The Lack of Effect of Basic and Acidic Fibroblast Growth Factors on the Naturally Occurring Death of Neurons in the Chick Embryo

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***In vivo* treatment of developing chick embryos with acidic and basic fibroblast growth factors (aFGF and bFGF) failed to affect the differentiation and survival of several populations of developing neurons in the CNS and PNS. All of the neuronal populations examined are known to undergo naturally occurring cell death, and they include spinal and cranial motoneurons, dorsal root ganglia, sympathetic ganglia, nodose ganglia, ciliary ganglia, and sympathetic preganglionic neurons in the PNS, as well as the accessory oculomotor nucleus, the isthmo-optic nucleus, and the brainstem auditory nuclei laminaris and magnocellularis in the CNS. Despite the lack of effect of bFGF on neuronal survival and differentiation, *in vivo* treatment increased the serum levels of bFGF and stimulated the proliferation of non-neuronal cells in the spinal cord. Therefore, although the administration of exogenous FGF to the developing chick embryo *in vivo* clearly has some biological activity in the CNS, it was nonetheless ineffective in promoting neuronal survival or differentiation. These data do not support the idea that FGF is a physiologically relevant neurotrophic agent in the developing avian nervous system.**

The heparin-binding family of growth factors has been suggested to regulate a variety of cellular and molecular events during vertebrate development (Burgess and Macaig, 1989). For example, some of the best-studied members of this family, basic and acid fibroblast growth factor (bFGF and aFGF), are reported to affect the induction, mitotic division, survival, morphology, cell–cell interactions, and differentiation of many mesenchymal and neuroectoderm derivatives (Walicke, 1989; Gospodarowicz, 1990; Westermann et al., 1990; Wagner, 1991). Notable among the myriad reported effects of aFGF and bFGF on neuroectoderm-derived cells (neurons and glia) is the ability of these agents to promote the survival of different types of developing neurons *in vitro* (e.g., Kalcheim, 1989; Walicke, 1989; Eckenstein et al., 1990; Matsuda et al., 1990; Westermann et al., 1990; Biederman et al., 1991; Sweetnam et al., 1991), including spinal

cord motoneurons (MNs; Unsicker et al., 1987; Arakawa et al., 1990; but see Dohrmann et al., 1987; Bloch-Gallego et al., 1991). FGFs have also been reported to prevent cell death and other regressive changes induced by axotomy, injury, or genetic mutations in retinal ganglion cells (Sievers et al., 1987; Mattson and Rychlik, 1990), photoreceptors (Faktorovich et al., 1990), hippocampal neurons (Barotte et al., 1989), preganglionic neurons (Blottner et al., 1989), dorsal root ganglion (DRG) cells (Otto et al., 1987), and dopaminergic neurons (Otto and Unsicker, 1990) of adult animals. Thus, FGF may be a trophic-like agent that regulates both the normal survival of developing neurons and the survival and maintenance of mature neurons following injury.

Because we have been interested in the trophic requirements of developing spinal cord MNs *in vivo*, we have begun to screen a number of putative neurotrophic agents, including FGF, for their effectiveness in reducing the normal cell loss observed during naturally occurring MN death (Oppenheim, 1991). In virtually all vertebrates that have been examined, approximately one-half of all postmitotic spinal MNs undergo degeneration and cell death during embryogenesis. The survival of a proportion of postmitotic MNs during this period is partly regulated by neuron–target interactions, and the amount of survival can be increased by *in vivo* treatment with muscle-derived as well as by non-muscle-derived molecules (Oppenheim et al., 1988, 1991; McManaman et al., 1990). In the present study, we have examined the effectiveness of FGFs in promoting the *in vivo* survival of spinal MNs as well as the survival of a variety of other neuronal populations that are known to undergo naturally occurring neuronal death in the chick embryo.

Portions of this work have been published previously in abstract form (Oppenheim et al., 1990).

Materials and Methods

Embryos and FGF treatment. Eggs were obtained as a generous gift from Hubbard Farms, Statesville, NC. The eggs were incubated in the laboratory at 37°C and 60% relative humidity and were automatically turned several times per day. On the day before they were to be used in an experiment, the eggs were removed from the incubator and a small window was made on the side of the egg over the embryo thereby exposing the vascularized chorioallantoic membrane (CAM). The window was then sealed with Parafilm (Sigma) and the egg returned to an incubator, where they were no longer turned. For most experiments reported here, embryos were treated daily from embryonic day 6 (E6) to E9 or from E9 to E13–E14 with aFGF or bFGF and then killed on E10 or E14–E15. In one experiment, embryos were treated daily from E8 to E13. In all of the above experiments, treatment consisted of daily application of either human recombinant aFGF, which was provided by Ralf Pettersson (Cao and Pettersson, 1990), or human recombinant bFGF, which was provided by California Biotechnology, Inc. (Mountain

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View, CA), or of control substances (saline, heparin, or cytochrome C, Sigma), onto the CAM. Although administration of putative trophic agents and other substances onto the CAM has been repeatedly shown to be an effective mode of treatment of chick embryos *in ovo* (e.g., Oppenheim et al., 1991), in one group of embryos FGF or control solutions were injected directly into the vitreous of the right eye *in ovo* on E10 or E11 and embryos were allowed to survive to E11 or E12, at which time they were killed and processed histologically for examination of cell number in the ciliary ganglion (CG; see below). In another group of embryos, bFGF (3–5 μ g) was injected intramuscularly into the right leg *in ovo* on E8 and the animals allowed to survive for 24 hr (E9), at which time they were killed, processed histologically, and examined for pyknotic motoneurons in the lumbar spinal cord (see below). Because heparin is known to protect aFGF (and to a lesser extent bFGF) from acid, heat, and protease degradation (Gospodarowicz, 1990), and because heparin or heparin-like molecules modulate biological activity and appear necessary for the functional binding of FGF to its receptors (Klagsbrun and Baird, 1991; Ruoslahti and Yamaguchi, 1991; Savona et al., 1991; Yayon et al., 1991), some of the treatment paradigms noted above included FGF treatment both with and without heparin. When heparin was used, it was administered at either 25 or 50 μ g per injection.

Radioimmunoassay and *in vivo* mitogenic assay. In order to examine whether FGF applied to the extraembryonic CAM of the avian egg had access to the embryo, a sensitive two-site radioimmunoassay was used to measure serum levels in the embryonic circulatory system on E8. Thirty minutes following the administration of 10 μ g or 20 μ g of bFGF (both with and without 25 μ g of heparin) onto the CAM, embryos were removed from the egg and decapitated and blood was obtained and pooled from six embryos per group. These samples were centrifuged (600 $\times$ *g* for 5 min) and the serum collected and stored at -70°C . The two-site ELISA assay used was a modification of Sato et al. (1989). Briefly, this assay employed glutaraldehyde-fixed Balb5C/3T3 cells as a heparin matrix to bind FGF in the first reaction and a labeled monoclonal antibody in the second reaction. Specific FGF was determined by subtracting the sample background obtained when the first FGF binding step is carried out in the presence of 10 μ g/ml soluble heparin. The minimum sensitivity of this assay is approximately 1 ng/ml (Sato et al., 1989).

In order to examine the mitogenic effect of FGF on spinal cord cells, an antibody to the thymidine analog 5-bromo-2'-deoxyuridine (BrdU; Becton-Dickinson) was used for immunocytochemical quantitation of proliferation in the lumbar spinal cord (Gratzer, 1982; Hayashi et al., 1988; Miller and Nowakowski, 1988). Embryos were administered bFGF (5 μ g) onto the CAM in the morning of E8 and E9. One hour after the second treatment on E9, they were given 10 μ g of BrdU onto the CAM (Sigma) and then killed 4 hr later. The spinal cord was removed and placed in Carnoy's fixative overnight, processed for paraffin histology, sectioned (10 μ m), and reacted immunohistochemically with the BrdU antibody (diluted 1:20) using a Vector ABC kit for diaminobenzidine (immunoperoxidase) immunoreactivity. Quantitation was carried out as described below.

Histology, cell counts, and morphometrics. Cell counts of spinal motoneurons, spinal sensory (DRG) and sympathetic ganglion (SG) neurons, and sympathetic preganglionic neurons were obtained from transverse sections (10–12 μ m) of the entire thoracolumbar region of the embryo. Motoneurons were counted in every tenth section through the entire lumbar spinal cord. Sensory and sympathetic neurons were counted in every fifth section through the third lumbar segmental ganglion (L3), and preganglionic cells were counted in every tenth section through the fifth and sixth thoracic segments (T5–6). Nodose and ciliary ganglia were dissected out and processed independently. In both ganglia, cells were counted in every fifth section. Neurons were also counted in every fifth section through several brain nuclei that have been previously shown to undergo naturally occurring cell death. These included the isthmo-optic nucleus, the cochlear nuclei laminaris and magnocellularis, the trochlear and abducens nuclei, the mesencephalic nucleus of the trigeminal nerve, the isthmo-optic nucleus, and the accessory oculomotor nucleus. All tissues were embedded in paraffin, sectioned, and stained with thionin as described previously (Chu-Wang and Oppenheim, 1978). Only cells that exhibited *all* of the following characteristics were included in the counts: large soma with substantial cytoplasm, large nucleus, and a single large clump of nucleolar material (Fig. 1). With these criteria, it was not necessary to apply correction factors for double counting (see Oppenheim et al., 1989). Quantitation of non-neuronal (glia and endothelial) cells involved counting all small cells in

the lumbar ventral horn or lateral motor column (LMC) in every tenth section. These non-neuronal cells were clearly distinguishable from both the large motoneurons and the few smaller interneurons present in the LMC (Fig. 1). Counts of BrdU-labeled cells were made from single transverse sections through segment L4 and included *all* heavily labeled cells in both the white and gray matter of the entire spinal cord section (Fig. 1). Degenerating (pyknotic) cells were identified using previously established criteria (Chu-Wang and Oppenheim, 1978). Pyknotic cells were counted in every tenth section through the LMC on either E8 or E9, and in every fifth section through CG on E12 following treatment with bFGF (5 μ g) onto the CAM from E6 to E8 (LMC), or E8 to E12 (CG), or following intramuscular injection on E8 (LMC).

Cell size was assessed by measuring the nuclear diameter or the soma diameter (or both) of CG cells and motoneurons in the lumbar LMC. Only those cells that met all of the same criteria for inclusion in the cell counts (see above) were utilized. Because nuclear diameter is proportionate to soma size among chick spinal motoneurons (R. W. Oppenheim, D. Prevette, and F. Fuller, unpublished observations), this measure is a reasonably accurate index of cell size. The outline of the nucleus or soma was drawn using a drawing tube, and the average of the longest and shortest diameter was estimated using a computerized morphometric program (SIGMASCAN). In all cases of cell counts and morphometric analyses, the observer was unaware (blinded) of the treatment status of each preparation being evaluated.

Results

Radioimmunoassay and mitogenic assay

Following the administration of exogenous human recombinant bFGF onto the vascularized CAM of E8 chick embryos, serum levels of immunologically active bFGF were significantly elevated (Table 1). Similar results were obtained regardless of whether bFGF was administered with heparin (25 μ g) or without. These serum levels are several orders of magnitude higher than the ED_{50} of the human recombinant bFGF used here for promoting the survival of avian ciliary neurons *in vitro* (i.e., 50 pg/ml; F. Fuller, unpublished observations).

BrdU is incorporated into the nuclei of S-phase cells, where it can be identified immunohistochemically by an anti-BrdU monoclonal antibody, and has been demonstrated to be a sensitive and reliable marker of proliferation in the vertebrate CNS, generating data that are in close agreement with those obtained using tritiated thymidine autoradiography (Miller and Nowakowski, 1988; Lundy et al., 1991). Following 2 d of treatment with 5 μ g of bFGF (E8 and E9), the number of BrdU-immunolabeled cells in the lumbar spinal cord was increased by approximately 30% (Table 2). Because partial hydrolysis to denature the DNA is necessary in preparing tissue for BrdU immunohistochemistry, it was not possible to identify distinct cell types in thionin-counterstained tissue. However, it is almost certain that the BrdU-labeled cells were non-neuronal. Studies using tritiated thymidine autoradiography have shown that neuronal proliferation has ceased by E7–E8 in the chick spinal cord (Fujita, 1964; Langman and Haden, 1970; McKay and Oppenheim, 1991). Furthermore, as indicated in Figure 1, many of the BrdU-labeled cells are located in regions devoid of neurons (white matter, dorsal root, glycogen body). Finally, whereas the number of MNs in the LMC was unchanged on E10 following treatment with bFGF (see below), counts of non-neuronal cells in the LMC were significantly increased (Table 2). Therefore, it seems quite likely that bFGF administered onto the CAM had access to cells including neurons in the CNS. Although we consider it unlikely, we cannot exclude the possibility that the amount of FGF in the nervous system was sufficient to have a mitogenic effect on non-neuronal cells but insufficient for affecting neuronal survival. However, the fact that the serum levels of FGF were

Figure 1. *A* and *B*, Transverse frozen sections ($15\ \mu\text{m}$) through the lumbar spinal cord of E9 control (*A*) and bFGF-treated (*B*) embryos examined with a monoclonal antibody to BrdU. Small dark-stained profiles distributed throughout the sections reflect BrdU-labeled cells in the S-phase of mitosis. *c*, central canal; *dr*, dorsal root; *gb*, glycogen body; *gm*, gray matter; *wm*, white matter. The large black dots in *A* indicate the boundary between white and gray matter. Scale bar, $210\ \mu\text{m}$. *C* and *D*, Transverse sections of E15 control (*C*) and bFGF-treated (*D*) CG stained with thionin (Nissl stain). *p*, a pyknotic neuron. Scale bar, $45\ \mu\text{m}$. *E* and *F*, Transverse sections through the lumbar ventral horn on E10 of control (*E*) and bFGF-treated (*F*) embryos. Asterisks indicate examples of small, non-neuronal cells included in the cell counts described in Materials and Methods. *mn*, motoneuron. Scale bar, $8\ \mu\text{m}$.

Figure 2. Mean ($\pm$ SD) number of MNs on E10 following treatment with various doses of bFGF from E6 to E9. Numbers in columns are sample sizes.

several orders of magnitude higher than the ED_{50} of FGF for promoting CG survival *in vitro* argues strongly against this possibility.

Neuronal numbers

Treatment *in vivo* (E6–E9) with a wide range of doses of bFGF had no apparent effect on the survival of spinal MNs irrespective of whether the bFGF was administered with (data not shown) or without heparin (Fig. 2). Heparin alone (25 μ g) was also ineffective (not shown). Direct injections of bFGF into the hindlimb on E8 failed to reduce the total number of pyknotic MNs present in the lumbar region 24 hr later (control, 84 ± 8 , $n = 4$, vs. bFGF-treated, 79 ± 11 , $n = 5$). Similarly, *in vivo* treatment (E9–E14) with several different doses of bFGF was also without effect on the survival of neurons in the CG (Fig. 3, Table 3). Moreover, treatment with bFGF from E8 to E13, following a protocol previously reported to prevent cell death in the chick CG *in vivo* (Dreyer et al., 1989), was also ineffective in promoting neuronal survival (Fig. 4). Injections of bFGF directly into the vitreous of the eye *in ovo* on E10–E11 also failed to alter the normal course of cell death in the CG (Table 3). Although aFGF was only administered at a single dose from E6 to E9 or from E9 to E14 (5 μ g/day) in the presence of heparin (25 or 50 μ g),

Figure 3. Mean ($\pm$ SD) number of ciliary neurons on E14 following treatment with various doses of bFGF from E9 to E13. Numbers in columns are sample sizes.

it also failed to promote the survival of spinal MNs, DRG cells, CG cells, or several populations of CNS neurons (Fig. 5, Table 4). The lack of a difference in the numbers of pyknotic cells per 1000 healthy neurons provides independent confirmation of the ineffectiveness of bFGF on either MN or CG survival [pyknotic MNs, E10: control, 16.6 ± 3.8 ($n = 8$) vs. bFGF, 15.3 ± 5.1 ($n = 6$); pyknotic CG, E12: control, 12.3 ± 3.4 ($n = 5$) vs. bFGF, 10.9 ± 3.3 ($n = 5$)]. Finally, treatment with bFGF (10 μ g) from E6 to E9 also failed to alter the number of surviving neurons in the SG, DRG, and nodose ganglia or in the sympathetic preganglionic nucleus of T_{erni} (Table 5), and treatment from E9 to E14 did not promote survival in several brain nuclei on E15 (Table 4). All of the populations of neurons examined are known to exhibit naturally occurring cell death during the treatment periods (Oppenheim, 1991).

Despite the complete lack of effect of either aFGF or bFGF on neuronal survival, it is still possible that these agents can influence other aspects of growth or differentiation. For instance, FGF has been shown to enhance the cholinergic development of rat spinal cord cells *in vitro* and chick CG cells *in vivo* (McManaman et al., 1989; Hill et al., 1991) and NGF is well known to affect neuronal size and process outgrowth (Snider and Johnson, 1989). However, measures of soma and nuclear size among MNs and CG cells were unchanged following treatment with either bFGF or aFGF (Table 6). Furthermore, cytological characteristics of these cells following thionin staining also appeared unaffected by treatment with either aFGF or bFGF (Fig. 1).

Table 1. Two-site radioimmunoassay of serum bFGF levels following *in vivo* treatment with bFGF in the chick embryo

Group	FGF (ng/ml)
Normal serum	<0.6
Saline	<0.6
Heparin (25 μ g)	<0.6
FGF (10 μ g + heparin)	53.0
FGF (20 μ g + heparin)	>75.0
FGF (10 μ g)	53.0
FGF (20 μ g)	>75.0

$n = 6$ for all groups. Because serum samples were pooled from all six embryos in each group, it was not possible to determine measures of variance. The values are average serum levels per embryo.

Table 2. Effects of bFGF on non-neuronal cells

	BrdU-labeled cells	Non-neuronal cells in LMC
Control	350 $\pm$ 46 (16)	18,764 $\pm$ 2041 (4)
bFGF	493 $\pm$ 63* (19)	28,508 $\pm$ 3825** (4)

Values are means $\pm$ SD of numbers of cells; sample sizes are given in parentheses. * $p < 0.001$; ** $p < 0.01$ (t tests).

Figure 4. Mean ($\pm$ SD) number of ciliary neurons on E14 following treatment with two doses of bFGF. bFGF or cytochrome C (Cyto-C) was administered on E8, E9, E11, and E13 as described in Dreyer et al. (1989). Numbers in columns are sample sizes.

Discussion

FGF and related heparin-binding polypeptide growth factors have been previously suggested to be candidate molecules that may act as neurotrophic survival agents in the developing nervous system. Evidence in support of this contention comes mainly from *in vitro* studies in which the survival of a wide variety of neurons is reported to be promoted by these agents (Walicke, 1989; Westermann et al., 1990). However, even when tested *in vitro*, not all types of neurons exhibit survival effects following treatment with FGF (Rydel and Greene, 1978; Lipton et al., 1988; Stemple et al., 1988; Walicke, 1988; Matsuda et al., 1990). In the chick embryo, some investigators have reported that FGF increases the survival or differentiation of CG, sensory ganglion, and SG neurons, as well as spinal MNs *in vitro* (Unsicker et al., 1987; Arakawa et al., 1990; Eckenstein et al., 1990; Grothe et al., 1991b), whereas other investigators have failed to find an effect of FGF on either cultured chick spinal MNs (Dohrmann et al., 1987; Bloch-Gallego et al., 1991) or cultured neural crest derivatives (Rydel and Greene, 1987; Unsicker et al., 1987; Stemple et al., 1988), including sympathetic, sensory, and chromaffin cells. Although some of these apparent discrepancies can probably be explained by differences in the age of the neurons or other methodological differences, others are more difficult to explain in this way.

Because putative trophic agents may promote neuronal survival *in vitro* by means that are not normally operative *in vivo*, we have chosen to examine the effects of FGF on the magnitude of naturally occurring cell death *in vivo* as an alternative and

Figure 5. Mean ($\pm$ SD) number of motoneurons (MNS), DRG, and CG neurons following treatment with aFGF (5 μ g) plus heparin (50 μ g). Numbers in columns are sample sizes.

more biologically relevant model for neurotrophic activity. One previous study has reported that bFGF treatment *in vivo* rescues chick ciliary neurons from naturally occurring cell death (Dreyer et al., 1989), a finding that is consistent with the observed survival effects of bFGF on ciliary neurons *in vitro* (Unsicker et al., 1987; Eckenstein et al., 1990). However, as shown here, we have been unable to replicate this finding (also see Hill et al., 1991). One major apparent difference between our study and that of Dreyer et al. (1989) is that neurons were always counted blind in the present study.

Both FGF protein and FGF receptors and their respective mRNAs are present in the nervous system of developing avian embryos (Mascarelli et al., 1987; Janet et al., 1988; Heuer et al., 1990; Kalcheim and Neufeld, 1990; Mitrani et al., 1990; Schnürch and Risau, 1991; Hondermarck et al., 1992). Chorio-allantoic fluid of E9 chick embryos also contains significant levels of biologically active (mitogenic) bFGF (Flamme et al., 1991), and developing limb buds and skeletal muscle also exhibit significant bFGF immunoreactivity (Kalcheim and Neufeld, 1990). Similar results have been reported for the nervous system and skeletal muscle of embryonic and postnatal mouse and rat (Janet et al., 1987; McManaman et al., 1989; Caday et al., 1990; Reid et al., 1990; Wanaka et al., 1990, 1991; Grothe et al., 1991a; Wilcox and Unnerstall, 1991). Thus, FGF and its receptor appear to be present at the appropriate ages and in the appropriate regions *in vivo* (PNS, CNS, or target regions) to play a role in neuronal survival (but see Heuer et al., 1990). However, it is also possible, even though it is present at the right time and place, that FGF is involved in aspects of neuronal or non-neuronal development other than survival. For example, FGF is known to act as a mitogen for a variety of precursor cell types, including glioblasts, osteoblasts, neuroblasts, myoblasts, capil-

Table 3. Effects of bFGF on ciliary neuron numbers (mean $\pm$ SD)

E12			E14			
Control (saline)	FGF		Control (saline)	FGF (5 μ g)	FGF + heparin (5 μ g + 50 μ g)	Heparin (50 μ g)
	2 μ g	5 μ g				
3191 $\pm$ 476	3229 $\pm$ 450	3025 $\pm$ 657	2876 $\pm$ 361	2820 $\pm$ 310	2916 $\pm$ 335	2905 $\pm$ 287
(11)	(11)	(15)	(5)	(5)	(5)	(6)

The number of embryos is given in parentheses.

Table 4. Cell numbers (mean $\pm$ SD) in several brain nuclei on E15 following daily treatment with bFGF or aFGF (5 μ g) from E9 to E14

Nucleus	Control (n)	Experimental (n)		% Normal cell loss between E10 and E15 ^a
		bFGF	aFGF	
Accessory oculomotor (Edinger-Westphal)	1155 $\pm$ 186 (4)	1080 $\pm$ 150 (6)	1030 $\pm$ 163 (5)	46%
Isthmo-optic	7855 $\pm$ 932 (4)	7710 $\pm$ 1003 (6)	7547 $\pm$ 1246 (5)	50%
Mesencephalic V	1442 $\pm$ 203 (4)	1367 $\pm$ 185 (6)	1356 $\pm$ 393 (5)	71%
Magnocellularis	3523 $\pm$ 576 (4)	3646 $\pm$ 497 (6)	3345 $\pm$ 531 (5)	18%
Laminaris	1525 $\pm$ 149 (4)	1439 $\pm$ 214 (6)	1431 $\pm$ 199 (5)	38%
Trochlear	678 $\pm$ 149 (4)	751 $\pm$ 122 (6)	647 $\pm$ 112 (5)	36%
Abducens	660 $\pm$ 93 (4)	705 $\pm$ 87 (6)	730 $\pm$ 150 (5)	44%

Sample sizes are given in parentheses.

^a Normative data on cell death between E10 and E15 are from unpublished observations (R. W. Oppenheim). However, these data are in close agreement with published data on cell death in these populations (see Oppenheim, 1981, 1991).

lary endothelial cells, hematopoietic cells, and neural crest-derived neuronal precursors (Pettman et al., 1985; Gensburger et al., 1987; Ferrara et al., 1988; Hatten et al., 1988; Stemple et al., 1988; Frenkel and Singh, 1989; Davis and Stroobant, 1990; Gabbinelli et al., 1990; Gospodarowicz, 1990; McKinnon et al., 1990; Eckenstein et al., 1991; Lundy et al., 1991; Morrison, 1991). Alternatively, FGF may only affect neuronal survival indirectly by increasing the availability of trophic agents derived from some of the above populations of non-neuronal cells (Engle and Bohn, 1991; Petroski et al., 1991). The *in vivo* model used in the present study has the advantage of being able to detect both *direct* and *indirect* effects of FGF on neuronal survival, but it also has the disadvantage of not being able to distinguish between these two modes of action. In view of the general lack of effect of FGF on neuronal numbers observed here, we conclude that in the present context FGF has neither direct nor indirect effects on neuronal survival *in vivo*.

Because FGF had no effect on the *in vivo* survival of any of the different populations of neurons examined in the present study, it is conceivable that the human recombinant FGF used here is not biologically active on neurons in the chick embryo. This seems exceedingly unlikely, however, since bFGF is known to be a highly conserved protein (Gospodarowicz, 1990) and at least the bFGF used here does, in fact, promote the survival of

chick CG neurons *in vitro* (see Materials and Methods). Alternatively, the main route of administration used by us, involving injection onto the CAM, may not provide access of FGF to the embryo. Clearly this is not the case, however, since administration onto the CAM resulted in biologically significant levels of bFGF in embryonic serum as measured by a sensitive two-site radioimmunoassay. Furthermore, embryos treated in this way, exhibited an *in vivo* mitogenic effect of FGF on spinal cord non-neuronal cells (glia), and this effect was observed both with and without added heparin. This *in vivo* effect on non-neuronal proliferation supports previous *in vitro* studies that found a mitogenic effect of FGF on glial precursors (Hatten et al., 1988; Davis and Stroobant, 1990; Gospodarowicz, 1990; Morrison, 1991). Finally, injections of bFGF with and without heparin directly into the embryo *in ovo* (limb bud or vitreous of the eye) also failed to affect the survival of either spinal MNs or ciliary neurons. In view of these various controls, we conclude that exogenous FGF administered to the chick embryo *in vivo* exhibits biological activity in the CNS (mitogenic for non-neuronal

Table 5. Neuronal numbers (mean $\pm$ SD) following *in vivo* treatment with bFGF (10 μ g)

Cell type	Control	bFGF
Sympathetic (L3)	10,675 $\pm$ 1011	10,820 $\pm$ 964
Preganglionic (T5-6)	3004 $\pm$ 180	3142 $\pm$ 297
Dorsal Root (L3)	10,033 $\pm$ 1006	9458 $\pm$ 875
Nodose	5317 $\pm$ 621	5195 $\pm$ 572

n = 5 for all.

Table 6. Cell size following FGF treatment

	MN (E10) nuclear diameter (μ m)	CG (E14)	
		Soma diameter (μ m)	Nuclear diameter (μ m)
Control	7.9 $\pm$ 0.8 (35)	14.8 $\pm$ 7.7 (170)	6.6 $\pm$ 1.0 (184)
bFGF ^a	8.2 $\pm$ 1.1 (102)	15.2 $\pm$ 6.5 (60)	6.4 $\pm$ 1.4 (182)
aFGF	8.0 $\pm$ 1.2 (35)	14.9 $\pm$ 6.1 (141)	6.8 $\pm$ 1.6 (140)

Sample sizes are given in parentheses.

^a Data were pooled from embryos receiving 0.2, 2.0, and 10.0 μ g bFGF. There were no significant differences between these groups.

cells) but is without effect on neuronal survival. Thus, it seems unlikely that FGF is a biologically relevant neurotrophic agent for any of the populations of developing neurons examined here. FGF also did not appear to affect the growth or cytological differentiation of either MNs or CG cells. However, we cannot exclude the possibility that FGF may regulate other aspects of neuronal differentiation. *In vitro* and *in vivo* studies have shown that FGF enhances cholinergic differentiation of spinal cord and ciliary neurons and promotes neuronal growth in a variety of different types of neurons (Walicke et al., 1986; McManaman et al., 1989; Westermann et al., 1990; Grothe et al., 1991; Hill et al., 1991). In view of the lack of effect of FGF on the *in vivo* survival of either avian MNs or CG cells, it is of some interest that *adult* rat MNs and *postnatal* chick CG fail to exhibit retrograde transport of aFGF or bFGF (Ferguson and Johnson, 1991; Hendry and Belford, 1991). By contrast, *adult* rat spinal MNs express high levels of aFGF mRNA and protein (Elde et al., 1991). MN-derived FGF could serve an autocrine or paracrine function or may be a source of retrograde trophic support for innervating interneurons.

The apparent differences in the *in vivo* versus *in vitro* effects of FGF on neuronal survival are, on the face of it, difficult to explain. It is possible that the positive effects *in vitro* reflect the response of injured neurons that are damaged or injured during processing for tissue culture. According to this view, only injured neurons would be responsive to the survival-promoting effects of FGF. Evidence in support of this view comes from the observation that some of the populations of developing neurons shown here to be unresponsive to FGF *in vivo* respond to FGF following injury in the adult animal (Otto et al., 1987; Sievers et al., 1987; Blottner et al., 1989). Moreover, bFGF protein and mRNA are rapidly increased in areas adjacent to a brain lesion in adult rats (Frautschy et al., 1991). In addition, we have found that bFGF treatment *in vivo* can prevent the induced death of spinal MNs following deafferentation in the chick embryo (Okado and Oppenheim, 1984; Yin et al., 1991), even though, as reported here, FGF has no effect on the *naturally occurring* death of spinal MNs. One interpretation of these results is that some populations of neurons may change their trophic dependency from non-FGF to FGF proteins between embryogenesis and maturity (e.g., Barde et al., 1988). It is also possible that developing neurons do require FGF for survival but that they develop *in vivo* in the presence of saturating concentrations of endogenous FGF such that their requirement is fulfilled and therefore additional exogenous FGF is ineffective. However, if this were the case, then there should be no naturally occurring cell death. Because aFGF and bFGF both lack a hydrophobic signal sequence required for secretion by the classical secretory pathway, it is also conceivable that these agents are only released in sufficient amounts to promote neuronal survival following tissue or cell injury. Accordingly, the present results as well as previous studies indicate that FGF may not be a biologically relevant trophic agent for the survival and maintenance of either developing or mature intact neurons. Rather, FGF may only become effective following neuronal damage and may therefore be a plausible candidate as a pharmacological, therapeutic agent for promoting the survival of neurons at risk following injury, in genetic mutations, or in neurodegenerative disease. Injured neurons may upregulate the expression of FGF or other neurotrophic factor receptors.

In conclusion, despite the increased serum levels of FGF and the significant mitogenic effect on non-neuronal cells in the spi-

nal cord of chick embryos following *in vivo* treatment with FGF, FGF failed to alter the survival or differentiation of several populations of neurons that exhibit naturally occurring or programmed neuronal death. These include spinal MNs, SG and parasympathetic ganglion cells, spinal sympathetic preganglionic cells, sensory neurons in the DRG and nodose ganglia, and several populations of neurons in the brain. On the basis of these consistently negative data, we conclude that FGF is unlikely to be a biologically relevant neurotrophic agent for the survival of developing neurons in the avian embryo.

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