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FGF2 regulates proliferation of neural crest cells, with subsequent neuronal differentiation regulated by LIF or related factors

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SUMMARY

Two of the key early events in the development of the peripheral nervous system are the proliferation of neural crest precursor cells and their subsequent differentiation into different neural cell types. We present evidence that members of the fibroblast growth factor family, (FGF1 or FGF2) act directly on the neural crest cells in vitro to stimulate proliferation in the presence of serum. These findings correlate with in situ hybridisation analysis, which shows FGF2 mRNA is expressed in cells both in the neural tube and within newly formed sensory ganglia (dorsal root ganglia, DRG) at embryonic day 10 in the mouse, when neural crest precursors are proliferating within the DRG. This data infers an autocrine/paracrine loop for FGF regulation of proliferation. Evidence supporting this notion is provided by the finding that part of the endogenous proliferative activity in the NC cultures is related to FGF. It was also found, in early neural crest cultures, that exogenous FGF completely inhibited neuronal differentiation, probably as a direct consequence of its mitogenic activity. In order to stimulate neuronal differentiation significantly, it was necessary to remove the FGF and replace it with leukemia inhibitory factor (LIF) or related factors. Under these conditions, 50% of the cells differentiated into neurons, which developed a sensory neuron morphology and were immunoreactive for the sensory markers CGRP and substance P. These data support a model of neural crest development, whereby multipotential neural crest precursor cells are stimulated to divide by FGF and subsequent development into sensory neurons is regulated by LIF or other cytokines with a similar signalling mechanism.

Key words: neural crest, proliferation, neuronal differentiation, FGF, LIF, sensory neuron

INTRODUCTION

The neural crest (NC) is a transient embryonic structure which gives rise to a multitude of cell types in vertebrates including most of the peripheral nervous system, melanocytes of the skin and many mesectodermal structures including muscle, cartilage and bone in the head and upper body. There is considerable evidence that individual NC cells are initially multipotential, although not necessarily having the capacity to differentiate into the full complement of NC cell types (Le Douarin and Dupin, 1993; Weston, 1986; Murphy and Bartlett, 1993). The NC cells migrate from the neural tube into discrete regions of the embryo where their proliferation and subsequent differentiation is regulated by factors presumably produced within the local environment.

There is increasing evidence that soluble growth factors play a role in the process of NC development (Marusich and Weston, 1991; Stemple and Anderson, 1993; Murphy and Bartlett, 1993). In studies of sensory development, we have shown that leukemia inhibitory factor (LIF) can act at the stage of primary differentiation of sensory neurons from their NC precursors (Murphy et al., 1991b, 1993). LIF belongs to a structurally related family of molecules (Bazan, 1991; Patterson and Nawa, 1993) including ciliary neurotrophic factor (CNTF), oncostatin M (OSM) and probably another factor, growth promoting activity (Leung et al., 1992), which share the same signalling mechanism (Gearing et al., 1992; Ip et al., 1992). It is unclear which of these factors may be operant in sensory neuron development in vivo.

Although LIF has a potent activity in the differentiation of sensory neurons, it has no effect on cell division of NC precursor cells (Murphy et al., 1991b). Presumably this proliferative phase, which is essential for the generation of the full complement of neurons, is controlled by other factors. One member of neurotrophin family, NT3, has been shown to stimulate proliferation of quail NC cells (Kalcheim et al., 1992). However, the degree of stimulation of the NC cells was quite low and NT3 may have affected only a small percentage of the cells.

Studies undertaken by Kalcheim and co-workers have implicated fibroblast growth factor 2 (FGF2) in NC development (Kalcheim, 1989). FGF2 can act as a survival factor for primary quail NC cells both in vitro and in vivo (Kalcheim, 1989). If silastic membranes are inserted between the neural tube and migrated NC cells of the dorsal root ganglion anlage, there is a selective death of these NC cells. Impregnation of these membranes with FGF2, which is present in the neural tube in vivo (Kalcheim and Neufeld, 1990; Drago et al.,

1991a), results in significant survival of the NC cells (Kalcheim, 1989). In mixed cultures of trunk NC cells and somite cells or in pure cultures of NC cells in a serum-free defined medium, FGF2 was found to act as a survival agent for NC cells, but no effect on proliferation could be observed (Kalcheim, 1989).

However, precursor cells in the neuroepithelium of embryonic day 10 (E10) mice do proliferate in response to FGF1 and FGF2 (Murphy et al., 1990; Drago et al., 1991a). In addition, a number of murine NC cell lines respond to FGF2 by proliferating and by changes in morphology (Murphy et al., 1991a). We have further investigated the role of FGF in the development of the NC. In cultures of NC cells derived from E9 mouse embryos, FGF2 stimulated the proliferation of greater than 90% of NC cells, dependent on the presence of serum. Subsequent treatment of these cultures with LIF resulted in differentiation of 50% of the cells into sensory-like neurons. Thus, FGF2 can act early in NC development as a proliferative factor and following treatment with LIF, there is a resultant large expansion in the sensory neuron population.

MATERIALS AND METHODS

Neural crest cultures

Neural crest cultures were prepared as previously described (Murphy et al., 1993). Growth factors were added as follows as follows: FGF1 and FGF2, 50 ng/ml or as otherwise stated (Boehringer Mannheim); LIF, 10^4 U/ml (recombinant murine LIF, produced at the Walter and Eliza Hall Institute of Medical Research; specific activity 10^8 units/mg); CNTF, 100 ng/ml (Pepro Tech, Rocky Hill, NJ); OSM, 100 ng/ml (supplied by D. Gearing, Immunex Corp, Seattle, WA), inositol hexakisphosphate, 25 μM ($InsP_6$, Sigma, Aus). Cultures were incubated in 5%CO_2/95% air at 37˚C. After 1 day, the wells were made up to 500 μl with the appropriate growth factor solutions. In some experiments, neural tubes were removed at this time.

Determination of neural crest cell number

NC cultures were incubated for 4 days after which the neural tubes and any surrounding neuroepithelial cells were scrupulously removed. The neuroepithelial cells form a continuous 'cobblestone' monolayer around the tube and are distinguishable from NC cells which have undergone an ectomesenchymal transformation prior to migration. These NC cells form a less organised 'fibroblastic' monolayer of cells circumscribing and clearly separable from the neuroepithelial cells. Further, these NC cells are specifically immunoreactive for B33 (data not shown), a monoclonal antibody previously shown to mark NC cells in vivo and in vitro (Stainier and Gilbert, 1991). The NC population was treated with 170 μl/well of trypsin versene solution (Commonwealth Serum Laboratories, Melbourne, Aust.) for 5 minutes at 37˚C, then triturated through a 200 μl pipette tip until a single cell suspension was obtained. The cells were then fixed in 1% formaldehyde, 2% glucose, 5 mM sodium azide and analysed by flow cytometry on a Becton Dickinson FACS II analyser and total cell number for each sample was determined. At least 5 samples were counted for each condition.

To assess the effect of FGF2 in the absence of the neural tube, the cells were cultured in Monomed medium with 10% FBS alone for the first 24 hours, then the neural tubes were removed, FGF2 added and the cultures incubated for a further 3 days, before being trypsinized and counted as described above.

Proliferation studies

Neural tubes were plated onto fibronectin-coated plastic 8-well slide chambers (Nunc, Naperville, Il). After 20 hours, [^{3}H]thymidine (1.5 μCi/ml) was added and incubation continued for 1 hour, after which the cultures were washed and fixed in 10% buffered formalin. The slides were allowed to air dry, dipped in photographic emulsion and autoradiographed for 2 to 4 weeks, developed and the percentage of NC cells which had incorporated [^{3}H]thymidine into their nuclei was calculated. Percentages represent the mean of at least 3 separate determinations in each of which a minimum of 1000 cells was counted. Bromodeoxyuridine (BrdU) was also used to label proliferating NC cells after 1 day in order to determine maximum degrees of proliferation. BrdU (10 nM, Amersham, Buck, UK) was added to the cultures for up to 8 hours, after which the cells were washed and fixed in ethanol/acetic acid (19:1) for 30 minutes at room temperature, and stained using immunoperoxidase (see below).

The ability of $InsP_6$ to block the effects of exogenous FGF was tested in cultures of E10 murine neuroepithelial cells: in agreement with Sherman et al. (1993) 25 μM $InsP_6$ was chosen as this concentration blocked the effects of 20 ng/ml FGF2 but not 1 μg/ml FGF2 in stimulating proliferation. Proliferation was demonstrated by the presence of large clusters of cells in the neuroepithelial cultures (Murphy et al., 1990).

In situ hybridisation

FGF1 and FGF2 cDNA clones were derived as described (Nurcombe et al., 1993). Reverse transcribed PCR generated products from murine brain mRNA were blunt end ligated into the *Sma*I site of pGEM3Zf (Promega) and authenticated by DNA sequencing. Plasmid DNA was linearised using *Eco*RI for antisense, sp6 RNA polymerase synthesised riboprobes and *Sal*I for sense, T7 polymerase synthesised riboprobes. Transcription in vitro of biotinylated probes was essentially as described in Riboprobe Gemini II System (Promega, Australia) with the replacement of rUTP with 1 μl of 2% BSA (Sigma). The DNA template was removed by the addition of 1 μl of RNase-free DNase (Promega) and incubation at 37°C for 15 minutes. The cRNA probes were precipitated by the addition of carrier tRNA or glycogen (Boehringer Mannheim), 1 volume of 5M ammonium acetate, 2 volumes of ethanol and cooling to −70°C for 30 minutes. Precipitate was pelleted by centrifugation at 15,000 revs/minute for 15 minutes, dissolved in 100 μl of 1 M ammonium acetate, precipitated and pelleted as above. The cRNA pellet was air dried, dissolved in 20 μl of DEPC-treated water and stored at −70°C. Probes were assessed for size and concentration by formaldehyde agarose gel analysis, and sense and antisense probes were adjusted to equivalent concentrations (usually between 50 and 100 ng) for use on tissue sections.

For in situ hybridisation, transverse serial 4 μm sections from 4% paraformaldehyde fixed E10 embryos were floated onto aminopropyltriethoxysilane coated slides and baked at 37°C overnight. Sections were dewaxed and rehydrated and then processed as follows: Sections were treated for endogenous peroxidase with 0.3% H_20_2 in methanol for 10 minutes and then rinsed with PBS. Sections were then treated in succession with 0.1 M glycine in phosphate-buffered saline (PBS) for 5 minutes; 0.3% Triton X100 in PBS for 15 minutes; 50 μg/ml proteinase K in 0.1 M Tris, 50 mM EDTA, pH 7.0 for 20 minutes at 37°C; rinsed in PBS; fixed in 4% paraformaldehyde for 5 minutes; washed in PBS, 0.25% acetic anhydride in 0.1 M Tris, ph 7.0 for 10 minutes; washed in PBS and then prehybridized in 50% formamide, 2× SSC, 0.3% BSA and 1 mg/ml tRNA for 60 minutes at 42°C. Riboprobe (50-100 ng) was melted at 70°C for 5 minutes in 20 μl hybridisation buffer (50% formamide, 2× SSC, 10% polyethylene glycol, 1× Denhardt's, 500 μg/ml tRNA, 0.25 M Tris, pH 7.0) cooled on ice for 5 minutes and 20 units of RNasin (Promega) was added. Probe mix (20 μl) was overlaid on sections which were then covered with a siliconised coverslip and sealed with rubber cement for overnight hybridisation at 42°C in a humidified chamber. Coverslips were removed by soaking in 2× SSC and sections were then washed and visualised for in situ hybridisation by treatment as follows: two washes in 2× SSC for 10

minutes at 37°C, two washes in PBS for 10 minutes at 37°C, once in 2% BSA in Tris-buffered saline (TBS, 0.1 M Tris, ph 7.4, 0.9% w/V NaCl) for 30 minutes; incubation in ABC reagent (Vectastain Kit, Vector Labs, CA) for 60 minutes; two washes in TBS, 0.3% Triton X-100 for 5 minutes and one in TBS for 5 minutes. Colour was developed with diaminobenzidine (0.5 μg/ml) and H_2O_2 (0.012%) in TBS. Development time was variable (2-15 minutes) and was monitored with a light microscope. Development was stopped by rinsing the slides in TBS, which were then dehydrated through an ethanol series and coverslipped for viewing using an Olympus BH2 microscope with differential interference contrast optics.

Immunohistochemistry

Cultures were stained for neurofilament ($_p$150 NF) using peroxidase as previously described (Murphy et al., 1993). To determine the presence of incorporated BrdU, fixed cultures were incubated with an anti-BrdU monoclonal antibody (undiluted; Amersham) for 1 hour, followed by detection with peroxidase. To stain for calcitonin gene related peptide (CGRP) or substance P, cultures were fixed in 4% formaldehyde with 15% saturated picric acid in PBS, pH 7.3, washed in PBS three times and incubated in a blocking solution of PBS containing 0.8% Tween 20, 2% FBS, 2% goat serum, 0.4% mouse serum, 0.1% NaN_3 for 30 minutes at room temperature. Cultures were then incubated overnight at 4˚C in blocking solution containing rabbit anti-CGRP antiserum (Murphy et al., 1991b) diluted 1:100, or a rabbit anti-substance P antiserum (Ausprep Pty Ltd, Melbourne) diluted 1:500. Binding was detected using biotin-conjugated second antibodies and peroxidase staining as described above.

Determination of neuronal numbers

The per cent of neurons within the NC cultures was estimated along 4 radii emanating from the centre of the culture, each separated by 90˚ from each other. Only neurons and cells within the NC part of the culture were counted. Total number of neurons in each culture was estimated by determining neuronal densities along four diameters of the culture and multiplying by the area of the culture assuming an elliptical outgrowth from the tube.

RESULTS

FGF stimulates NC cell proliferation

Soon after plating E9 murine neural tubes migration of NC cells began and continued for at least the next 24 hours. With the addition of FGF2 to these cultures, migration of the cells proceeded and by 24 hours there were both more cells and their morphology was different to that seen in serum alone. After 1 day in culture, cells in FGF-treated cultures had a uniform round, flat morphology, whereas cells in serum alone cultures were more heterogeneous in appearance, and contained elongated bipolar cells and more fibroblastic cells in addition to the rounded cells (Fig. 1). By 4 days of culture, there were about 8 times as many NC cells in the FGF2 cultures compared to controls (Fig. 2). In order to determine whether the increase in cell number was independent of the presence of the neural tube, the tubes were plated in the presence of serum alone and left for 24 hours to allow NC migration to occur. The neural tube was then removed from the cultures and FGF2 was added. A similar increase in cell number (7- to 8-fold) was observed after 3 days in FGF2-treated cultures compared to cultures incubated in 10% serum alone, which indicates that FGF2 could act directly on the NC cells (Fig. 2). However, there were more cells in cultures where the neural tube was present, both in the presence and absence of FGF. Part of the difference was probably caused by the loss of NC cells when the neural tube was removed, which results in a lower starting number of NC cells. In addition, neural-tube-derived factors, including FGF itself, may have stimulated the increase in cell number.

In order to examine whether FGF2 was exerting a prolifer-

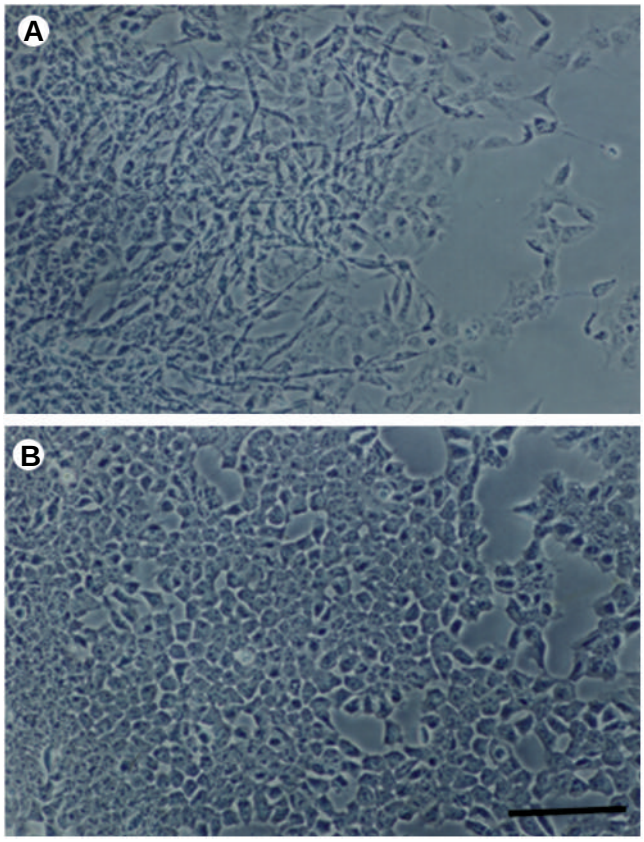

Fig. 1. Effect of FGF on morphology of cells in neural crest cultures. Neural tubes were plated in the presence of (A) 10% serum alone or (B) FGF2 plus 10% serum and the resultant neural crest outgrowths were photographed under phase contrast after 24 hours. Bar, 100 μm.

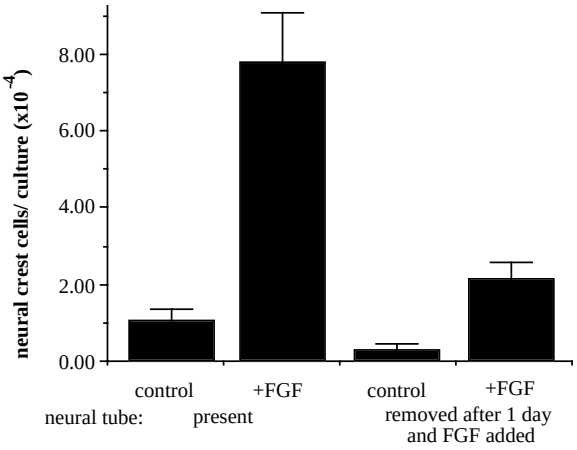

Fig. 2. Effect of FGF on NC cell number. NC cell numbers were determined following incubation of cultures in the presence (+ tube) or absence (– tube) of neural tube as described in materials and methods. Results are expressed as mean±s.d.

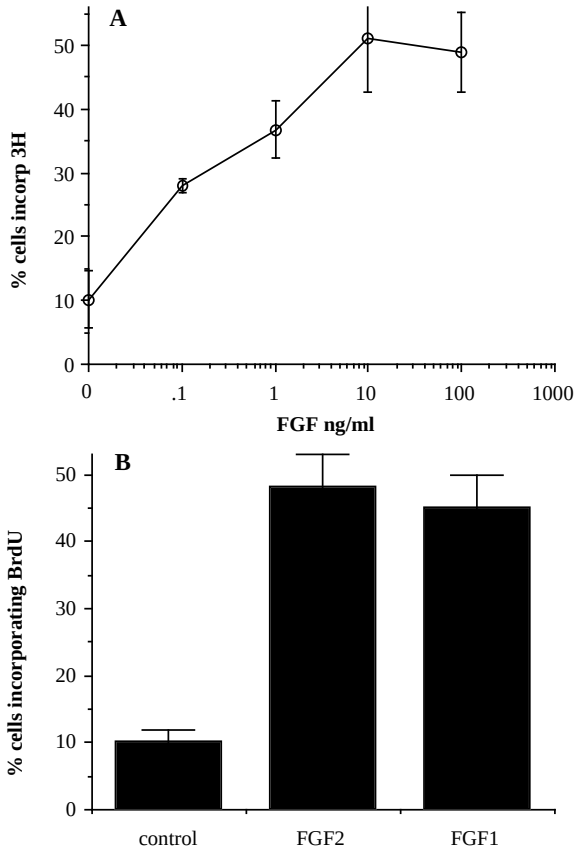

Fig. 3. Effect of FGF on NC cell division. (A) Effect of FGF2 concentration on NC cell division. NC cells were cultured for 20 hours with specified concentrations of FGF in the presence of 10% serum, pulsed with [^{3}H]thymidine for 1 hour and the percent of cells incorporating [^{3}H]thymidine were determined for each culture as described in Materials and Methods. (B) Comparison of FGF1 and FGF2 in stimulation of NC cell division. NC cells were cultured for 20 hours in either FGF1 or FGF2 at 50 ng/ml, pulsed for 1 hour with [^{3}H]thymidine and the per cent of cells incorporating label was determined.

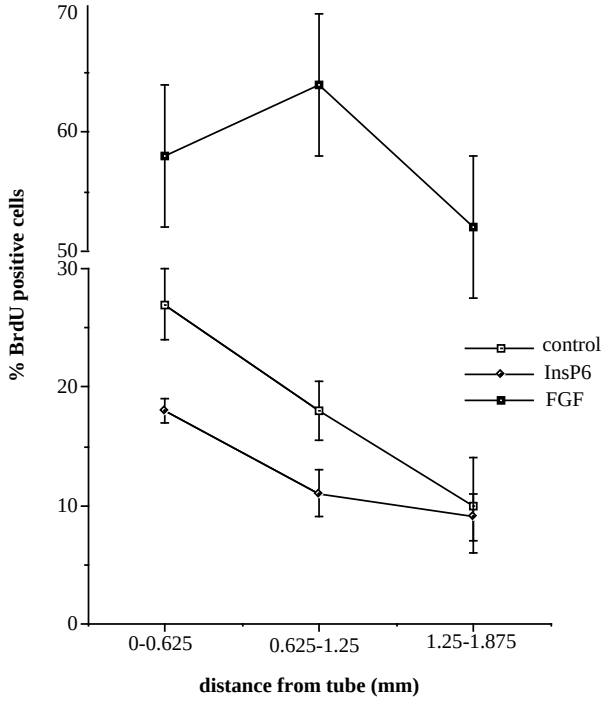

Fig. 4. Influence of distance from the neural tube on NC proliferation and the effects of $InsP_6$. Neural tubes were plated in medium containing 10% FBS alone; plus FGF2 (100 ng/ml); or plus $InsP_6$ (25 μm). After 20 hours, BrdU was added, incubation continued for 2 hours, then the cells were fixed and stained for incorporated BrdU. The per cent of cells that had incorporated BrdU was then determined at different distances from the neural tube.

ative effect on the NC cells, FGF2 was added to the cultures at the time of plating and, after 24 hours, the cultures were pulsed with [^{3}H]thymidine for 1 hour (see Fig. 3A). At an FGF2 concentration of 50 ng/ml, 50% of the NC cells in the culture incorporated [^{3}H]thymidine, compared to 10% or less in control cultures (Fig. 3). Incubation of the FGF2-treated cultures for 8 hours resulted in 94±4% of the cells incorporating label compared to 31±7% (mean±s.d., n=4) in serum alone. A titration of FGF2 showed that there was maximal incorporation of [^{3}H]thymidine at 10 ng/ml and half maximal stimulation at approximately 200 pg/ml (Fig. 3A). These concentrations are similar to those observed in FGF2 stimulation of neuroepithelial cultures (Murphy et al., 1990) as well as other cell culture systems where FGF2 has been shown to act directly (Gospodarowicz, 1987).

The stimulation of proliferation of neural crest cells was not unique to FGF2 but was also shared by at least one other member of the FGF family, FGF1. In the presence of 50 ng/ml FGF1, 50% of the neural crest cells incorporated label in a one hour incubation, similar to FGF2 (Fig. 3B).

A titration of serum in the presence of maximal concentrations of FGF2 (50 ng/ml) showed that there was a serum requirement for proliferation (Fig. 5). In the absence of serum, 7% of the cells incorporated [^{3}H]thymidine during one hour pulse, compared to 50% in 10% serum (Fig. 5). In the absence of both FGF2 and serum, very few NC cells were present on the fibronectin substrate 24 hours after plating out the neural tubes and none of these cells incorporated [^{3}H]thymidine. This may be due to a lack of survival of the cells under these conditions, similar to that described in the avian NC (Kalcheim, 1989).

Endogenous FGF-like activity is present in the neural crest cultures

As noted above, neural-tube-derived factors, possibly including FGF itself, may have contributed to neural crest cell proliferation. An analysis of the proliferative index of the neural crest cells as a function of distance from the neural tube showed that, in the presence of medium containing 10% serum alone, proliferation was higher in cells closer to the neural tube (Fig. 4), further suggesting that the neural tube may have been a source of proliferative factors. The addition of FGF to the cultures increased the proliferation index to the same level at any distance from the tube, which established that there was no fundamental difference in the ability of these cells to respond to growth factors. The possibility that the proliferation-promoting activity associated with the neural tube was related to FGF was examined by incubating neural tube containing cultures with inositol hexakisphosphate ($InsP_6$), which blocks the binding of FGF2 to its extracellular receptors (Sherman et al., 1993). This resulted in a significant reduction

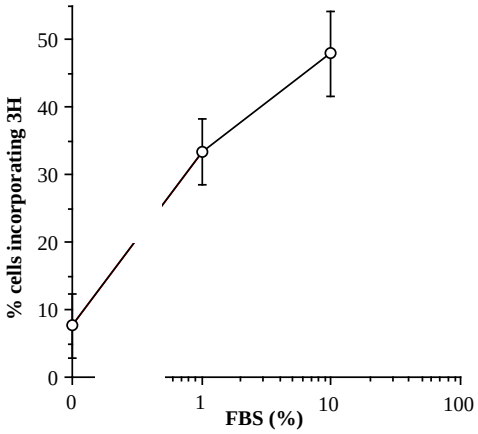

Fig. 5. Effect of FBS on NC cell division in the presence of FGF. NC cells were cultured for 20 hours in the presence of 50 ng/ml FGF and the per cent of cells incorporating [^{3}H]thymidine were determined as described Fig. 3.

in the degree of proliferation of cells closest to the neural tube, but the effect decreased with distance from the tube and at the edge of the culture, proliferation was unaffected by the addition of $InsP_6$ (Fig. 4). These findings are thus consistent with the notion that there is endogenous FGF-like activity in these cultures, concentrated around the neural tube.

FGF2 mRNA is expressed specifically in the neural tube and developing DRG

If the responses that we observed with FGF in our in vitro assays reflect an activity of FGF in vivo, then it would be expected that there would be a source of FGF available to the NC cells at the time of proliferation. To look for possible sites of synthesis of FGF in the embryo, we conducted in situ hybridisation studies of sections of the trunk region of E10 mice with FGF1 and FGF2 RNA probes. FGF2 mRNA is obvious in both the neuroepithelial cells of the embryonic spinal cord and the neural-crest-derived cells of the dorsal root ganglia (Fig. 6B). It is clearly present at high concentrations in the proliferating cells of the ventricular zone (VZ) of the embryonic spinal cord while the maturing cells of the lateral motor column (LMC) and the marginal zone (MZ) appear negative (6D). At high magnification (6E) the perinuclear capping of the nuclei in the peripheral ganglia is clearly visible. Similar staining of sections through the telencephalon show FGF2 mRNA is present throughout the length of the neural tube at this time (results not shown) and these results complement an immunohistochemical study which localised FGF2 protein in the neuroepithelium of the neural tube at this time (Ford et al., 1994). As both sense and antisense mRNA for FGF2 normally co-exist in tissues (Kimelman and Kirschner, 1989, Zuniga et al., 1993) the FGF1 sense cRNA probe was used as the control for non-specific background hybridisation. The probe is of similar length and biotin content to the FGF2 probes and is clearly negative (Fig. 6C).

LIF and related factors stimulate neuronal differentiation in FGF-treated NC cultures

To examine neuronal differentiation in the neural crest cultures, we stained the cultures for the presence of 150×10^3 M_r neurofilament (p150 NF), which is expressed in newly differentiated neurons derived from the neural crest. After 1 day, in cultures treated with medium containing 10% serum alone, an average of 150 cells per culture stained for p150 NF (average of 3 determinations) and had a bipolar morphology as

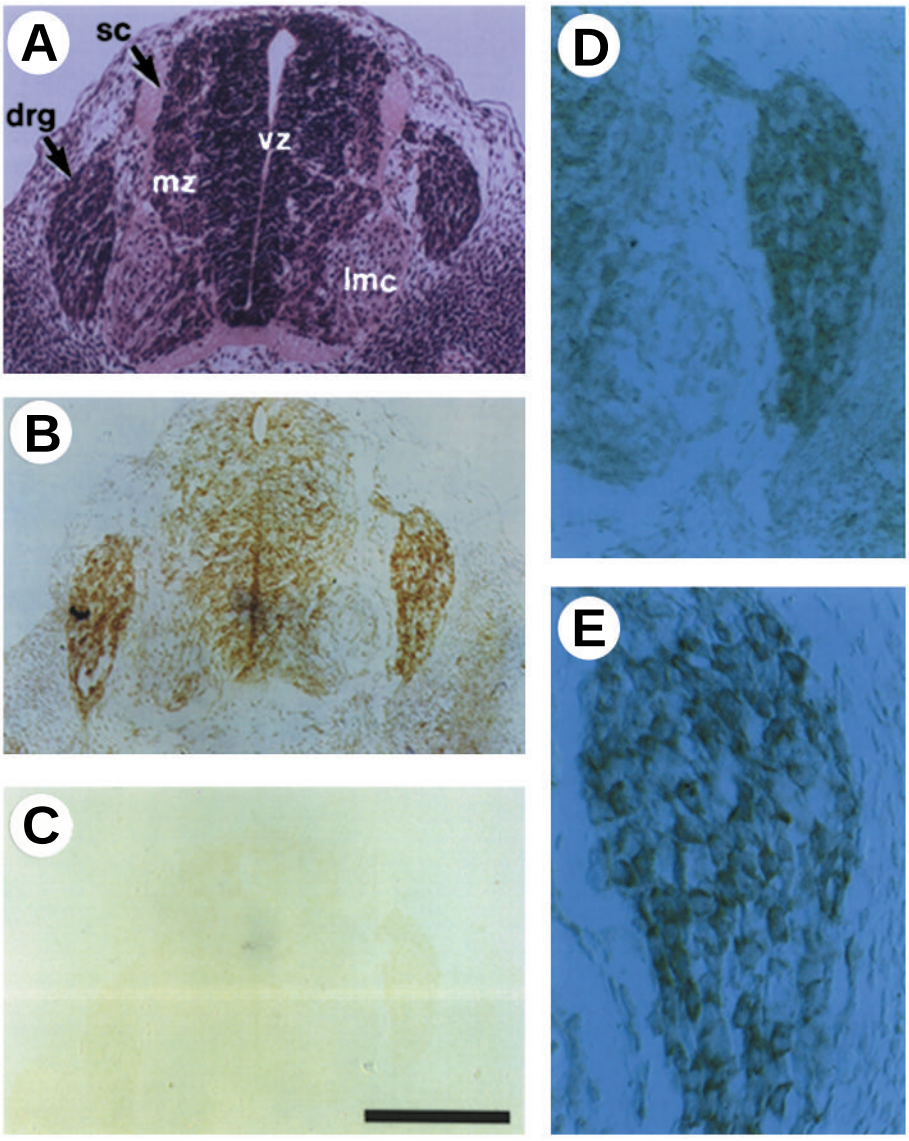

Fig. 6. Expression of FGF2 mRNA in embryonic day 10 spinal cord and dorsal root ganglia. Bright-field illumination (B,C) with differential interference contrast optics (A,D,E) (A) H&E-stained section; (B) FGF2 antisense biotinylated riboprobe; (C) negative control-FGF1 sense probe; (D) FGF2 antisense probe and (E) FGF2 antisense probe. SC, spinal cord; DRG, dorsal root ganglion; VZ, ventricular zone; MZ, marginal zone; LMC, lateral motor column. Bar (A-C) 200 μm , (D) 100 μm and (E) 50 μm.

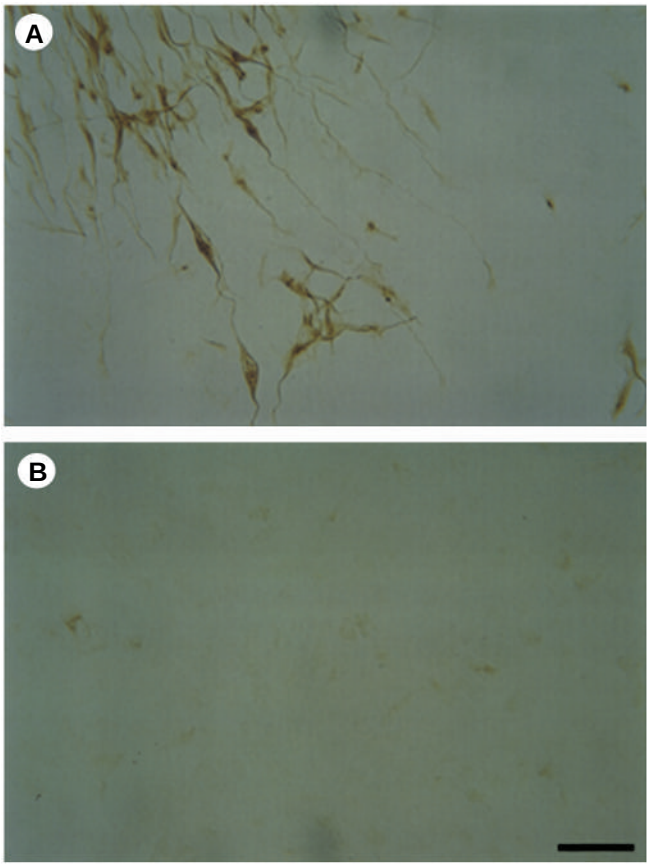

Fig. 7. Inhibition of neuronal differentiation in early neural crest cultures by FGF. Neural crest cultures were established as described in Fig. 1, fixed after 24 hours and stained for the presence of p150 NF as described in Materials and methods. Shown are bright-field photomicrographs of NC cultures incubated in (A) serum alone or (B) FGF2. Unstained cells are not readily visible as cultures were photographed under conditions which best demonstrate immunostaining; see Fig. 1 for phase contrast view of cultures. Bar, 50 μm.

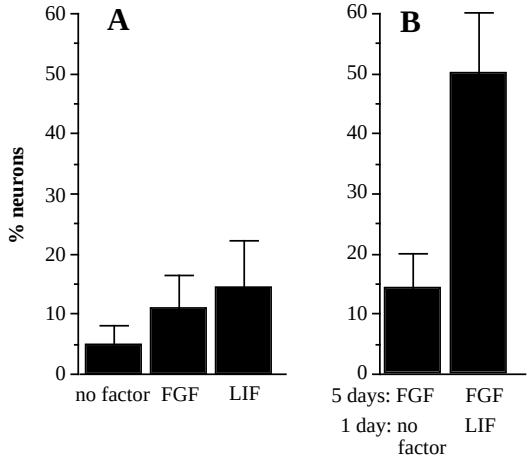

Fig. 8. Neuronal differentiation in NC cultures treated with FGF and LIF. (A) NC cells were cultured in the presence of the neural tube and specified growth factors for 6 days, fixed and stained for the presence of $_{p}$150 NF and the percentage of neuron-like $_{p}$150 NF^{+} cells was determined for each condition as described in Materials and Methods. (B) NC cultures were incubated for 5 days in the presence of FGF, washed and fresh medium with or without LIF was added. After a further 24 hours, the per cent of neurons in the cultures was determined as described in A.

described above (Fig. 7A and see Fig. 1). The staining also revealed fine processes on these cells, showing that they were probably newly differentiated neurons. However, in the presence of FGF, none of these cells were present in the cultures and there was almost no staining with p150 NF (Fig. 7B), indicating that there were no neurons in these cultures.

In serum alone cultures, the number of neurons did not increase significantly over 7 days (number of neurons at day 1 =155; at day 7 =190; average of 3 determinations), which suggests that there was little new primary neuronal differentiation after the first day of culture. However, the neurons in these cultures matured morphologically by becoming spherical, increasing cell body size and having finer, longer processes (Fig. 9A). In the FGF2-treated cultures, the majority of cells remained NC-like in appearance although staining for $_{p}$150 NF showed immature neuron-like cells began to appear in the cultures after 3 to 4 days. After 6 days of culture, 11% of the cells in the FGF2-treated cultures both stained positively for $_{p}$150 NF and resembled immature neurons (Figs 8A, 9B). However, there were very few clearly defined mature neurons in these cultures. A proportion of the flatter, NC-like cells in all cultures also stained positively for $_{p}$150 NF (Fig. 9), but the staining was less intense than in the neuronal cells, and the number of NC-like cells which stained positively was quite variable and showed no observable correlation with culture conditions.

FGF may interfere with neuronal differentiation by promoting cell proliferation and thus removal of the proliferative stimulus might permit more differentiation. However, following removal of FGF2 after 5 days, there was no major increase in the percent of neurons in the cultures (Figs 8B, 9C).

As LIF has been previously shown to stimulate neuronal differentiation in NC cultures (Murphy et al., 1991b, 1993) it was added to the cultures after removal of the FGF2. Under these conditions, many of the cells differentiated into neurons (Figs 8B, 9D): after 24 hours in LIF, 50% of the cells in these cultures were $_{p}$150 NF^{+} and were spherical with fine processes (Figs 8B, 9D), clearly indicative of a neuronal phenotype. Two other members of this family of factors were tested for their ability to stimulate neuronal differentiation, CNTF and OSM. Both of these factors were able to stimulate neuronal differentiation to a similar extent, in cultures which had prior treatment with FGF2 (Fig. 10). This finding is consistent with the shared signalling mechanism of these factors.

Cultures that were not treated with FGF at all, but were treated with LIF at the time of plating, resulted in only 15% of neural crest cells differentiating into neurons (Fig. 8A). Thus, sequential treatment with FGF2 followed by LIF results in a significantly greater proportion of cells differentiating into neurons than treatment with LIF alone. In addition, the total number of neurons that developed in sequentially treated cultures was greatly increased over cultures treated with LIF alone. In cultures which were treated with FGF2 for 5 days and then switched to LIF, 44,000±13000 (mean±s.d., n=4) neurons developed. By comparison, only 1,300±550 neurons developed in cultures treated with LIF continuously for 6 days. This 34-

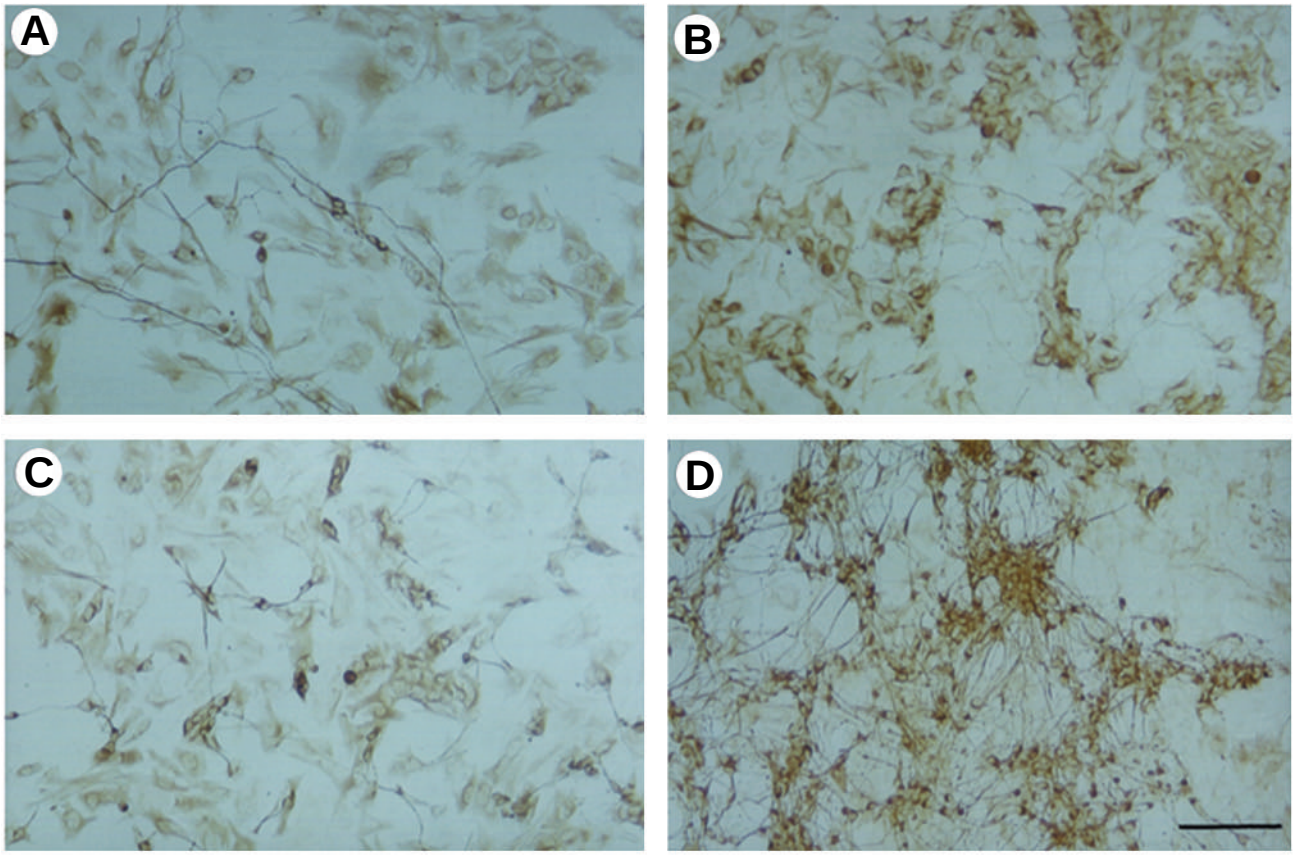

Fig. 9. Appearance of NC cultures following incubation in FGF and LIF. NC cells were cultured for 6 days in medium containing 10% serum alone (A) or FGF (B). Other cultures were incubated in FGF for 5 days and then cultured for 1 day in medium containing 10% serum alone (C) or LIF (D). Cultures were stained for $_{p}$150 NF and photographed as described in Fig. 6. Photomicrographs show representative areas of each culture. Scale bars, 100 μm; scale in B, C and D are the same.

fold increase is the combination of an approximate 10-fold increase in number due to proliferation with a 3- to 4-fold shift in the percentage of neurons arising.

Sensory-like neurons develop in cultures sequentially treated with FGF2 followed by LIF

As previously reported, neurons that develop in cultures continuously treated with LIF tend to cluster and acquire characteristics of sensory neurons such as a large cell body with monopolar or bipolar processes and immunoreactivity for calcitonin gene related peptide (CGRP) and substance P (Murphy et al., 1991b). Neurons with the same morphology developed in cultures that were first treated with FGF2 and then switched to LIF. Staining of these cultures for CGRP showed positive staining in most of the cell bodies and processes (Fig. 11A). In addition, substance P immunoreactivity was found in varicosities within neuronal processes as well as cell bodies (Fig. 11B). Neurons of similar morphology, expressing CGRP, were also found in cultures in which the neural tube was removed prior to addition of FGF and subsequent treatment with LIF (data not shown). This is in agreement with our previous findings that LIF can stimulate the generation of sensory-like neurons in the absence of the neural tube, or neural tube-derived factors (Murphy et al., 1991b).

DISCUSSION

FGF stimulates the proliferation of NC cells

In the present study, we have shown that at least 94% of NC cells that have migrated from the trunk regions of the neural tube are in division in the presence of FGF. FGF increases the percentage of NC cells in division in a titratable fashion and thus is most likely acting to stimulate the proliferation of the NC cells. Another interpretation of these results is that FGF may not directly stimulate proliferation but that the increase in the percentage of cells labelled with [^{3}H]thymidine or BrdU is a specific survival effect of FGF on dividing cells. However, we believe that FGF is directly acting as a proliferative agent for the following reasons: First, 80% of NC cells incorporate label following long-term incubation of serum alone cultures with [^{3}H]thymidine (Murphy et al., 1991b), indicating that the majority of NC cells divide (albeit slowly) with no added FGF. Thus, FGF is not maintaining cycling cells but stimulates an increase in their rate of proliferation. Second, there was no observable effect on NC survival following blocking any endogenous FGF with $InsP_6$. Third, all cells in the neural crest population appear to respond to FGF by assuming a small rounded morphology, whereas cells in serum alone cultures are quite heterogeneous, which suggests that FGF affects most

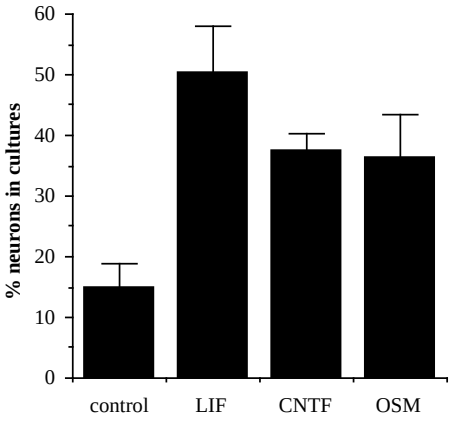

Fig. 10. Effects of CNTF and OSM on neuronal differentiation in FGF-treated NC cultures. NC cultures were incubated for 5 days in the presence of FGF2, washed and fresh medium containing 10% serum alone, or with LIF, CNTF or OSM was added. After a further 24 hours, the per cent of neurons in each culture was determined as described in Fig. 7.

cells in the NC population, not just the cycling cells. Finally, no neuronal differentiation is seen in early NC cultures in the presence of FGF. This is not due to a specific inhibition of neuronal survival, as neurons are observed at later times in FGF-treated NC cultures, but is probably due to FGF's proliferative effects and should not occur if FGF were merely a survival agent for cycling cells.

Serum is also required for NC proliferation; factors in serum that may be responsible include the insulin-like growth factors (IGFs) and, in particular IGF1, which is required for FGF2 regulated proliferation of neuroepithelial cells (Drago et al., 1991a). However, other factors must also be required for NC division, since our serum-free medium contains enough insulin to bind to the IGF1 receptor and produce a biological signal. In addition, these findings are consistent with previous studies (Kalcheim; 1989), which found that in the absence of serum, FGF2 had no proliferative activity but acted as a survival factor.

NC cells appear to consist of populations of multipotential stem cells, neural precursor cells (which can give rise to all NC-derived neurons and glia) and melanocyte precursors (as discussed by Le Douarin and Dupin, 1993). Given that FGF2 stimulates 94% of NC cells to divide, it may act on all NC cell types. Although our results demonstrate a preponderance of sensory-like neurons could be generated from these cultures following FGF treatment, this does not necessarily indicate that FGF was specifically stimulating the proliferation of a pool of sensory precursor cells, as it has been shown that the majority of sensory neurons are derived from multipotential stem cells (Sieber-Blum, 1989). Thus FGF may be acting to expand a multipotential stem cell pool. Previous results with subcloning of neuroepithelial cells show that FGF can stimulate proliferation of multipotential cells within this population (Kilpatrick and Bartlett, 1993).

The effects of FGF on the NC cells are independent of the presence of the neural tube, since upon its removal after 24 hours, the cell number increases by a similar proportion to cultures in which the neural tube is present. Thus, the effect of FGF2 on the NC cells neither requires other cell types in the cultures, nor is it dependent on factors that may be synthesised by other cells. This is in contrast to the effects of NT3, which weakly stimulates the proliferation of isolated NC cells but, in the presence of somite cells, more strongly stimulates proliferation in the NC (Kalcheim et al., 1992). Recent data indicate that NT3 stimulates sympathetic neuroblast proliferation by promoting precursor survival (DiCicco-Bloom et al., 1993) and thus it may be possible that a part of the effect seen in NC cultures is due to a similar mechanism.

Does FGF2 have a role in proliferation of NC cells in vivo?

FGF2 immunoreactivity has been detected in the basement membrane around the dorsal neural tube (Kalcheim and Neufeld, 1990) as well as in association with a heparan sulphate proteoglycan (HSPG) within the neuroepithelium (Ford et al., 1994). This correlates with the expression of FGF2 mRNA within the neural tube from E9 (Drago et al., 1991a). Our in situ hybridisation analysis shows specific expression of FGF2 within the neural tube and developing dorsal root ganglia at E10. Within the DRG, most of the cells are labelled with antisense probe and are probably synthesising FGF2. This corresponds to a time when most of these DRG cells are proliferating (Lawson et al., 1974) suggesting autocrine/paracrine regulation of proliferation within the DRG. Likewise, the cells in the neural tube that are positive for FGF2 mRNA are predominantly proliferating cells. The finding of an endogenous FGF-like proliferative activity in our NC cultures correlates with this in situ hybridisation data and these results together add further support to idea that FGF2 is produced by the cells and regulates proliferation in vivo. FGF2 activity may also be regulated through specific interaction with HSPG (Nurcombe et al., 1993; Ford, 1994).

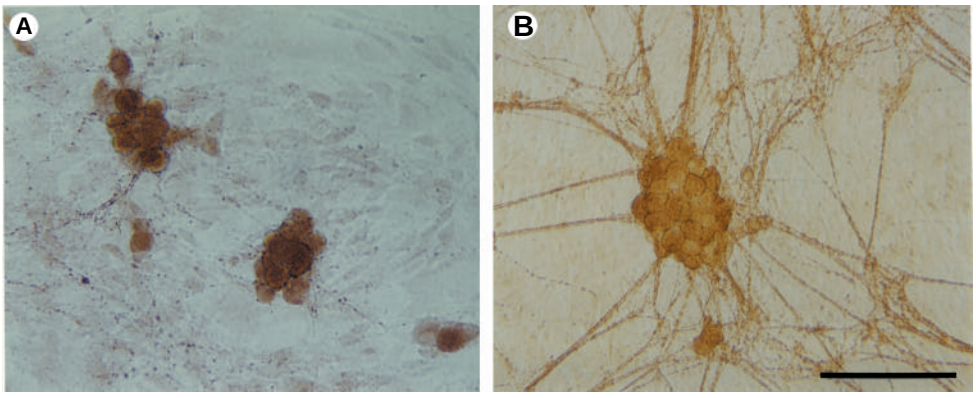

Fig. 11. Phenotype of neurons in NC cultures following treatment with FGF and LIF. NC cultures were sequentially treated with FGF for 4 days, followed by LIF for 14 days, fixed and stained with antibodies for Substance P (A) or CGRP (B) as described in Materials and Methods, and photographed. Scale bars, 100 μm.

Studies of the localisation of the FGF receptor also support a role for FGF in the early phases of sensory development. In situ hybridisation studies in the chicken show a significant level of FGFR mRNA in NC cells from 55 hours to 3.5 days, as well as in a subpopulation of sensory ganglion cells from E3.5 to E5 (Heuer et al., 1990), periods of active proliferation of NC cells and sensory neuroblasts (Carr and Simpson, 1978). In the rat, FGFR mRNA is present in the DRG from at least E12 (Wanaka et al., 1991), a period of maximal proliferation of sensory neuroblasts (Lawson et al., 1974).

FGF influences neuronal differentiation

Consistent with previous observations (Boisseau and Simonneau, 1989; Bannerman and Pleasure, 1993), there is no neuronal differentiation in the presence of FGF in early NC cultures, possibly because it maintains the cells in division. However, FGF does influence the differentiation of NC cells as prior treatment with FGF2 results in 50% of the cells differentiating into neurons when followed by treatment with LIF, a significantly higher proportion than that which differentiate in LIF alone (15%). Sequential treatment with FGF2 followed by LIF results in the generation of up to 50,000 neurons from a single neural tube explant, about half of the 100,000 sensory neurons that would be generated from this region of the crest. Brill et al. (1992) reported that in serum-free conditions and in the presence of BHK21 cells, FGF2 causes up to a 4-fold increase in the number of neurons arising from NC cells. These findings suggest that FGF2 stimulates neuronal differentiation through interaction with this cell line or factors produced by this cell line. In our cultures, FGF2 may stimulate the production of other factors in the NC cultures which then prime some of the cells so that they differentiate under appropriate conditions. We have previously proposed such an FGF2-induced cascade to explain the effects of FGF2 on the differentiation of neuroepithelial cells, and have shown that FGF2 upregulates the synthesis of the neurite promoting molecule, laminin, in addition to stimulating proliferation (Drago et al., 1991b).

The role of LIF or related factors in neuronal differentiation and sensory development

The phenotype of the neurons generated following LIF treatment in this and our previous studies (Murphy et al., 1991b) is most consistent with the sensory lineage: relatively large (≥20 μm), spherical and uni or bipolar, similar in size to dorsal root ganglion neurons of newborn rodents (Lawson et al., 1974), staining positively for CGRP and substance P. These neurons also express mRNA for *Pax 3*, a homeodomain-containing gene which has so far only been found within sensory neurons within the peripheral nervous system (Kobler, S., Murphy, M. and Bartlett, P. F., unpublished observations). However, since there are no definitive markers for sensory neurons, we cannot definitively ascribe a lineage to the neurons generated in our NC cultures. However, we have shown that LIF can act specifically at the stage of differentiation of sensory neurons from their precursors in dorsal root ganglia cultures (Murphy et al., 1993).

Two related factors, which signal through the same receptor system, CNTF and OSM, shared the capacity of LIF to stimulate the development of neurons in these cultures and thus it remains to be determined which of these factors may contribute to neuronal development in vivo. Initial findings of mice deleted for either LIF or CNTF did not report any defects in sensory development (Stewart et al., 1992; Masu et al., 1993) although more recently studies indicate that there are changes in sensory neurons in mice deleted for the LIF gene (Patterson et al., Soc. Neurosci. abstract 1993). We have also detected LIF mRNA in the developing DRG at a time when neuronal differentiation is occurring (Murphy et al., 1993), suggesting that LIF might play a role in sensory development. Likewise, in situ hybridisation analysis for CNTF alpha receptor expression showed high levels in the developing DRG (Ip et al., 1993), implying a role for this receptor component in sensory neuron development. Preliminary observations of CNTF receptor knockout mice show that there are sensory deficits probably within the DRG (G. Yancopoulos, personal communication).

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