BHk-21-derived cell lines that produce basic fibroblast growth factor, but not parental BHK-21 cells, initiate neuronal differentiation of neural crest progenitors

Gilat Brill1, Nora Vaisman2, Gera Neufeld2 and Chaya Kalcheim1,*

1Department of Anatomy and Embryology, The Hebrew University-Hadassah Medical School, Jerusalem 97010 - POB 1172, Israel
2Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel

*Author for correspondence

Summary

We present evidence that basic fibroblast growth factor (bFGF)-producing cells stimulate primary differentiation of neurons from neural crest progenitors. Baby hamster kidney (BHK-21) cells were stably cotransfected with plasmid pSV2/neo, which contains the gene conferring resistance to the neomycin analog G418 and expression vectors containing the human bFGF cDNA. Various clones, which differed in their bFGF production levels, were isolated. Homogeneous neural crest cells were cultured on monolayers of bFGF-producing, BHK-21-derived cell lines. While the parental BHK-21 cells, which do not produce detectable bFGF, had poor neurogenic ability, the various bFGF-producing clones promoted a 1.5- to 4-fold increase in neuronal cell number compared to the parental cells. This increase was correlated with the levels of bFGF produced by the different transfected clones, which ranged between 2.3 and 140 ng/mg protein. In contrast, no stimulation of neuronal differentiation was observed when neural crest cells were grown on monolayers of parental BHK cells transfected with plasmid pSV2/neo alone, or on a parental BHK-derived clone, which secretes high amounts of recombinant vascular endothelial growth factor (VEGF). Furthermore, the neuron-promoting ability of bFGF-producing cells could be mimicked by addition of exogenous bFGF to neural crest cells grown on the parental BHK line. A similar treatment of neural crest cells grown on laminin substrata, instead of BHK cells, resulted in increased survival of non-neuronal cells, but not of neurons (see also Kalcheim, C. 1989, Dev. Biol. 134, 1-10). Taken together, these results suggest that bFGF stimulates neuronal differentiation of neural crest cells by a cell-mediated signalling mechanism.

Key words: baby hamster kidney cells, dorsal root ganglia, peripheral nervous system, sensory neurons, sympathetic ganglia, trophic factors, vascular endothelial growth factor.

Introduction

Research in the past few years on the ontogeny of the neural crest has led to the notion that this embryonic structure is composed both of multipotent progenitor cells as well as of cell subpopulations with restricted developmental capacity. Local environmental cues, encountered by the crest cells during separation from the neural tube, migration and gangliogenesis, may play an important role in determining the fate of the multipotent neural crest cells, as well as in initiating differentiation of the committed progenitors (see Anderson, 1989; Le Douarin, 1990; Weston, 1991, for reviews).

The environment in which neural crest-derived dorsal root ganglia (DRG) develop is composed of the neural tube and the adjacent somitic mesoderm. It is already well established that the neural tube is required for regulating the early stages of neural crest development into DRG (Teillet and Le Douarin, 1983; Le Douarin, 1984; Kalcheim and Le Douarin, 1986). The effect of the central nervous system (CNS) primordium appears to be mediated by factors produced at the appropriate developmental time. Three such factors have been already identified: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and basic fibroblast growth factor (bFGF).

BDNF (Barde et al., 1982 and see also Barde, 1989 for review) has been shown to promote the survival of neural crest cells in vivo (Kalcheim et al., 1987), and their differentiation into Substance-P immunoreactive neurons in culture (Kalcheim and Gendreau, 1988). More recently, it has been shown that BDNF, but not nerve growth factor (NGF), influences the commitment of pluripotent neural crest cells to develop along the sensory lineage (Sieber-Blum, 1991). NT-3, a homologue of BDNF and NGF (Hohn et al., 1990; Maisonpierre et al., 1990b; Rosenthal et al., 1990), is expressed at high levels in the early CNS (Maisonpierre et
al., 1990a) and is a mitogen for cultured neural crest cells (Kalcheim et al., 1992).

The third molecule, bFGF, is synthesized in a variety of tissues, including the brain. bFGF controls both the proliferation and the differentiation of a wide range of mesodermal and neuroectodermal cells (for reviews see Rifkin and Moscatelli, 1989; Gospodarowicz, 1990). The role of bFGF in maintaining the differentiated state of neurons is substantiated by the findings that it promotes survival and neurite outgrowth from cultured neurons of various CNS regions (Morrison et al., 1986; Unsicker et al., 1987; Hatten et al., 1988; Walicke, 1988), and from peripheral neurons of the ciliary ganglion (Unsicker et al., 1987).

bFGF has also been implicated in the early stages of nervous system development. Two cell lines have been utilized to demonstrate the neurogenic effect of bFGF. The first are the neural crest-derived rat pheochromocytoma (PC12) cells, which stop dividing and begin differentiating upon treatment with bFGF (Togari et al., 1985; Wagner and D’Amore, 1986; Neufeld et al., 1987; Rogelj et al., 1989). The second example refers to sympathoadrenal precursors isolated from E14.5 rat adrenal glands, which have been immortalized with a recombinant murine retrovirus containing the v-myc oncogene (Birren and Anderson, 1990). These immortalized bipotential precursors cells can be driven to differentiate into neurons by treatment with bFGF (Birren and Anderson, 1990).

Little information is, however, available concerning the possible effects of this growth factor on primary differentiation of neural crest progenitors. It has been recently suggested by Stocker et al. (1991) that bFGF influences commitment to melanogenesis of a bipotent precursor of the Schwann cell-melanocyte lineage still present in E7 quail DRG and peripheral nerves. We have demonstrated that neural crest-derived cells of the E3 chick DRG anlagen, which were experimentally separated from the neural tube, can be temporarily rescued by treatment with bFGF (Kalcheim, 1989). Furthermore, in culture, this growth factor promotes the survival, but not the proliferation, of a subpopulation of neural crest cells with non-neuronal morphology that expresses the HNK-1 epitope (Kalcheim, 1989). In line with this effect, biologically active bFGF is expressed in avian neural tubes as early as day 3 of embryonic development (E3) (Kalcheim and Neufeld, 1990). Moreover, a subpopulation of avian migrating neural crest cells expresses transcripts of a bFGF receptor in a transient manner (Heuer et al., 1990).

The above-mentioned studies suggest that exogenous bFGF influences differentiation of various neural crest-derived cells. However, it is not clear whether soluble bFGF is present at all in vivo, in those areas containing neural crest precursor cells. This is partly because the native bFGF lacks a signal sequence for secretion (Abraham et al., 1986). In spite of this fact, the factor is released from cells by an unknown mechanism, as it has been shown that bFGF is deposited into the extracellular matrix (ECM) produced by endothelial cells in vivo and in vitro (Baird and Ling, 1987; Vlodavsky et al., 1987; Cordon-Cardo et al., 1990). Furthermore, complexes of bFGF with a heparan sulfate proteoglycan can be released into the extracellular milieu and such complexes are detected on cell surfaces (Saksela and Rifkin, 1990; Bashkin et al., 1991; Brunner et al., 1991). Other studies have demonstrated that the \(18 \times 10^3 \ M_r\) form of bFGF is localized to intracellular domains such as the cytoplasm of muscle cells and young neurons (Joseph-Silverstein et al., 1989; Kalcheim and Neufeld, 1990). Furthermore, various groups provided evidence that bFGF can be translocated into the nucleus of various bFGF-producing cells (Bouche et al., 1987; Baldin et al., 1990; Tessler and Neufeld, 1990).

It is therefore conceivable that bFGF is compartmentalized in distinct intracellular domains, as well as in the ECM, and that bFGF derived from each of these domains exerts different biological functions on responsive cells. Such a model could be proposed for the development of subpopulations of neural crest cells that migrate on specific ECM substrata, and closely interact with the neural tube and/or somite cells during migration and organogenesis (Le Douarin, 1982; Teillet et al., 1987; Perris et al., 1989). To begin testing such a hypothesis, we have established a coculture system of pure neural crest cells with various baby hamster kidney (BHK) cell lines that have been transfected with expression vectors containing the cDNA of the human bFGF gene, and investigated the effects of these cells on neural crest differentiation. The advantage of these cocultures over previous experimental paradigms, in which the effect of soluble factor was tested, is that this system may better resemble natural intercellular interactions by offering the possibility of responding to the factor via cell-cell and cell-matrix contacts.

In the present work, we report that bFGF-producing cells initiate the differentiation of neural crest cells into neurons. This is in addition to the previously reported effect of exogenous bFGF on survival of neural crest-derived, non-neuronal cells (Kalcheim, 1989). Moreover, this neurogenic effect can be reproduced by addition of exogenous factor to crest cells growing on BHK cells devoid of intrinsic bFGF activity, but not on laminin substrata alone. This novel effect of bFGF-producing cells on the survival and/or neuronal differentiation of neural crest cells may be mediated by a factor that associates with the cell membrane or with the ECM.

Materials and methods

Plasmids and cell lines

The construction of pbFGF has been described in detail elsewhere (Neufeld et al., 1988). This plasmid contains the cDNA encoding human bFGF under the control of the metalotionein IIA promoter and the SV40-derived enhancer. This plasmid was used to transfect the parental BHK-21 cells from which clones B19 and B14 were derived. To limit the amounts of the bFGF produced in transfected cells, we have also used plasmid pbFGF-SV which was derived from pbFGF by deletion of the SV40-derived sequences using digestion with HindIII. This plasmid was introduced into the BHK-21-derived cell line B50. Plasmid pbsFGF was kindly provided by Dr J. Abraham (California Biotechnology Corp., Sunnyvale, USA), and its construction was described in detail elsewhere (Blam et al., 1988). This plasmid resembles pbFGF except that a growth hormone-derived signal sequence for secretion was fused to the 5′ end of the bFGF cDNA. This plasmid was introduced into BHK-21 cells to produce the Bsp cell line. The con-
tubes at 45 hours, as previously described (Loring et al., 1981). The clusters were pooled in SFRI and seeded in a final volume of 150 µl SFRI medium onto the BHK cells. Crest clusters were cultured either as whole explants (5 per well) or after mechanical dissociation with the aid of glass micropipettes with a tip opening of 30-50 µm. In the case of dissociated neural crest cells, each well received the equivalent of 10 clusters (approximately 2000 cells). In some experiments, the effect of exogenous bFGF on neural crest cells grown on the parental BHK cell line was tested. To this end, recombinant human bFGF (0.1 and 1 ng/ml, Takeda Inc., Osaka, Japan) was added at the time of addition of the neural crest cells to the BHK lines. Cocultures were fixed one day later and processed for neuronal quantification as described below.

Transfection of BHK-21 cells with bFGF expression vectors

BHK-21 cells were co-transfected using the CaPO4 co-precipitation method with the plasmid pSV2/neo, and either pbFGF or pbFGF-SV or pbFGF as described (Neufeld et al., 1988; Tesseler and Neufeld, 1990). To determine the amount of bioactive bFGF produced by the transfected clones of cells, 0.5 ml of PBS containing 0.1 mM phenylmethyl-sulfonyl fluoride was added to a 6 cm dish containing confluent cells (5 × 10^6 cells). Cells were scraped using a rubber policeman, transferred to a sterile glass tube chilled on ice, and sonicated at 4°C for 90 seconds in a model G112SP1 bath sonicator. The sonicated sample was centrifuged at 12,000 g for 15 minutes. Aliquots of supernatant containing predetermined amounts of protein were assayed for the presence of bFGF as described below.

Determination of bFGF in cell extracts

Cultures of bovine aortic arch-derived endothelial cells were established and maintained as previously described (Schweigerer et al., 1987; Vlodavsky et al., 1980). Endothelial cells were seeded in 24-well cluster plates (5000 cells/well). Samples (10 µl), which contained increasing concentrations of cell extracts or of pure recombinant human bFGF, were added to the cells every other day. The amount of protein in the samples was determined using the Bio-Rad quick protein assay. Cell numbers were determined in a coulter counter after 5 days in culture. The concentration of bFGF in the cell extracts was determined by comparison to log-dose curves of pure bFGF. Using this method, it was determined that clones B50, B14 and B19 produce 2.3, 14 and 140 ng of bFGF per mg protein of cell extract. No bFGF (or acidic FGF) could be detected by this method in cell extracts derived from parental BHK-21 cells. These results were confirmed using a radioimmunoassay for bFGF which was performed as described by Schweigerer et al. (1987).

Cell cultures

Cultures of BHK cells

Parental and transfected BHK cells were seeded at a density of 0.5 × 10^6 or 1 × 10^6 cells/ml in a 12 mm circle made in the center of untreated 35 mm culture dishes (Nunc). Culture medium consisted of a mixture of F-12 and Dulbecco's modified Eagle Medium (DMEM) (1:1) supplemented with 5% special newborn calf serum, L-glutamine (2 mM), and a mixture of penicillin, streptomycin and amphotericin at final concentrations of 100 units/ml, 10 µg/ml and 2.5 µg/ml, respectively (all tissue culture products were purchased from Biological Industries Inc., Kibutz Bet Haemek). Cultures were incubated for two days prior to the addition of neural crest cells. Before cocultures were established, BHK cells were washed three times with serum-free medium (SFRI Laboratoire, Berganton, France).

Cultures of neural crest clusters

Neural crest clusters were isolated from explanted quail neural tubes at 45 hours, as previously described (Loring et al., 1981). The clusters were pooled in SFRI and seeded in a final volume of 150 µl SFRI medium onto the BHK cells. Crest clusters were cultured either as whole explants (5 per well) or after mechanical dissociation with the aid of glass micropipettes with a tip opening of 30-50 µm. In the case of dissociated neural crest cells, each well received the equivalent of 10 clusters (approximately 2000 cells). In some experiments, the effect of exogenous bFGF on neural crest cells grown on the parental BHK cell line was tested. To this end, recombinant human bFGF (0.1 and 1 ng/ml, Takeda Inc., Osaka, Japan) was added at the time of addition of the neural crest cells to the BHK lines. Cocultures were fixed one day later and processed for neuronal quantification as described below.

[^H]thymidine incorporation and autoradiography

Cocultures of neural crest cells with the BHK-parental or the BHK-50 cell lines were labeled by the addition of 1.25 µCi/ml [^H]methyl thymidine (specific activity, 40-60 Ci/m mole; Amer sham) for one day, starting at the time of addition of dissociated neural crest cells to the 2-day-old BHK cultures. At the end of incubation, cultures were fixed with Bouin’s fluid, stained with the HNK-1 antibody (see below) and processed for autoradiography as described elsewhere (Kalcheim, 1989).

Fixation and immunofluorescence

1- or 2-day-old cultures were fixed in Bouin’s fluid for 10 minutes, washed in phosphate-buffered saline (PBS), pH 7.3, and subjected to immunocytchemical labeling. Neural crest cells and their derivatives in neural crest-BHK cocultures were distinguished by immunostaining with the monoclonal antibody HNK-1 (Abo and Balch, 1981), followed by a goat anti-mouse second antibody coupled to fluorescein isothiocyanate, as previously described (Teillet et al., 1987). As seen in Fig. 3, the HNK-1 epitope is expressed exclusively by the neural crest cells and not by the BHK cells of the underlayer.

Quantification and data analysis

Three morphologically distinct cell types expressing the HNK-1 epitope were separately considered in the cocultures, as follows: differentiating neurons in general had round and phase-bright cell bodies with one or more processes with a length of at least three cell diameters. The neuronal identity of these cells was confirmed by positive immunostaining with antibodies against the 200 × 10^3 Mr form of neurofilament proteins (Amersham), and with the A2B5 monoclonal antibody that recognizes a neuronal ganglioside (Vogel and Weston, 1990) (data not shown). A subpopulation of neuroblasts was characterized by their round appearance. Neuroblasts were either devoid of neurites or had a short process whose length did not exceed two cell diameters. Like differentiated neurons, only about 10% of these progenitors incorporated labeled thymidine into their nuclei, suggesting that this subpopulation is composed mostly of post-mitotic, committed cells (data not shown). The remaining neural crest-derived cells in the cultures had polymeric appearance and were generally designated as non-neuronal cells.

Quantification of the number of neuroblasts, neurons and non-neuronal cells in all the types of cocultures was performed using a Zeiss Axioscope microscope equipped with epiphilourescence and phase optics. A total of 350-1050 cells of each category was counted in 40-140 microscopic fields per dish. Experiments were repeated at least twice, in duplicate or triplicate cultures, for each experimental condition.

The proliferation of neural crest cells in crest-BHK cocultures was calculated as the percentage of HNK-1-positive cells with [^H]thymidine grains over their nuclei. Only cells with more than 10 silver grains per nucleus were considered as labeled.

Results are expressed either as the average number of cells per microscopic field (±s.d. of duplicate or triplicate cultures), or as the average percentage of neural crest cells with thymidine grains (±s.d. of duplicate cultures). Cultures were photographed on Tmax film (Kodak) at 400 ASA.
Results

bFGF-producing cells enhance survival and initiate neuronal differentiation of neural crest cells

Cocultures of neural crest with BHK cells expressing varying amounts of bFGF were used as a model system for analyzing the effects of this factor on neural crest differentiation. 45 hour clusters containing only neural crest cells (Loring et al., 1981) were added on top of 2-day-old BHK cultures, and observed one or two days later. As early as one day after this association was performed, 67-87.5% of the clusters growing on BHK cells that were transfected with either the native bFGF cDNA (B50, B19) or with the cDNA, to which a signal peptide sequence has been fused (Bsp), revealed the presence of an intense neuronal outgrowth from the central explant (Table 1 and Fig. 1B). In addition, other neurons migrated away from the cluster and extended long processes, which organized into neuronal networks, as shown in Fig. 1B. In contrast, clusters grown on top of the parental BHK line producing no detectable bFGF, revealed the presence of many pycnotic cells, and only a few round cells devoid of neurite-like processes or with very short unipolar or bipolar neurites were evident (Fig. 1A). Quantification of these explants revealed that none of the seven clusters examined showed any significant neurite outgrowth one day after coculture, and only one cluster of the seven explants (14%) showed neurite outgrowth on day two (Table 1).

Table 1. The effect of bFGF-producing cells on neuronal survival and differentiation in cultures of neural crest clusters

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clusters with neurite outgrowth (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 1</td>
</tr>
<tr>
<td>BHK-parental</td>
<td>0.0 (n=7)</td>
</tr>
<tr>
<td>BHK-B50</td>
<td>87.5 (n=8)</td>
</tr>
<tr>
<td>BHK-B19</td>
<td>66.7 (n=9)</td>
</tr>
<tr>
<td>BHK/pbsFGF</td>
<td>71.4 (n=7)</td>
</tr>
</tbody>
</table>

BHK cells were cultured for two days prior to the addition of the neural crest. Clusters of neural crest cells were excised at 48 hours from cultured neural tubes as described in Materials and Methods, and tested for neurite outgrowth one and two days after coculture with the BHK cells. Only explants with extensive neuronal outgrowth were considered as positive (compare panels A and B in Fig. 1).

n, total number of clusters scored for each treatment.

In contrast to the stimulation in neuronal differentiation observed after 24 hours in all the cocultures that contained
bFGF-producing cell lines, after two days in coculture only the B50 cell line, a low bFGF producer (2.3 ng bFGF/mg protein), revealed a durable neuronal phenotype (85.7%). However, most clusters growing on either the B19 (140 ng bFGF/mg protein) or the Bsp lines showed significant degeneration, and the number of such explants exhibiting neuritic outgrowth declined to control levels (Table 1).

Cultures of dissociated neural crest cells derived from 2-day-old clusters, and grown on top of different bFGF-producing lines, were used as a model system to quantify the effects of cell-derived bFGF on the survival and differentiation of distinct subsets of neural crest cells. Neural crest-derived cells expressed the HNK-1 epitope and were distinguished as neurons, neuroblasts or non-neuronal cells (Fig. 2). Neural crest cells growing for one day on the parental BHK line were mainly polymorphic in appearance. These cultures also contained round, neuroblast-like cells sometimes bearing short processes, but only few had neurons (Figs 3A, 4). A 2- to 4-fold increase in the number of neurons per field was obtained in the cocultures of neural crest cells with the various transfected lines, as compared to the control BHK cells (Figs 3, 4A). The B50 cell line, producing relatively low levels of bFGF, specifically stimulated neuronal survival and differentiation, without affecting the number of cells with non-neuronal morphology (Fig. 4). The increase in neurons in these cocultures was accompanied by a 20% average decrease in the number of neuroblasts compared to the parental line (not shown). The other lines tested, which produce higher bFGF levels, enhanced both the number of neurons as well as of non-neuronal cells (Fig. 4B), in agreement with our previous findings (Kalcheim, 1989).

Exogenously added bFGF mimics the neurogenic effect of bFGF-producing cells

The ability of exogenous bFGF to promote the survival and differentiation of neural crest cells into neurons was tested. Recombinant factor was added to neural crest cells growing on a monolayer of parental BHK-21 cells, which do not produce any detectable levels of bFGF (Neufeld et al., 1988) and have a very low neurogenic potential (see Figs 1, 2 and 3). As shown in Fig. 5, a concentration of 0.1 ng/ml bFGF was without effect, but 1 ng/ml bFGF produced a 50% increase in the number of neurons developing in the cocultures, compared to untreated cocultures of neural crest growing on the parental line (Fig. 5, first bar). This experiment was repeated three times with similar results.

In contrast, bFGF (1 ng/ml) added to pure neural crest cells grown on laminin did not significantly enhance the number of neurons when compared to the untreated cells (data not shown, and see also Kalcheim, 1989). Interestingly, the percentage of neurons of the total neural crest population cultured on laminin substrata was extremely low after one day of culture, and it ranged between 1.5 and 2.3% in untreated and bFGF-treated cells, respectively. This proportion increased to 7.5-10% when neural crest cells were cultured on the parental BHK line, and up to a maximum
Fig. 3. The effect of bFGF-producing cell lines on dissociated neural crest cells. 2-day-old dissociated neural crest clusters were cultured on top of a monolayer of (A,B) parental BHK cells, or (C,D) B14 cells. At the end of 24 hours of incubation in serum-free SFRI medium, cocultures were fixed and stained with the HNK-1 antibody as described in Materials and methods. Many crest-derived neurons with long processes (arrows) in addition to non-neuronal cells are seen on the B14 cell line (C). In contrast, fewer neural crest-derived cells with non-neuronal morphology can be observed on the parental line (A). (B and D) Phase contrast. Bar, 50 µm.
of 30% when grown on the various bFGF-producing lines. These results suggest that cell-cell contacts more efficiently mediate both the basal and the bFGF-induced neuronal differentiation of neural crest cells.

We have tested additional BHK-derived cell lines for their ability to stimulate neuronal differentiation of crest cells. The Bneo cells, which contain the pSV2/neo plasmid (Southern and Berg, 1982) and produce no detectable bFGF, were tested to control for the possible changes

Fig. 4. Quantification of the effect of bFGF-producing lines on the survival and/or differentiation of neurons and non-neuronal cells from neural crest clusters. Results were collected from 1-day-old cocultures of dissociated neural crest cells growing on the parental BHK line (Bpar), or on various lines of BHK cells that were transfected with native bFGF cDNA (B50, B14, and B19), or with the cDNA coding for bFGF to which a signal peptide sequence for secretion has been fused (Bsp). Quantification of the number of HNK-1 immunoreactive cells with neuronal or non-neuronal morphology was performed as described in Materials and methods. Results represent the average ± s.d. of triplicate cultures.

Fig. 5. Soluble bFGF can mimic the neurotrophic effect of bFGF-producing lines when added to neural crest cells cultured on top of parental BHK cells. Bar graph representing the number of neurons per microscopic field which develop after one day in the cocultures. Bpar, no bFGF; bFGF (0.1) and (1.0), recombinant human bFGF at 0.1 and 1.0 ng/ml, respectively. Results represent the average ± s.d. of triplicate cultures.

Fig. 6. The neurogenic effect of the transfected lines is specific for bFGF-expressing cells. Bar graph representing the number of HNK-1-immunoreactive neurons per microscopic field that develop after one day in the cocultures. Neural crest cells growing on the parental BHK line (Bpar); on the bFGF-expressing B14 line (B14); on the Bneo line (transfected with the pSV2/neo vector carrying resistance to neomycin, Bneo); and on a BHK line expressing VEGF (Vegf). Results represent the average ± s.d. of duplicate cultures.
caused to the BHK cells upon transfection. A second specificity control was the BHK cell line expressing vascular endothelial growth factor (VEGF) (Peretz et al., 1992) instead of bFGF. None of these lines stimulated the survival and differentiation of neural crest progenitors into neurons (Fig. 6) or non-neuronal cells (not shown) above control values. In the same experiment, the B14 line, producing 14 ng bFGF/mg protein, promoted a 3.0-fold increase in the amount of neuronal cells over the respective value in the parental line. These results suggest that the neurogenic effect observed with the bFGF-producing cells is specifically due to the expression of bFGF protein by the active cells.

Neural crest cells responsive to the bFGF-producing cells are postmitotic precursors

2-day-old dissociated clusters were cultured for 24 hours on top of parental BHK cells or on a monolayer of transfected B50 cells in the presence of [3H]thymidine. HNK-1 immunoreactive neurons and non-neuronal cells with or without thymidine grains over nuclei were scored in the control and transfected cultures. In this experiment, the total number of neurons increased by 2.4-fold (0.43±0.17 and 0.18±0.04 neurons/field, respectively) in cocultures on the B50 cells compared to the parental BHK line. However, the great majority of progenitors were already postmitotic at the time of explantation on the BHK lines because an average of 97% and 93.5% of the neurons growing on top of the parental or the B50 cells, respectively, did not incorporate thymidine into nuclei after a one-day pulse. These results suggest that bFGF initiates neuronal differentiation of already committed neural crest precursors.

Discussion

We present evidence that several BHK lines, which produce different amounts of bFGF, stimulate associated neural crest cells to survive and/or differentiate into neurons. This effect is specific to the bFGF-producing cells because neither the parental line, nor vector-transfected cells, nor BHK cells expressing an irrelevant factor had any detectable influence on neuronal development. Moreover, the addition of soluble bFGF to neural crest cells grown on the parental BHK line, but not on laminin, also stimulated the appearance of morphologically distinct neurons. Altogether, these results suggest that the neurogenic effect observed is mediated by bFGF.

A certain relationship was observed between the bioactivity of the different factor-producing lines and the level of bFGF production. For example, the B50 cell line, producing low amounts of bFGF (2.3 ng/mg protein) had a moderate effect on neuronal number, but no effect at all on the number of non-neuronal cells (Fig. 4). The other lines had a stimulatory effect on both neurons and non-neuronal cells, with a maximal activity on neuronal differentiation of the B14 cell line that produces 14 ng bFGF/mg protein. These results indicate that survival of non-neuronal cells and neuronal progenitors may be regulated by different mechanisms, both activated by bFGF. These results also suggest that low factor concentrations are sufficient to elicit neuronal differentiation, but that there is an optimal concentration of factor above which neuronal development declines in this system. In agreement with the results of the dissociated neural crest cells, it was also observed that, whereas the effect of low bFGF-producer lines on whole clusters was stable over time (as extensive neurite outgrowth was observed after one as well as two days of coculture), the neurotropic effect of high factor producers, such as the B19 and the Bsp lines, significantly declined with the age of the cocultures (Table 1). Cytotoxic effects of high concentrations of bFGF were already reported for other systems. For example, the proliferation of endothelial cells is progressively stimulated in response to increasing concentrations of bFGF and reaches a maximum at a factor concentration of 5 ng/ml. Exposure to higher factor concentrations results in an increasing inhibitory effect (Gospodarowicz et al., 1986).

Previous work from our laboratory has shown that treatment of neural crest cells cultured on a laminin substratum with exogenous bFGF promoted an increase in the survival, but not in the proliferation, of crest-derived cells with non-neuronal morphology (Kalcheim, 1989). In agreement with these observations, we find that coculturing neural crest cells with bFGF-producing cells such as the B14, B19 and Bsp lines, causes an increase in the number of neural crest-derived, non-neuronal cells expressing the HNK-1 epitope (Fig. 4). The state of commitment of this cell population generically termed “non-neuronal” is unclear. At least a part of this non-neuronal cell subpopulation may be composed of multipotent precursor cells, as 20-40% of them retain mitogenic capacity after one day of culture on laminin or on any line of BHK cells tested (see Kalcheim, 1989, and unpublished data). This cell subset may also contain neuronal precursors able to differentiate into neurons under appropriate conditions (see below).

In addition to their effect on crest-derived, non-neuronal cells, all the lines producing bFGF that were tested in the present study stimulated the survival and/or morphological differentiation of neurons. Moreover, soluble bFGF added to crest cells growing on the parental BHK line had a significant neurogenic effect (Fig. 5). This is in contrast with the lack of effect of soluble factor on neuronal differentiation of crest cells cultured on laminin (unpublished observations and see also Kalcheim, 1989). Two possibilities could account for the differences observed. The first possibility is that the conditions used in the early experiments (laminin substratum and serum-free Basic Brazeau medium) are not compatible with the survival of neuronal progenitors. As a consequence of that, bFGF had no effect on neuronal development. This possibility seems unlikely because the addition of BDNF to neural crest cells grown under similar culture conditions stimulated neuronal differentiation (Kalcheim and Gendreau, 1988).

The second possibility is that neuronal precursors are equally present in both experimental systems. However, neuronal differentiation from neural crest cells requires proper presentation of bFGF to the responding cells. According to this view, the neurogenic effect of the bFGF-producing cell lines may not merely be mediated by soluble factor released from these cells. In support of this possibility is the observation that bFGF could not be detected
in the medium fraction of the biologically active, transfected lines (Neufeld et al., 1988). Therefore, the observation that exogenous bFGF promotes neuronal differentiation of neural crest cells growing on BHK cells, but not on laminin, suggests that these cells provide a better spatial substratum than purified laminin. Effective presentation by the BHK lines of either BHK-derived factor, or exogenously added bFGF could be achieved in several ways, such as binding to cell surface heparin-like molecules (Neufeld et al., 1987; Bashkin et al., 1991; Brunner et al., 1991; Yayon et al., 1991), or association with specific ECM components secreted by the cell lines (Baird and Ling, 1987; Neufeld et al., 1987; Rogelj et al., 1989; Vlodavsky et al., 1987, 1991).

In addition, the possibility should be considered that different mechanisms mediate the response of distinct cell subsets to the same growth factor. For example, the survival of non-neuronal cells could be directly controlled by binding of factor to specific receptors on the neural crest cells, whereas neuronal differentiation might involve the activity of secondary molecules produced by the BHK cells upon stimulation with bFGF. For example, a transmembrane signal could be activated in the BHK cells either upon binding of the factor to its receptors on the BHK cells (Neufeld and Gospodarowicz, 1985), or due to the intracellular accumulation of factor within the producing cells (Neufeld et al., 1988). Along this line, it has been recently proposed that transmembrane signalling mediated by N-CAM and N-Cadherin can induce transcription-independent neuronal differentiation of PC12 cells (Doherty et al., 1991).

The nature of the response of different neural crest subsets to bFGF may also depend on the state of commitment of the responding cells. We find that about 95% of the neurons present in the BHK-neural crest cocultures have differentiated from postmitotic precursors, and they are evident in the cocultures as early as one day after seeding. These data suggest that this subpopulation is restricted in its developmental potential to give rise to cells with neuronal traits such as a characteristic bipolar appearance of neurites (see Figs 2 and 3), and neurofilament and A2B5 immunoreactivities (unpublished data). The role of bFGF-producing cells would then be a permissive one, i.e. that of initiating the expression of a neuron-specific developmental repertoire. The present report is the first to address the role of bFGF on primary neuronal differentiation of neural crest cells. We are now attempting to characterize the phenotypic identity of these early neuronal cells. Others have reported an inductive effect of bFGF on neural crest-derived cells of the sympathetic/adrenal lineage present in rat adrenal glands. These bipotential precursors were immortalized with a murine retrovirus containing the v-myc oncogene and were driven to differentiate along the neuronal pathway upon treatment with bFGF, but not NGF (Birren and Anderson, 1990). Similarly, PC12 cells can be induced to differentiate as neurons if stimulated with either bFGF or with NGF (Greene and Tischler, 1982; Togari et al., 1985; Wagner and D’Amore, 1986; Neufeld et al., 1987; Rogelj et al., 1989).

It is interesting to mention that bFGF-stimulated neuronal differentiation in the present system takes place from neural crest cells that developed in association with neural tubes for 48 hours. This observation shows that neurogenic precursors are able to survive in relatively “old” neural crest clusters and differentiate into neurons when given the appropriate signal. This view is in apparent contrast with the observations of Vogel and Weston (1988). According to the concept proposed by these authors, the neurogenic potential of some neural crest cells is only transient, as neurons can develop in cultures derived from 1-day-old clusters, but not from older ones such as those used in the present experiments. A possible explanation for this discrepancy resides in the different culture conditions used. Whereas Vogel and Weston (1988) used a medium highly enriched in embryo extract and serum, we used a serum-free preparation. The first of the two conditions may be progressively detrimental for progenitor cells with neurogenic potential. In support of this possibility, previous works have demonstrated that culturing neural crest cells in serum-free medium is compatible with the differentiation of Substance P-expressing neurons, a subpopulation that derives from early committed precursors within the neural crest (Ziller et al., 1987). However, culturing crest cells in serum- and chick embryo extract-containing medium promotes cell proliferation, which is a prerequisite for the differentiation of melanocytes and adrenergic cells developing under these conditions, but causes the progressive and irreversible loss of progenitors with the capacity to give rise to Substance P-expressing neurons (Ziller et al., 1983, 1987). If this analogy is correct, then subjecting 2-day-old clusters to each of the above culture conditions should provide a test for the idea of the existence of the neural crest of transient, as opposed to more stable, precursors with neurogenic potential.

Using serum- and chick embryo extract-enriched cultures could also provide a test for the effect of bFGF-producing cells on the development of additional crest cell subpopulations, such as melanocytes. This is because it was found that bFGF influences the commitment to melanogenesis of progenitors present in quail DRG (Stocker et al., 1991).

The question is raised of the significance to embryonic development of the association in culture of bFGF-producing cells with neural crest progenitors. The contacts between neighbouring cells in the cocultures closely resemble natural intercellular interactions in the developing organism. The neurogenic effect observed in the cocultures, but not in cultures of crest cells grown on laminin in the presence of soluble bFGF, are evidence that these close contacts more effectively mediate the transmission of specific biological signals (see also Roberts et al., 1988 for stromal cell-mediated hemopoiesis). Although the mechanism(s) that mediate the effect of bFGF on neural crest cells are still unknown, the cocultures developed in the present experiments provide us with a convenient model system to investigate further whether the active factor is associated with the cell membrane or the ECM, and whether it stimulates neural crest differentiation in a direct manner or through the activation of secondary signals in the BHK cells. Any of the above mechanisms can be of relevance to the understanding of neural crest development in vivo, because neural crest cells giving rise to DRG migrate adjacent to the neural tube (Teillet et al., 1987). CNS-derived bFGF may be suitably presented to these crest cells in the
form of membrane-bound or ECM-associated factor. Moreover, as we already suggested (Gvirtzman et al., 1992; Kalcheim et al., 1992), the effects of this molecule can be directly exerted on the neural crest progenitors or alternatively, may be mediated by somite cells that are permissive to neural crest migration (Rickmann et al., 1985; Bronner-Fraser, 1986; Newgreen et al., 1986; Loring and Erickson, 1987; Teillet et al., 1987) and proliferate in response to bFGF (Kalcheim, 1989).

We thank Dr I. Vlodavsky for helpful suggestions and critical reading of the manuscript. This work was supported by grants from the National Council for Research and Development and the Commission for European Communities, the Familial Dysautonomia Foundation and the Israel Academy of Sciences and Humanities to C.K. G.N. was supported by grants from the Tobacco Research Council, The Israel Ministry of Health and the US-Israel Binational Foundation.

References


Birren S. and Anderson, D. J. (1990). A v-myel immortalized sympathoadrenerginal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. Neuron 4, 189-201.


(Accepted 7 May 1992)