25226–25233.
[18] Loeb, J.A. and Fischbach, G.D. (1995) J. Cell Biol. 130, 127–135.
[19] Sandrock Jr., A.W., Dryer, S.E., Rosen, K.M., Gozani, S.N., Kramer, R., Theill, L.E. and Fischbach, G.D. (1997) Science 276, 599–603.
[20] Meyer, D. and Birchmeler, C. (1995) Nature 378, 386–390.
[21] Burden, S. and Yarden, Y. (1997) Neuron 18, 847–855.
[22] Wen, D., Suggs, S.V., Karunagaran, D., Liu, N., Cupples, R.L., Luo, Y., Janssen, A.M., Ben-Baruch, N., Trollinger, D.B., Jacobsen, V.L., Meng, S.Y., Lu, H.S., Hu, S., Chang, D., Yang, W., Yanigahara, D., Koski, R.A. and Yarden, Y. (1994) Mol. Cell. Biol. 14, 1909–1919.
[23] Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D., Shepard, H.M., Kuang, W., Wood, W.I., Goeddel, D.V. and Vandlen, R.L. (1992) Science 256, 1205–1210.
[24] Marchionni, M.A., Goodearl, A.D.J., Chen, M.S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Denehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J.B., Hsuan, J.J., Totty, N.F., Otsu, M., McBury, R.N., Waterfield, M.D., Stroobant, P. and Gwynne, D. (1993) Nature 362, 312–318.
[25] Yang, J.F., Ng, Y.P., Pun, S., Ip, N.Y. and Tsim, K.W.K. (1997) FEBS Lett. 403, 163–167.
[26] Busis, N.A., Daniels, M.P., Bauer, H.C., Pudimat, P.A., Sonderegger, P., Schaffner, A.E. and Nirenberg, M. (1984) Brain Res. 324, 201–210.
[27] Daniels, M. and Hamprecht, B. (1974) J. Cell Biol. 62, 691–699.
[28] Christian, C.N., Daniels, M.P., Sugiyama, H., Vogel, Z., Jacques, L. and Nelson, P.G. (1978) Proc. Natl. Acad. Sci. USA 75, 4011–4015.
[29] Wallace, B.G. (1989) J. Neurosci. 9, 1294–1302.
[30] Ng, Y.P., Pun, S., Yang, J.F., Ip, N.Y. and Tsim, K.W.K. (1997) Mol. Cell. Neurosci. 9, 132–143.
[31] Sanes, J.R., Johnson, Y.R., Kotzbauer, P.T., Mudd, J., Hanley,

T., Martinou, J.-C. and Merlie, J.P. (1991) Development 113, 1181–1191.
[32] Sambrook J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[33] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[34] Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R. and Jessen, K.R. (1995) Neuron 15, 585–596.
[35] Brockes, J. and Kintner, C.R. (1986) Cell 45, 301–306.
[36] Shah, N.M., Marchionni, M.A., Isaacs, I., Stroobant, P. and Anderson, D.J. (1994) Cell 77, 349–360.
[37] Lee, K.F., Simon, H., Chen, H., Bates, B., Hung, M.C. and Hauser, C. (1995) Nature 378, 394–398.
[38] Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995) Nature 378, 390–394.