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# CELLULAR DISTRIBUTION, SUBCELLULAR LOCALIZATION AND POSSIBLE FUNCTIONS OF BASIC AND ACIDIC FIBROBLAST GROWTH FACTORS

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**Abstract**—The distribution in the rat nervous system of acidic and basic fibroblast growth factors (FGFs) was analysed by a combination of biochemical and anatomical methods. Acidic FGF (aFGF) was found to be present exclusively in specific neuronal populations, such as motor neurons and basal forebrain cholinergic neurons. Basic FGF (bFGF) was found in astrocytes and in neurons in hippocampal area CA2. Within labelled astrocytes and CA2-neurons, bFGF was detected in both the cytoplasm and the nucleus. The levels of intracellular bFGF were manipulated by antisense oligonucleotide treatment of cultures of developing neural crest cells. Results indicated that the amount of melanogenesis in the cultures is likely to be regulated by intracellular, possibly nuclear bFGF.

The family of FGFs† consists of at least seven different members, all encoded by different genes [1]. All FGFs show substantial homology in their core regions, which exhibit about 50% amino acid sequence identity. All FGFs appear to bind with relatively high affinity to heparin, thus they are also referred to as heparin binding growth factors. Some members of the family also contain amino acid sequences homologous to nuclear translocation signals, and some FGFs have been reported to be present in the nucleus [2–4]. A major difference between family members is whether they either lack or contain a traditional hydrophobic signal peptide sequence, which is thought to be necessary for the sorting of polypeptides into the traditional secretory pathways [5]. It is of interest in this respect that the first two FGFs to be isolated, aFGF and bFGF, do not contain such a signal peptide [1]. These two FGFs are relatively abundant in the CNS, possibly because they accumulate intracellularly, which may not occur in the case of other FGFs that are efficiently secreted from cells by traditional pathways. Indeed, mRNA coding for FGF-5, an additional member of the FGF family which contains a signal peptide, is known to be expressed in the CNS [6]. However, steady state levels of FGF-5 protein levels in CNS appear to be minor (in fact FGF-5 protein has so far not been described in CNS), possibly due to the high turnover of secreted FGFs.

As their name implies, FGFs were first purified as agents that promote mitogenesis in fibroblasts, but it has now become clear they they are mitogens for a variety of mesodermal and neuroectodermal cell types [1]. In addition, FGFs can promote the survival of many different peripheral and central neuronal populations [7–9], and may also affect differentiation in a variety of cell types [10–12]. The wide array of FGF effects is to a large extent mediated by a family of sequence-homologous transmembrane receptors. This FGFR family currently consists of at least four different genes, that can be differentially spliced to give rise to a large variety of proteins that may differ in their binding affinity for different FGFs [13]. Common features of all the receptors include the presence of two or three extracellular immunoglobulin-like domains, one of which is thought to bind FGFs, and an intracellular tyrosine kinase domain, which is involved in signalling. Interestingly, in order for FGFs to activate the tyrosine kinase receptor, heparan sulphate must be present on the surface of the cell expressing the FGFR [11], suggesting that a cell surface heparan proteoglycan may bind FGF and present it in an active conformation to the FGFR.

Besides effects that are mediated through cell surface FGFRs, the intracellular stores of non-secreted FGFs may also serve specific biological roles. This hypothesis is supported by observations that bFGF is imported into the nucleus of cells at specific stages of the cell cycle [2], where it may regulate the expression of identified RNAs [3]. In addition, intracellular bFGF may affect differentiation in specific cell types [14].

Taken together, it is clear that more knowledge concerning the precise cellular distribution and subcellular localization of FGFs is needed for a rational design of drugs that interact with and exploit the biological effects of FGFs. The following is a review of the authors efforts to define such a distribution in the nervous system, and includes one example where intracellular bFGF may play a role in the cellular differentiation in the development of

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† Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; aFGF, acidic fibroblast growth factor, bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; PDGF, platelet derived growth factor; NGF, nerve growth factor; PNS, peripheral nervous system; NC, neural crest; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; CM, conditioned medium.

the neural crest. This focus of the review is by necessity narrow, and citations referring to broader or differing viewpoints are given where possible.

## RESULTS

### *The distribution of FGFs in the nervous system*

Observations that aFGF and bFGF are relatively abundant in nervous tissue and that these FGFs have potent and broad spectrum neurotrophic activity serves as motivation for further investigations of the role of these factors in nervous tissue. Initially, a biological assay based on measuring the stimulation of mitogenesis in cultured AKR-2B cells was used to determine the levels of FGF-like bioactivity in extracts prepared from different areas of adult rat CNS [15, 16]. In this assay, bFGF is fully active whether heparin is present or not, whereas aFGF is active only in the presence of heparin. Thus, the assay allowed the discrimination of aFGF-like versus bFGF-like bioactivity. In the normal adult CNS, levels of heparin-independent activity were distributed relatively evenly, but no heparin independent activity was found in the PNS. The distribution of heparin-dependent activity, however, showed great variation, with high levels present in sciatic nerve and spinal cord, intermediate levels in midbrain, low levels in forebrain, and was not detectable in cerebral cortex. Interestingly, transection of the peripheral sciatic nerve lead to a complete disappearance of heparin-dependent bioactivity in the disconnected distal stump, whereas transection of the central optic nerve lead to a moderate increase in heparin-independent bioactivity.

The mitogenic assay used for these studies, however, is not specific for FGFs, thus the observations described above were confirmed by western-blot experiments using antibodies that specifically recognized either aFGF or bFGF and showed no cross-activity between the two FGFs. As predicted, protein bands immunoreactive for bFGF (18–23 kDa in molecular mass) were found in similar levels throughout the CNS and were absent from the PNS, and the levels of an aFGF immunoreactive protein (16 kDa) followed closely the distribution of heparin-dependent activity. This strongly supported the concept that the AKR-2B bioassay was able to differentiate and predict the respective levels of aFGF and bFGF, as long as the levels of these FGFs are sufficiently higher than those of other mitogens, such as EGF or PDGF, which may interfere with the assay.

Taken together, the data described above, especially the differential changes in FGF levels observed in peripheral versus central nerve after lesion, led to the hypothesis that aFGF was localized in specific sets of neurons, such as motoneurons (having axons in the sciatic nerve), whereas bFGF seemed more likely to be present in non-neuronal cells specific to the CNS. This hypothesis was tested by immunohistochemical localization of aFGF and bFGF in paraformaldehyde fixed rat CNS [4, 17]. Due to the substantial sequence homology between FGFs, a thorough characterization of the antibodies to be used was needed, in order to assure the monospecificity of these reagents. The monoclonal mouse antibody specific to bFGF employed in the western blot experiments was found to be useful for the localization of bFGF, but none of several available aFGF antibodies exhibited the requisite combination of specificity and histochemical sensitivity. We thus expressed human recombinant aFGF in *E. coli*, purified it to homogeneity and prepared several antisera to this preparation. All antisera were found to exhibit some degree of cross-reactivity between aFGF and bFGF, however, such cross-reactivity could be removed by pre-absorption with bFGF.

Using the bFGF-specific monoclonal antibody, we found bFGF imnmunoreactivity to be present in astrocytes throughout the rat CNS (Fig. 1), and to be absent from the vast majority of neuronal populations. A prominent exception were pyramidal neurons in the CA2 area of hippocampus which were also strongly labelled. More faint labelling was also seen in neurons in the singulate cortex. Interestingly, the distribution of label in both astrocytes and the CA2 hippocampal neurons appeared to be more intense over the nuclei of stained cells and more faint in the cytoplasm, an observation that was confirmed by electron microscopic inspection (Fig. 2). Three different forms of bFGF are known all, originating by differential initiation of translation from a single species of mRNA [18, 19]. Only the two larger of these forms (21 and 23 kDa) contain nuclear translocation sequences, thus we investigated whether it was just these two forms that were present in the nuclei of labelled cells. Rat CNS extracts were separated into fractions enriched for cytoplasm or nuclei, followed by analysis of bFGF-forms by western blot. As predicted, only the 21 and 23 kDa species were found in the nuclei, whereas cytoplasm appeared to contain the additional 18 kDa form. Using the better resolution of staining seen in astrocytes in culture, we found that bFGF immunoreactivity appeared to accumulate in discrete small areas within the nuclei of non-dividing type-1 astrocytes. It will be of interest to determine whether such accumulation occurs in a cell-cycle specific manner, as has been suggested in studies on cells transfected with a bFGF expression vector [2].

The localization of bFGF in astrocytes, together with the demonstrated mitogenic and differentiation-promoting effects of bFGF on these cells, suggest that this factor may act as an autocrine growth regulator for them. It will be of special interest to determine whether increased activity of the FGF/FGFR system may be involved in the formation of astrocytomas.

Immunoreactivity for aFGF was observed in distinct neuronal populations only, as described in detail in Ref. 17. Most intensely stained were motoneurons and sensory neurons, confirming the high levels of aFGF observed in peripheral nerve with other methods, as described above. However, discrete neuronal populations in the mid and forebrain also contained detectable aFGF-immunoreactivity. Most notably, these included the basal forebrain cholinergic neurons and neurons in the substantia nigra. Thus, many neuronal populations known to be affected by slowly progressing neurodegenerative diseases, such as amytrophic

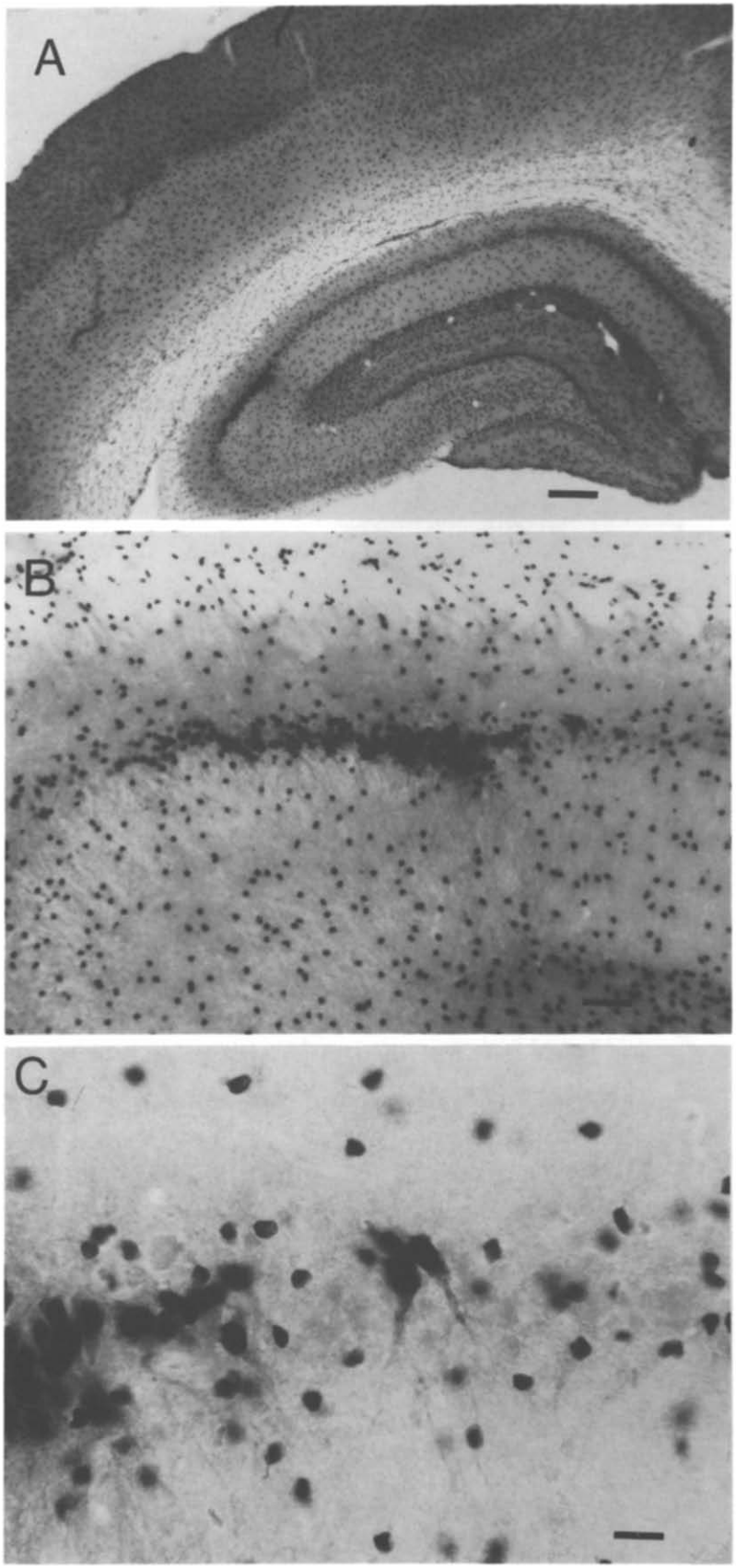


Fig. 1. Immunohistochemical localization of bFGF in a coronal section through adult rat cerebral cortex and hippocampus, using an immunoperoxidase method. Note the even distribution of labelled astrocytes throughout the section and the prominent staining of neurons in area CA2 of hippocampus (panel A, bar = 120 $\mu$m). Panels B (bar = 50 $\mu$m) and C (bar = 20 $\mu$m) show area CA2 at progressively higher magnification. Note the intense staining of the nuclei within labelled neurons.

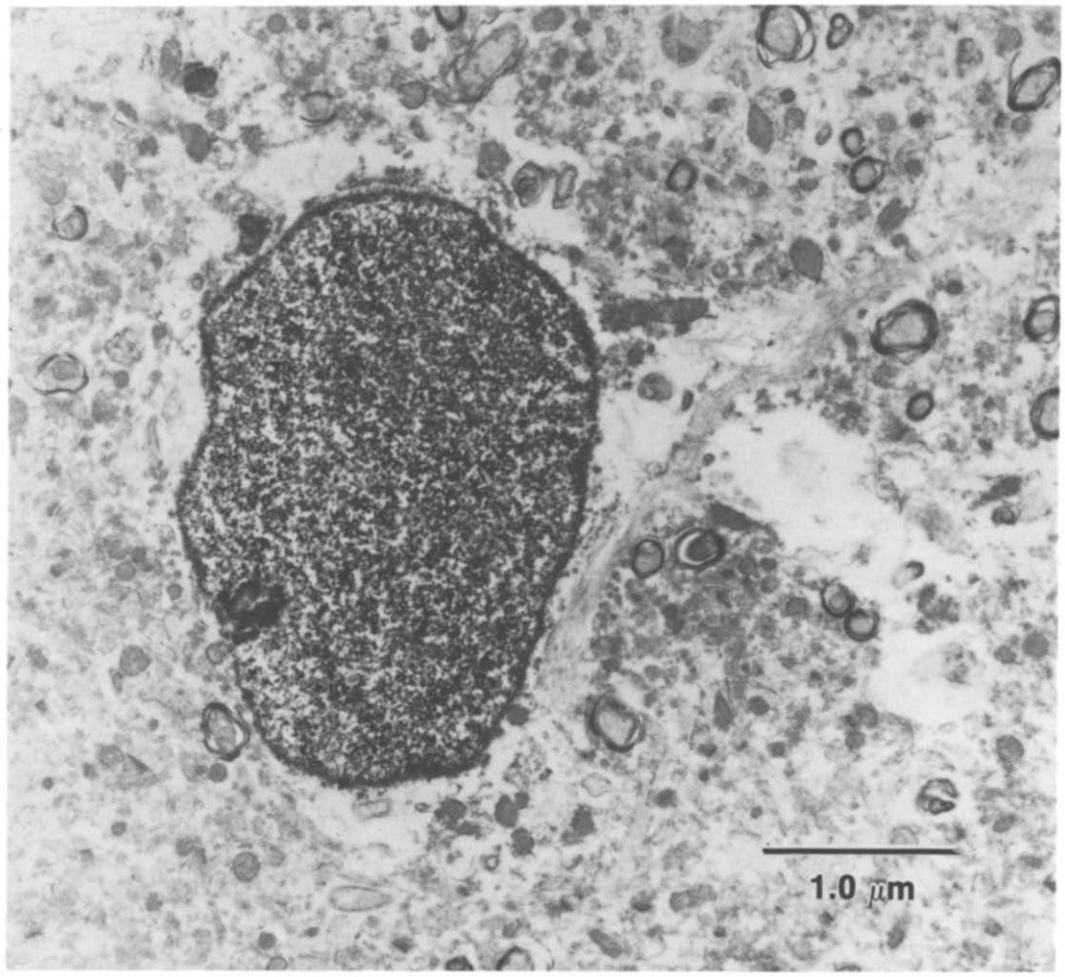


Fig. 2. Electron microscopic analysis of bFGF immunoreactivity in a hippocampal astrocyte. The nucleus of this cell is strongly stained. The cytoplasm of this cell, which is mostly collapsed into a small area around the nucleus, is also labelled. The less than optimal ultrastructural preservation of this specimen is due to the mild fixation protocol needed to preserve bFGF immunoreactivity.

lateral sclerosis (ALS, affecting motoneurons), Parkinson's disease (affecting neurons in the substantia nigra), and Alzheimer's disease (affecting basal forebrain cholinergic neurons), appear to contain aFGF. The survival of these neurons can be promoted by FGFs [20], and it is thus possible that aFGF may act as an unusual autocrine neurotropic factor for populations at risk from degenerative disease. Clearly, the biology of aFGF in these neurons will be different from that of well characterized polypeptide neurotrophic factors, such as NGF, as indicated by the great abundance of aFGF in the cytoplasm of neurons producing the factor. We have estimated, for example, that the concentration of aFGF in motoneurons is at least 1000-fold greater than the abundance of NGF in cells synthesizing this factor. This may simply reflect the absence of a signal peptide in aFGF, leading to its accumulation in a cytoplasmic compartment. Such an abundant cytoplasmic factor would be in an excellent position to signal an even minute leak in the plasma membrane, and to immediately initiate repair, a mechanism that may be of great importance especially for vulnerable neurons with long projections, such as motoneurons, basal forebrain cholinergic and substantia nigra neurons.

Direct nuclear effects of aFGF have also been suggested [21], but we have been unable so far to reproduce these findings. In addition, our immunohistochemical observations indicate that aFGF immunoreactivity is not present in the nucleus of most labelled neuronal populations. There may be, however, a small group of exceptions, including large aFGF-positive neurons in the caudate, which, examined so far with only the light microscope, may contain nuclear aFGF. Thus, extended studies are required to determine whether aFGF is likely to have, besides its role in activating transmembrane receptors, also a direct nuclear function.

It should be pointed out here that several different investigators have studied the distribution of FGFs in the nervous system. Some of the published reports

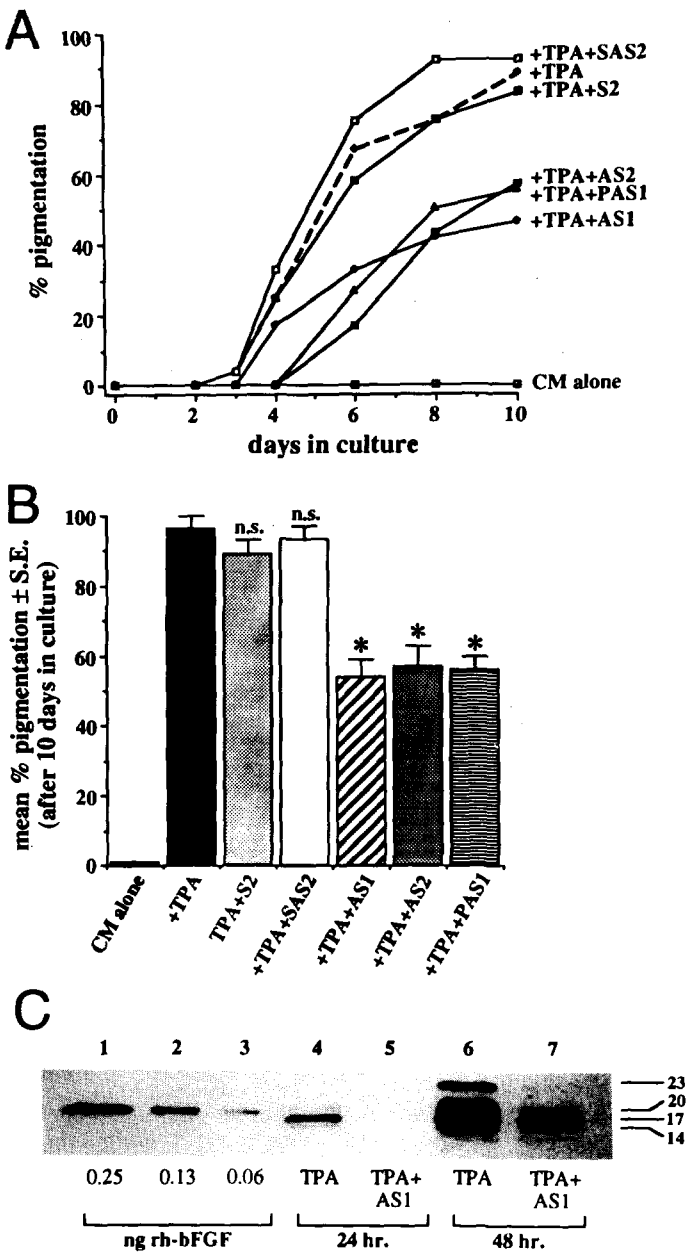


Fig. 3. Effects of bFGF-antisense oligonucleotides on TPA-induced pigmentation and bFGF expression in peripheral nerve explant cultures. Explants were grown for various periods of time in CM supplemented with 1.0 $\mu$M TPA in the presence or absence of 50 $\mu$M bFGF oligonucleotides (antisense oligonucleotides "AS1" or "AS2", scrambled antisense oligonucleotide "SA2", sense oligonucleotide "S2") or 10 $\mu$M of phosphorothioate-modified bFGF antisense oligonucleotide ("PAS1"). Cultures were then either scored microscopically for the presence of pigment cells or assayed for bFGF expression using protein immunoblot analysis. (A) Time-course of pigmentation in one representative experiment. Note that the antisense and modified antisense oligonucleotides delayed the initial onset of TPA-induced pigmentation and reduced the final level of pigmentation, whereas the sense and scrambled antisense oligonucleotides had no effects in this regard. (B) Means (±SEM) of per cent pigmentation after 10 days of culture from four separate experiments. Note that there was no statistically significant difference in pigmentation between cultures treated with TPA alone and cultures treated with either TPA + S2 or TPA + SAS2. All three antisense oligonucleotides, however, significantly inhibited TPA-induced pigmentation to a similar degree. (C) Protein immunoblot analysis of bFGF expression in peripheral nerve explant cell lysates treated with either TPA alone (lane 4 and 6) or TPA ± AS1 (lanes 5 and 7). Equal amounts of cellular protein (20 $\mu$g) were added to each of lanes 4–7. Note that AS1 blocked bFGF expression after 24 hr, and continued to inhibit up to 80% of bFGF expression after 48 hr. Lanes 1–3 represent serial dilutions of the 18 kDa bFGF used as controls. n.s. = not significant; * = $P < 0.05$. Reproduced from *Development*, in press [14] with permission.

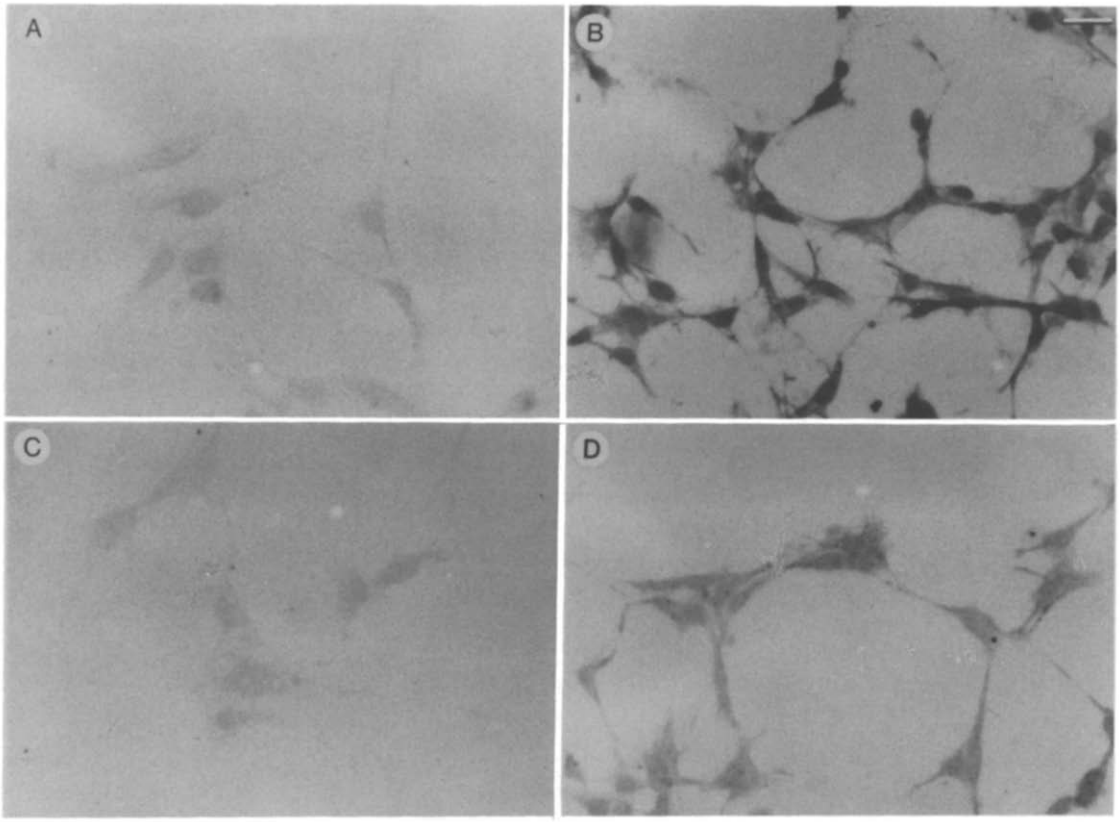


Fig. 4. Effects of bFGF antisense oligonucleotides on the immunocytochemical localization of bFGF in cultured peripheral nerve explants. Peripheral nerves from stage 32–33 quail embryos were cultured for 2 days in either CM alone (panel A), CM + 1.0 $\mu$M TPA (panels B and C), or CM + TPA + 50 $\mu$M AS1 (panel D). Cultures were then fixed and stained for bFGF immunoractivity using a 1:150 dilution of monoclonal anti-bFGF antibody DE6, and then examined and photographed under bright-field photomicroscopy. (A) bFGF immunostaining in cells cultured in CM alone. Note the low level of bFGF immunoreactivity in the cell nuclei and cytoplasm. (B) bFGF immunostaining in cells cultured in CM ± TPA. Note the presence of bFGF immunoreactivity in cell nuclei and a slightly lower levels in the cytoplasm. (C) Negative control for antibody specificity. These cultures were grown in CM ± TPA, but were analysed using DE6 pre-absorbed with an excess of bFGF. Note that staining was reduced to background levels. (D) bFGF immunostaining in cells cultured in CM + TOA + AS1. Note that the bFGF antisense oligonucleotides reduced the overall levels of TPA-augmented bFGF expression, but that immunostaining could still be detected in both cell nuclei and cytoplasm. The bar in the upper right corner represents 30 $\mu$M. Reproduced from *Development*, in press [14] with permission.

are in clear conflict with the findings described above [22–24]. It is our opinion that these discrepancies are mostly due to differences in the specificity of probes, such as antibodies, used for the localization (see Ref. 17 for a detailed discussion of this point). It is thus of importance to confirm immunohistochemical localization by other, independent techniques. For example our findings using western blots and bioassays, as described above, correlate well with the immunohistochemical localization given, which strongly supports the notion that the immunohistochemical staining observed faithfully reflects the distribution of bFGF and aFGF. In addition, other reports confirm this pattern of distribution [25, 26].

*Intracellular effects of FGF in the differentiation of melanocytes*

The NC is a transient vertebrate embryonic structure which contains precursor cells for a variety of different cell types, including peripheral neurons, Schwann cells and melanocytes. These precursors migrate from the NC to their destination and differentiate into the appropriate phenotypes [see Ref. 27]. The precursor cells appear to be pluripotent and seem to become committed to their final phenotype via a series of developmental restrictions which progressively limit their potential fates [28]. The mechanisms governing these restriction steps are not well characterized, but it is of interest in this respect that bFGF appears to promote the development of melanocytes in cultures consisting of precursor cells that normally would differentiate into Schwann cells [29]. This effect can be elicited by the addition of extracellular bFGF to the culture medium, but our recent studies have provided evidence suggesting that intracellular bFGF, synthesized by the differentiating NC cell themselves,

may also be able to promote melanocyte differentiation [14]. This interpretation is based on the observations that addition of the phorbol ester TPA to the cultures resulted in a large increase of bFGF expression and a concommitant increase in melanocyte differentiation. Addition of bFGF-blocking antibodies to the TPA-stimulated cultures, which blocked the effect of added extracellular bFGF, was unable to reduce the stimulation of melanocyte differentiation by TPA. Treatment of TPA-stimulated cultures with bFGF antisense oligonucleotides led to a marked reduction of bFGF-protein in the cultures (Figs 3 and 4), and significantly reduced the stimulation of melanocyte differentiation. These data suggest that the TPA-induced high intracellular levels of bFGF alone can be sufficient to regulate differentiation, even in the absence of extracellular bFGF.

## DISCUSSION

Above, we have hypothesized that bFGF and aFGF may serve an unusual autocrine function in the growth and maintenance of astrocytes and neurons. Together with the additional data derived from the studies on melanocyte differentiation, it may be speculated that the intracellular stores of FGF and the stimulation of the transmembrane tyrosine kinase FGFRs may in the end act in synergy on the same set of genes that promote mitogenesis, differentiation and neutrophic effects. Clearly much further work is needed to test this idea critically.

The observations discussed above also point to two pathological conditions where either interference with FGF function or the augmentation of FGF function may be of benefit in treating the condition. First, it is possible that over-expression or increased release of bFGF (or, alternatively, constitutive activation of FGFRs) may be involved in the formation of astrocytomas. Second, an increased supply of extracellular bFGF is known to promote neuronal survival after some types of CNS injury. Thus, agents that block FGF action would be of potential interest in the treatment of astrocytomas, whereas FGFs, FGF-derivatives or agents that augment FGF action may be of use in the treatment of CNS injury and degenerative diseases. A potential problem with these approaches is that the effects of such treatments are likely to include undesired side effects, due to the multiple biological effects of the FGFs. For example, treatment of astrocytomas with FGF-blockers might interfere with the normal neurotrophic maintenance function of FGFs, thus producing concommitant neuronal degeneration. On the other hand, augmenting FGF action after CNS injury in order to promote neuronal regeneration, may also result in the concommitant stimulation of mitogenesis of astrocytes. This could in turn result in the formation of a glial scar, which inhibits regeneration. These possible complications point out that drugs affecting FGF related mechanisms, in order to be clinically useful, should be designed to allow the specific targeting to either neurotrophic or mitogenic effects. This may be possible by designing drugs that will selectively interact with individual FGF, FGFR or heparan sulphate proteoglycan family members.

These suggestions so far deal with the development of drugs affecting the extracellular, transmembrane receptor loop of FGF action. It is intriguing to speculate that an intracellular synergistic FGF loop (see above) may also be important for some of the actions of FGFs, thus opening yet more avenues for drug development. However, more substantial information about the biological importance of such an intracellular loop and about the molecules involved in this loop will be needed before drugs affecting this mechanism can be developed.

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