Neurotrophin 3 is a mitogen for cultured neural crest cells
(avian embryo/dorsal root ganglia/laminin/peripheral nervous system/trophic factors)

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ABSTRACT Neurotrophin 3 (NT-3) promotes the survival and induces neurite outgrowth from a subset of neural crest (NC) and placode-derived neurons. We now report that this growth factor regulates the proliferation of cultured NC progenitor cells grown in a serum-free defined medium. In cultures of somites containing NC cells at migratory stages, NT-3 promotes a 2- to 8.4-fold increase in the number of NC cells incorporating [3H]thymidine into nuclei and a 1.8- to 4.8-fold increase in NC cell number compared to controls without added factor. NT-3 also promoted, to a lesser extent, the proliferation of NC cells in homogenous cultures established from NC clusters. In addition to its effect on NC cells, NT-3 was mitogenic to somite cells in the mixed NC/somite cultures. These data demonstrate that NT-3 can act directly on the NC cells. They also indicate that the response of NC cells to NT-3 may be modulated by the presence of somitic cells. We suggest that NT-3 may be one of the central nervous system-derived factors that mediate NC cell proliferation in vivo.

The development of neural crest (NC) cells into dorsal root ganglia (DRG) is modulated by both the neural tube and the adjacent somitic mesoderm. The crucial role played by the central nervous system (CNS) primordium during the early phases of sensory ganglion development, originally proposed by Le Douarin (1), is now substantiated by three lines of evidence. First, back transplantation of peripheral ganglia into the migratory routes of NC cells has shown that only sensory ganglion cells from the donor embryos contributed to the development of neurons in the host DRG. Moreover, sensory neurons develop only in ganglia located in the proximity of the CNS (2, 3). Second, deprivation of contact between NC cells and the tube, achieved by neural tube removal after NC migration into the somite (4) or by interposing an impermeable membrane between the neural tube and the DRG anlagen (5), led to the selective death of the NC cells. Third, treatment of NC cells with specific neural tube-derived factors such as brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF) stimulated their survival in vivo (5–7). Moreover, in culture, BDNF promoted the survival and differentiation of NC progenitors that become substance P-immunoreactive neurons (8). In a recent report, this last finding was confirmed and extended to show that the commitment of NC progenitors to the sensory fate is influenced by BDNF (9). The addition of bFGF to cultured NC cells was found to stimulate the survival, but not the proliferation, of a subpopulation of precursors with non-neuronal morphology (7), whose fate has not yet been determined.

The somites, serially repetitive mesodermal structures located on both sides of the neural tube, determine the segmental organization of the peripheral nerves and ganglia (10–12). Whereas NC cells freely migrate into the rostral half of each somite, the caudal halves are inhibitory for NC migration (13–17). Replacement of the somites by a mesoderm composed of only rostral somitic halves leads to the formation of continuous and unsegmented DRG and sympathetic ganglia (11, 18). Thus, ganglion segmentation is likely to be determined by the inhibitory properties of the alternating caudal somitic halves.

In addition to regulating segmentation, the somitic mesoderm is involved in controlling the size of early DRG. Within a mesoderm composed of only rostral somitic halves, the volume, number of cells, and proliferative activity of DRG progenitors is significantly higher than in the contralaterally located normal ganglia (19). We have proposed two possible mechanisms to account for the effect of rostral somitic mesoderm on the sensory ganglia. First, the somite provides mitogenic stimuli to the NC cells. Second, NC proliferation is induced by factors released from the neural tube, and the somites play a modulatory role on the activity of these factors. In this case, the formation of a continuous area of contact between crest cells and the neural tube as a result of the graft of rostral mesoderm would provide greater accessibility of CNS-derived factors to the developing DRG. The modulation exerted by the rostral somite would therefore result from the permissiveness of this mesoderm to NC migration or to other, more specific interactions taking place between rostral somitic cells and NC cells upon neural tube stimulation.

In search of such putative factors, we have investigated the effects of neurotrophin 3 (NT-3) on the proliferation of NC cells. NT-3 is a 13.6-kDa basic polypeptide that is ñ50% identical to both nerve growth factor (NGF) and BDNF (20–22). NT-3 transcripts can be detected in many adult tissues such as brain, heart, skin, and muscle (20–23), suggesting that it may act as a target-derived factor for both central and peripheral projections. Indeed, NT-3 promotes the survival of neurons in peripheral dorsal root, nodose, and sympathetic ganglia (20–22). In this respect, NT-3 shows the broadest spectrum of activity on neurons, compared to NGF and BDNF (reviewed in ref. 24). Furthermore, NT-3 was shown to induce a mitogenic response in 3T3 fibroblasts through the tyrosine kinase receptors trk and trkB (25–27). In addition, NT-3 mRNA is abundant during early embryonic stages, suggesting that it plays a role in the formation of the nervous system.

In the present report we find that NT-3 is mitogenic to NC cells grown as homogeneous cultures and that its effect is significantly larger when migrating NC cells are grown together with somite cells. This finding suggests that during normal development the number of NC cells that populate the

Abbreviations: BBM, basic Brazeau medium; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNS, central nervous system; DRG, dorsal root ganglia; NC, neural crest; NGF, nerve growth factor; NT-3, neurotrophin 3; PBS, phosphate-buffered saline.

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DRG is regulated by interactions with CNS-derived factors and the somitic mesoderm.

MATERIALS AND METHODS

**Cells and Cell Cultures.** Culture substrates. Culture dishes were incubated with either laminin (L-2020; Sigma; 25 μg/ml) or fibronectin (F-4759; Sigma; 50 μg/ml) diluted in phosphate-buffered saline (PBS) for 2 hr, followed by washing twice with culture medium (8).

**Cultures of somites containing migrating NC cells.** The trunk region was excised from somite levels 15–20 of 25-somite stage quail (Coturnix coturnix japonica) embryos. These levels of the quail correspond to the migration of NC cells into the rostral portion of the corresponding somites and between adjacent segments (17). Tissues were transferred into 25% pancreatic in PBS for 5–10 min at room temperature until the somites containing NC cells detached from the neural tube, as described (8). Isolated somites were washed successively in PBS and Dulbecco’s modified Eagle’s medium (GIBCO) containing 10% newborn calf serum and finally pooled in serum-free basic Braeau medium (BBM). Somites were mechanically dissociated, and the equivalent of 8 somites was plated in a volume of 150 μl in 12-mm diameter circles made in the center of 35-mm wells. Purified recombinant human NT-3 was prepared as described elsewhere (22). The factor was added at the appropriate final concentrations to cell suspensions before seeding.

**Cultures of NC clusters.** Neural tubes were explanted from 15- to 20-somite stage quail embryos as described (28). NC clusters were isolated from explanted quail neural tubes at 45 hr. The clusters were pooled in BBM and cells were mechanically dissociated. The equivalent of 10 clusters (~2000 cells) was cultured in a final volume of 150 μl in BBM or BBM containing NT-3 in the center of laminin-coated dishes (see above).

**[^H]Thymidine Incorporation and Autoradiography.** Both types of NC cultures were incubated in the absence or presence of NT-3 for 24 hr. Cultures were then labeled by the addition of 1.25 μCi of [methyl-^3H]thymidine (specific activity, 50 Ci/mmol, 1 Ci = 37 GBq) or [3H]thymidine (specific activity, 1 Ci = 37 GBq) in Dulbecco’s modified Eagle’s medium per ml for 40 min. At the end of incubation, cultures were fixed with 4% formaldehyde in PBS. Cultures of NC cells prepared from clusters were directly processed for autoradiography (7), whereas neural crest/somite cultures were first stained with the HNK-1 antibody (see below).

**Immunofluorescence.** NC cells in NC/somite cultures were distinguished by immunostaining with the monoclonal antibody HNK-1 (29), followed by a goat anti-mouse second antibody coupled to fluorescein isothiocyanate, as described (7). All cultures, whether labeled or not with the HNK-1 antibody, were stained with the Hoechst nuclear stain (Serva; 1 μg/ml) for 15 min at room temperature, after development of the autoradiographs.

**Quantification and Data Analysis.** The proliferation of NC cells in crest/somite cultures was calculated as the percentage of HNK-1-positive cells with [^3H]thymidine grains over their nuclei (see Fig. 1 A and C). Somite cells were defined as all Hoechst-positive, HNK-1-negative cells in the somite/crest cultures (see Fig. 1). The proportion of the last type of cells bearing silver grains over nuclei was considered as the measure for somite proliferation. NC cells derived from clusters were visualized by Hoechst staining. Only cells with >10 silver grains per nucleus were considered as labeled.

Quantification of the data was performed with a Zeiss Axioscope microscope equipped with epifluorescence and phase optics. A total of 400–2500 cells of each category was counted in 40–140 microscopic fields per dish. Experiments were repeated at least three times in triplicate cultures for each experimental condition. Results are expressed either as the average number of cells per field (±SD of triplicate cultures) or as the average percentage of NC or somite cells with thymidine grains (±SD of triplicate cultures). Significance of the results was determined by using two-tailed, unpaired Student’s t test. Cultures were photographed on TMax film (Kodak) at 400 ASA.

**RESULTS**

**NT-3 Stimulates the Proliferation of NC Cells in Mixed NC/Somite Cultures.** Somites were excised from embryonic day 2 embryos at stages corresponding to the advanced migration of NC cells (17). As previously shown (8), NC cells were stained with the HNK-1 antibody, whereas the underlying mesodermal cells remained unstained (Fig. 1). HNK-1-immunoreactive cells were polymorphic in appearance. The number of phase-bright neurons with processes was extremely small in the present cultures.

Dissociated somites containing NC cells were grown on laminin substrates in the presence or absence of NT-3 for 1 day. At the end of this period, 2.5–7.5% of the HNK-1-positive NC cells grown in the absence of NT-3 incorporated [^3H]thymidine during a 40-min pulse, whereas 14–22% of the NC cells in NT-3-treated cultures incorporated the label (Figs. 2A and 3). Moreover, 24 hr after stimulation with NT-3 at 10 ng/ml, a significant increase in the number of HNK-1-immunoreactive NC cells was measured in the factor-treated as compared to control cultures (Fig. 2A and 2B).

The mitogenic effect of NT-3 on NC cells was observed whether cells were cultured on laminin or on fibronectin substrates (Fig. 3). In the absence of exogenous NT-3, the percentage of thymidine-incorporating NC cells of the total HNK-1-immunoreactive population was slightly higher in cultures grown on laminin as compared to fibronectin (7.5% ± 0.36% compared to 3.8% ± 1.4%, respectively). In the presence of the factor, a 2-fold increase in the number of thymidine-incorporating NC cells was already measured at an NT-3 concentration of 0.1 ng/ml in cultures grown on laminin-coated dishes, whereas a 10-fold increase in factor concentration was necessary to see a similar effect on NC cells grown on fibronectin (Fig. 3). These data suggest that laminin modulates the response of NC cells to NT-3.

The mitogenic effect of NT-3 was not restricted to NC cells. Fig. 2B shows that in the presence of NT-3 at 10 ng/ml a 3-fold increase in the percentage of somite cells incorporating thymidine and a 2-fold increase in somite cell density were observed. This experiment was repeated four times with thymidine incorporation values ranging between 1.4- and 5.7-fold over those of untreated controls.

**NT-3 Stimulates the Proliferation of NC Cells in Homogeneous Cultures.** Most NC cells developing from the dissociated clusters were polymorphic in appearance. Only a few neurons differentiated by 24 hr in BBM. To investigate whether NT-3 can directly stimulate NC cells to proliferate, the factor was added to freshly prepared cell suspensions at the time of seeding. Mitogenic activity was measured 1 day later. A moderate but significant increase (1.7-fold over control values)
in the number of cells incorporating thymidine was measured at an NT-3 concentration of 10 ng/ml, whereas a concentration of 0.1 ng/ml was ineffective and a concentration of 1.0 ng/ml was only slightly mitogenic (1.3-fold over control) (Fig. 4). Likewise, an increase of 2.2-fold in the number of cells per field was measured only for the high factor concentration, whereas the addition of the factor at 0.1 and 1.0 ng/ml had no effect on the field density (Fig. 4). This is in contrast to the results obtained in the NC/somite cultures where, at any concentration of factor tested (0.1–10 ng/ml), there was a stimulation in the number of NC cells counted by 24 hr of culture (data not shown and see also Fig. 2).

**Fig. 1.** Mixed NC/somite culture showing HNK-1 immunostaining of NC cells (A) combined with Hoechst nuclear staining (B) and autoradiography after [3H]thymidine labeling (C). Arrows point to the few HNK-1-positive cells that display thymidine grains over their nuclei. Arrowheads show two Hoechst-positive, HNK-1-negative somitic cells with thymidine grains. Note that in B the silver grains partially cover the Hoechst-stained nuclei. (Bar = 40 μm.)

**Fig. 2.** Effect of NT-3 on the incorporation of [3H]thymidine and cell number in 1-day-old NC/somite cultures. Cultures were grown for 1 day in the presence of NT-3. At the end of the incubation, they received a 40-min pulse of [3H]thymidine, followed by fixation, HNK-1 immunostaining, autoradiography, and Hoechst staining of nuclei after development of the autoradiographs. (A) NT-3 stimulates the proportion of HNK-1-immunoreactive NC cells that incorporate thymidine (thy+/total) (P < 0.005) and the total number of HNK-1-positive cells per field (P < 0.002) when compared to the respective untreated controls. (B) The number of thymidine-incorporating and total somite cells is also significantly augmented by NT-3 when compared to controls without added factor (P < 0.04 and P < 0.008, respectively). Values represent the means ± SD of triplicate cultures.

**Fig. 3.** Effect of NT-3 on the percentage of HNK-1-positive cells incorporating [3H]thymidine (thy+/total, %) in NC/somite cultures grown on laminin or fibronectin substrates. One-day-old control and NT-3 (0.1 and 1.0 ng/ml)-treated cultures were processed for immunofluorescence and autoradiography as described in Materials and Methods and in the legend to Fig. 1. All values represent the means ± SD of triplicate cultures.
are excised from the embryos and immediately exposed to the factor. In contrast, cluster-derived NC cells developed on explanted neural tubes for 45 hr before being transplanted to NT-3. Thus, cluster-derived cells may be much "older" than migratory cells at the time of treatment and consequently may be less responsive.

Our previous in vivo studies have demonstrated that, opposite a mesoderm composed exclusively of rostral half-somites, there is a stimulation in the total number of NC cells that incorporate thymidine on embryonic day 3-4, as well as in the total number of cells that populate the nascent ganglia (19). We then put forward the hypotheses that the rostral somite grafts are (i) mitogenic to the NC cells that migrate through this mesoderm or (ii) provide the NC cells with a greater accessibility to neural tube-derived mitogen(s). Three lines of experiments now sustain the second hypothesis.

First, the implantation of a foreign mesoderm, the lateral plate mesoderm, in place of the normal somites, results in a dramatic increase in early DRG growth, similar to the results obtained upon grafting the rostral somite halves (G. Gvirtzman, R. S. Goldstein, and C.K., unpublished results). Both the rostral somite and the lateral plate mesoderm support NC migration (30) and a consequent increase in DRG size. In contrast, other environments that inhibit NC penetration do not stimulate growth of the sensory ganglia (G. Gvirtzman, R. S. Goldstein, and C.K., unpublished results). These data suggest that the mitogenic effect on NC cells originally measured in multiple rostral somitic grafts may not be due to a rostral somite-derived mitogen. They stress, indeed, the importance of an environment permissive to NC migration in providing the early-formed DRG with the possibility to grow.

Second, cultured cells derived either from whole epithelial somites or from rostral or caudal somitic halves failed to stimulate the proliferation of NC cells grown on top of them (R. Goldstein, D. Naor, and C.K., unpublished results).

Third, the present results provide direct evidence that NT-3 stimulates NC cells to proliferate in vitro. Moreover, as the response of the NC cells to NT-3 is enhanced in mixed crest/somite cultures compared to homogeneous NC cells, the hypothesis that somite cells play a modulatory rather than a direct mitogenic role in NC proliferation is favored.

Somitic cells in mixed NC/somite cultures also proliferate in response to NT-3. This response is permissive to NT-3, suggesting that a molecule(s) derived from the NT-3-stimulated mesodermal cells could behave as a secondary signal to account, at least partially, for their modulatory activity on NC proliferation.

The biological activity of NT-3 that we detected at such young stages of embryonic development may be correlated with the observation that this factor is expressed very early and at high levels in the CNS compared with the other members of the neurotrophin family (23). To substantiate our findings, it will be necessary to demonstrate the expression of NT-3 at the mRNA and protein levels in the embryonic day 2-4 CNS primordium. The finding that NT-3 is also mitogenic to somite cells raises the possibility that this factor may act as a more general mitogen during early embryonic development and thereby may be found in additional embryonic structures, in line with its expression in several tissues in adult rodents (20-23).

The mitogenic and neurotrophic effects of NT-3 on NC cells and on peripheral neurons may be additionally regulated by specific basement membrane glycoproteins, such as laminin, as was shown for NGF and BDNF (6, 31, 32). This assumption is based on the observation that low concentrations of NT-3 are sufficient to elicit an increase in the number of thymidine-incorporating cells in NC cells cultured on laminin, whereas a much higher concentration of factor is necessary to obtain a similar effect on NC cells grown on fibronectin (see Fig. 3). The presence of NT-3 does not affect...
the attachment of cells to either substrate, as measured 3 hr after plating (C.C. and C.K., unpublished results). However, differences in the threshold of the response to NT-3 of NC cells grown on either substrate might somehow be related to the fact that the basal level of cell attachment is higher on fibronectin than on laminin (D. Naor and C.K., unpublished observations).

The present results demonstrate that NT-3 is a mitogen for trunk NC cells. They do not exclude, however, the possibility that the survival of NC cells, whether mitotically active or not, is also affected by this factor. In previous experiments, we have demonstrated that bFGF, another CNS-derived molecule, is a survival factor for a subpopulation of NC-derived cells. This conclusion is based on the finding that the total cell number, but not the number of thymidine-incorporating NC cells, is enhanced upon bFGF treatment (7).

The effects of NT-3 on survival and neurite outgrowth from DRG, sympathetic ganglia, and nodose neurons, as well as its mitogenic activity on their progenitor cells, suggest that this factor can act on cells at different times of development. The nature of the response (proliferation, differentiation, etc.) will then depend on the state of commitment of the responding cells at the time of testing and probably also on the way the factor is presented to the cells. The ability of a single factor to elicit multiple types of responses has also been reported for NGF, a survival factor for NC-derived neurons [see the review by Barde (24)] and a mitogen for cultured chromaffin cells (33) and for neuronal stem cells of embryonic rat striatum (34). Furthermore, bFGF, a known mitogen for many cell types (35, 36), is also a survival-promoting molecule for some peripheral and central neurons (37–39).

A property common to BDNF and NT-3 is that both factors are ligands for the trkB tyrosine kinase receptor, whereas NGF is not (40, 41). The possibility is then raised that the early responsiveness acquired by NC cells to NT-3 (the present data) and to BDNF (6, 8), before responding to NGF (5, 42), is correlated with the temporal sequence of expression of these specific high affinity receptors by NC progenitors.

The mitotic response of NC cells to NT-3 in vitro may reflect the potential mitogenic role of NT-3 on precursor cells of the peripheral ganglia in the embryo. Further studies are needed to elucidate the patterns of expression of this factor to the early embryos, its specificity of action towards NC cells, and the possible modulation exerted by the somitic mesoderm on the response of NC cells to NT-3.

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