REGULATION OF THE EARLY DEVELOPMENT OF THE NERVOUS SYSTEM BY GROWTH FACTORS

Perry F. Bartlett, John Drago, Trevor J. Kilpatrick, Linda J. Richards, Peter Wookey and Mark Murphy

The Walter and Eliza Hall Institute of Medical Research, and The Cooperative Research Centre for Cellular Growth Factors Parkville, Victoria 3050, Australia

INTRODUCTION

The nervous system of vertebrates begins as a thickening of the dorsal ectoderm early in embryogenesis which subsequently involutes to form the neural tube consisting of a single layer of epithelium (neuroepithelium); from this develops the entire central nervous sytem (CNS). Another population of cells bud off from the dorsal portion of the tube just prior to closure, the neural crest; these cells give rise to all the cells of the peripheral nervous system (PNS), and to many structures in the facial and branchial regions of the developing embryo.

At the simplest level, neural development can be considered as a process whereby epithelial like cells proliferate and ultimately differentiate into cells of the nervous sytem: neurons and glia. The magnitude of this process is best illustrated by the fact that the 100 billion neurons found in the human brain are derived from approximately 125 thousand primitive cells present at the early stage of neural tube formation. The regulation of this process of growth and differentiation has been thought of as being both intrinsic, genetically predetermined within individual cells, and to be due to enviromental or epigenetic factors. The distinction between the two types of regulation is often not clear and cell phenotype, as will be discussed, may often depend on their interplay.

Beyond understanding how individual cells are regulated to divide and differentiate lies the question of how organisation and pattern formation occurs in the nervous system. This question has been examined in detail in invertebrates, especially in the fruit fly, Drosophila. Here it has been elegantly shown, mainly by the use of developmental mutants, that the segmentation observed in both the nervous system and body compartments are regulated by a family of genes (homeobox), which code for proteins sharing a common amino acid sequence (homeodomain) that bind to regulatory elements in DNA (See Ingham, 1988 for review). Similar genes have been identified in most vertebrate species, including man, and their distribution within the developing nervous system has suggested that they may play a part in determining segmentation. However, segmentation is not at all obvious in the developing neural tube of animals such as chicken or mice, and that which does occur in the spinal cord is secondary to somite formation.

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Recently, it has been shown by Lumsden et al., (1989, 1990) that the series of folds that appear in the developing hindbrain of chicks, called rhombomeres, does indeed represent true segmentation of the neuroepithelium. They have shown that rhombomere boundaries restrict the migration of clones of neurons and demarcate the origin of the nerve roots of many of the cranial nerves. Pairs of rhombomeres also correspond to the branchial arches that give rise to head and neck structures. The significance of this latter observation is that neural crest cells give rise to much of the mesenchyme of this region and these crest cells have been shown to migrate within these boundaries suggesting that they carry preexisting information concerning their ultimate cellular phenotype.

Of particular interest is that boundaries of rhombomeres correspond to boundaries of expression of many of the homeobox genes as well as other genes coding for DNA binding proteins such as Krox-20 (See Wilkinson and Krumlauf, 1990 for review), implying that these genes are directly responsible for the observed segmentation. The importance of this gene expression to neural development in higher vertebrates awaits gene knockout experiments. Early results indicate that gene ablation leads to developmental defects which do not correspond to the extent of gene expression: loss of Hox-1.5 does not interfere with cranial nerve development even though it is expresed in all rhombomeres (Chisaka and Capechi, 1991). Thus, it may be that the system is degenerate in higher vertebrates, with no single gene determining regional specificity. Recent experiments favour another interpretation, that families of homeobox genes share the same distribution within the hindbrain but may determine the fate of only some of the cells within that region (Hunt et al., 1991). This would account for the loss of only some of the derivatives of a particular rhombomere.

It is notable that expression of genes coding for growth factors such as FGF like molecules (Int-2) and Steel factor are also regionally restricted in the nervous system (Matsui et al., 1990). It could be argued that these two categories of regionally distributed genes represent the two possible mechanisms that influence neural development: one acting directly as a DNA binding proteins that influence cell growth and phenotype within a particular area, and the second, also regionally expressed, influencing the same characteristics by regulating the cell through receptor-ligand interactions. How one type of interaction influences the other and in what sequence are major questions for the 90s.

This review details the evidence, principally gained from in vitro cell culture, in favor of the concept that the regulation of proliferation of precursor cells in the developing CNS and PNS is largely due to growth factors produced within the nervous system. It also suggests that there are quite separate signals for precursor proliferation from those which are required for differentiation.

EARLY EVENTS IN THE FORMATION OF THE CNS

From studies primarily carried out in amphibians it appears that neural induction begins at the time of gastrulation and is due to interactions with the underlying mesoderm. The classical studies of Mangold (1933) also indicated that mesoderm was important in specifying the subsequent phenotype of the neurectoderm: anterior mesoderm induced forebrain whereas posterior produced spinocaudal structures. Although it has been suggested that soluble factors mediate induction their indentity remains unclear. Recent experiments have shown that protein kinase activators such as phorbol ester can act as inducing agents (Davids et al., 1987).

Once induced the number of cells in the neural plate does not change appreciably as the process of tube formation proceeds. Proliferation commences after tube closure in the midline and gives the neuroepithelium of the neural tube a columnar appearance. At a macroscopic level swellings begin to appear in the rostral region of the tube that represent the three major areas of the brain: the forebrain (cerebral cortex and basal ganglia), the midbrain, and the hindbrain (medulla, pons and cerebellum). As mentioned earlier within the hindbrain there are additional folds called rhombomeres which have been shown to represent segmental areas of the neuroepithelium. the remainder of the neural tube gives rise to the spinal cord.

As the stratification of the cells proceeds, cell division is primarily restricted to the neuroepithelium adjacent to the cavities that will ultimately form the ventricles, although mitotically active cells can also be found in the other areas including the external granular layer of the cerebellum.

There appear to be two distinct phases of proliferation in the CNS: the first peaking some 3 to 4 days after neural tube closure in the mouse (Angevine and Sidman, 1961) and which correlates with the neuronal precursor division; and the second which occurs later and is associated with glial cell formation.

The major questions that arise when examining these early events are: What controls the proliferation of the neuroepithelial cells? What regulates the generation of cell lineages? If the cells of the neuroepithelium are multipotential, can subsequent differentiation be influenced by epigenetic factors?

REGULATION OF PRECURSOR PROLIFERATION IN THE CNS

One of the hallmarks of early neural development is the enormous degree of proliferation that occurs in the neuroepithelium. We have endeavored to explore the possibility that this could be regulated by epigenetic factors including soluble growth factors and components of the extra-cellular matrix. In order to examine this, new in vitro assays were developed in which pure populations of neuroepithelial cells (Drago et al., 1991a), prepared from mouse neural tubes at the time of closure, were cultured in the presence or absence of a wide range of soluble growth factors. It was found that both acidic and basic fibroblast growth factors (a,bFGF) were unique as they were potent stimulators of neuroepithelial cell division as assessed by tritiated thymidine incorporation (Murphy et al., 1990). Other factors including nerve growth factor (NGF), epidermal growth factor (EGF) and insulin-like growth factors (IGF-I,II), previously shown to be associated with embryonic neural tissue (Nexo et al., 1980; Sara et al., 1981), were ineffective in stimulating cell division. It was subsequently shown that FGF stimulation was dependent on the presence of IGF-I and especially the truncated form of IGF-1, which is primarily found in developing brain (Drago et al., 1991b). These studies demonstrated that FGF acted predominantly as a proliferative agent (see later for discussion on differentiation), whereas IGF-I was primarily a survival agent for the precursors. Recent experiments using clones of neuroepithelial cells support this conclusion, as they continue to proliferate in the presence of FGF without overt signs of differentiation. Differentiation can be induced in these populations by specific signals (Kilpatrick et al. manuscript in preparation).

What relevance does this in vitro data have to the regulation of precursors in vivo? To answer this question directly would require the

ablation of these growth factors in the tissue at the relevant time. Although such technologies as gene ablation by homologous recombination, anti-sense mRNA or ribozyme provide potential tools to answer the question directly they are still at an early stage of development. We can determine, however, whether the factors are present in the tissue at the appropriate time. mRNA for both BFGF and IGF-I was detectable in freshly isolated neuroepithelial cells, implying that these factors are produced endogenously by the precursor populations (Drago et al., 1991).

This idea was compatible with the observation that the requirement for growth factors in vitro could be largely overcome if the cells were plated at high cell density; implying that there was a limiting amount of factors produced by the cells. This proved to be correct as blocking antibodies specific for either bFGF or IGF-I totally inhibited the survival or growth of the precursor population (Drago et al., 1991b). Thus, it appears that the neuroepithelial cells may be regulated by an autocrine or paracrine mechanism, which is in stark contrast to that previously postulated as the mechanism for neurotrophic factor action on developing neurons; where it has been largely attributed to external or target derived factors.

If precursor cells produce their own stimulatory factors then how is the system regulated? One likely regulatory mechanism is the binding of FGF to heparan sulphate proteoglycans which are known to be present in the basement membrane with which the dividing precursors are all in contact. Recently, we have shown that there is FGF bound to the ECM of neuroepithelial cells and that there is an enormous excess capacity of the low affinity FGF receptor, provided by a heparan sulphate proteoglycan, compared to the high affinity receptor on the neuroepithelial cells (Nurcombe and Bartlett, manuscript submitted). This would result in the vast majority of the secreted FGF (the exact mode of secretion is still unclear because FGF does not have a leader sequence) being bound to the ECM and only accessible after cleavage of the core protein.

Another molecule to which FGF can bind via its attachment to heparin is the laminin molecule. Laminin is a large multidomain molecule expresssed early in development and is found in abundance in the basal lamina next to the proliferating neuroepithelial cells (Tuckett and Morris-Kay, 1986). It has been shown to promote survival and neurite extension in cultured neuronal cells (Cohen et al., 1986) and to be involved in the transdifferentiation of cultured retinal pigment cells into neurons (Reh et al., 1987). It was also found to stimulate the survival and proliferation of neuroepithelial cells in vitro and to be additive with FGF (Drago et al., 1991c). However, in contrast to FGF, the action of laminin was dependent on cell aggregation, a response which laminin promoted. It therefore appears that laminin responsiveness may result from its ability to promote cell-cell contacts that may in turn upregulate other endogenous stimulatory molecules.

ARE NEURAL PRECURSORS IN THE CNS MULTIPOTENTIAL?

As much of the data, until recently, relies on in vitro cultures to answer the above question it is important to establish how accurately the system reflects the in vivo situation. It has been shown that the spectrum of cell phenotypes cen indeed be generated from neuroepithelial cells in vitro (Abney et al., 1981; Bailey et al., 1987). More surprisingly, perhaps, is the finding that in high density cultures the appearance of specific phenotypic markers coincides temporally with their appearance in vivo (Saleh and Bartlett, 1989; Abney et al., 1981). This has given rise to the concept of an inbuilt biological clock that is

independent of its enviroment. However, it should be noted that this phenomenon has been largely established with high density cell culture and there is evidence from our laboratory using neuroepithelial clones that this mechanism does not operate without exogenous stimuli. Another example is the precursor cell in the optic nerve, termed the O-2A precursor, which has the capability of differentiating into either an oligodendrocyte or a type II astrocyte in vitro, but does not recapitulate the in vivo sequence unless the appropriate growth factors are present (Raff, 1989). This observation again stresses the importance of the interrelationship between intrinsic and extrinsic factors in the development of the nervous system and highlights the difficulty in interpreting ill-defined culture systems.

Beside the O-2A precursor cited above, there are several in vitro studies that strongly suggest that cells of developing CNS can give rise to cells of various phenotypes. It was shown in clonal assay of blast cells obtained from the rat hippocampus that about one fifth of the clones contained cells of the two major types in the CNS: neurons and glia (Temple, 1989). Recent work in our laboratory also suggests that some of the clones derived from neuroepithelial cells also have the ability to differentiate into neurons and astrocytes (Kilpatrick and Bartlett, manuscript in preparation).

A recently devised method based on the ability of retroviruses to insert into dividing cells and act as a marker of their progeny has led to the fate mapping in several vertebrate CNS systems. Two groups have shown that in the developing retina a single precursor can give rise to an array of glial and neuronal types (Turner and Cepko, 1987; Wetts and Fraser, 1988). In contrast, workers using a similar approach in the cortex of mice and rats failed to observe mixed progeny. This may indicate either that there are no multipotential cells in the cortex or that there is heirarchy whereby cells become progressively more committed with developmental age. The latter explanation appears more likely from studies that use markers to distinguish committed precursor populations.

It has been known for some time that the brain is unique as it does not express Class I MHC molecules constitutively. It was subsequently shown that this molecule could be induced by the lymphokine, interferon gamma (Wong et al., 1984). However, it is now known that neurons do not express this molecule under any circumstances (Bartlett et al., 1989) and it was established that cells in the developing neuroepithelium that were not inducible with the lymphokine gave rise to neurons exclusively when selected by cell sorting and were cultured (Bartlett et al., 1990). These committed precursors were not found, however, when the earliest precursor cells were examined, implying that commitment only began to occur after proliferation of the neuroepithelium was underway. From this type of phenotype study it was possible to construct a heirachy of commitment which originates with the multipotential cell, flows into the restricted glia or neuronal precursor and ends with the fully differentiated cell.

Another approach to investigating the differentiation potential of cells in the developing CNS is to generate cell lines from the precursor populations. This was first achieved by infecting neuroepithelium with a retrovirus containing the c-myc gene, chosen for its ability to promote proliferation rather than differentiation (Bartlett et al., 1988). One of the lines generated, 2.3D, grew as a stable neuroepithelial cell line and was found not to spontaneously differentiate. Subsequently, it was found to respond to FGF by rounding up and putting out neurite-like processess that stained with the neuron specific intermediate filament marker, neurofilament. Some of the cells in this cloned cell line expressed the glial specific marker, glial fibrillary acidic protein (GFAP) (Bartlett et

al., 1988). Thus, there can be no doubt that epithelial cells thus immortalised retain the ability to differentiate down both major CNS pathways.

FACTOR REGULATION OF NEURAL DIFFERENTIATION IN THE CNS

One of the problems in identifying factors that may act as differentiating agents is trying to separate them from the agents that stimulate survival and proliferation. As has been noted, cells cultured at sufficiently high cell density or with factors such as FGF proceed to differentiate, but it is far from clear exactly what signals are driving that differentiation. Originally we had thought that FGF was a differentiating agent (Murphy et al., 1990). However, even in spite of the observation with the cell line 2.3D, this conclusion is less clear. Recent experiments indicate that when precursors were plated at low cell density on monolayers of astrocytic cells and grown in serum-free medium, fewer neurons differentiated in the presence of FGF than in medium alone (Kilpatrick and Bartlett, manuscript in preparation). This implies that there are positive signals for differentiation that are quite separate from proliferative stimuli. Some of these signals are being identified in the neural crest (see below). However, in the CNS such factors remain uncharacterised.

THE PERIPHERAL NERVOUS SYSTEM

The peripheral nervous system is distinguished from the central nervous system on the basis of both anatomical and developmental criteria. Anatomically, the PNS neuronal cell bodies lie outside the brain and spinal cord and can be divided functionally into three major systems: sensory, autonomic and enteric. The autonomic nervous system can be further subdivided on the basis of anatomical, physiological, and pharmacological grounds into the sympathetic and parasympathetic subgroups. The enteric nervous system is considered a separate entity as it can function independently of the central nervous system.

Developmentally, all these structures have the same embryonic origin, the neural crest. As discussed above, the neural crest forms as a group of cells at the dorsal aspect of the neural plate, and just prior to neural tube closure, they bud off from the plate and migrate to specific locations in the embryo. At these locations, the neural crest cells then differentiate into the component ganglia of the PNS. The PNS is almost exclusively derived from the neural crest, except for some cranial ganglia which are derived from a related structure, the ectodermal placodes. The cell types derived from the neural crest are not restricted to neural cells but also include mesenchymal elements of the head and face, melanocytes of the skin, the adrenal medulla, meninges in the pro- and mesencephalon, corneal endothelium and the large arterial walls from the aortic arches (see Le Douarin and Smith, 1988). The generation of such an array of different phenotypes raises questions fundamental not only to neurobiology but to the field of vertebrate development in general. Are the crest cells a homogeneous population of pluripotent precursor cells or are they a heterogeneous population of committed progenitors? If there is evidence for commitment of these cells, what determined that commitment initially? How do the neural crest cells migrate through the embryo to their correct location? What controls the proliferation of the neural crest cells enabling their numbers to increase from a few thousand cells to a few million? What controls the subsequent differentiation of these crest cells into their differentiated phenotypes? As will become evident

below, some of these questions have been investigated extensively, whereas others have hardly been touched upon.

COMMITMENT AND MULTIPOTENTIALITY OF THE NEURAL CREST

One of the early problems in the study of neural crest ontogeny was to determine where regional populations of crest cells migrate to, and then to identify the phenotype of their progeny. This was achieved by the use of chick-quail chimeras: regions of the chick neural tube were replaced with quail neural tube, including the neural crest, taken from the same region at a similar stage of development (Le Douarin, 1982; 1986; Le Douarin and Smith, 1988). These chimeras remain viable at least until after birth and the quail cells could be distinguished from the host by its nuclear heterochromatin pattern. Over a period of ten years or more this approach led to the construction of a fate map of the neural crest. This map showed that there were defined regions of the crest which gave rise to particular ganglia and other neural crest derived structures. The fate map generally supports the idea that most neural crest cell migration is lateral and therefore the resultant neural crest derivatives reflect their position along a rostro-caudal axis. For example, the adrenal medullary cells originate from the spinal neural crest between the level of somites 18-24; the spinal neural crest caudal to somite 5 gives rise to the ganglia of the sympathetic chains; and the ciliary ganglion is derived from the mesencephaic neural crest; all of the meso-ectodermal derivatives are derived from the rostral regions of the neural crest and are mainly located in the head and neck.

The fate map described above only addresses questions of normal development as all grafting experiments were placed isotopically, and thus only identical regions of the crest were grafted. To ask whether neural crest cells were lineage restricted according to regional placement, experiments were performed using heterotopically placed grafts. It was found that it was the location of the grafted cells within the chimeric embryo, and not their origin, that ultimately determined their developmental fate. For example, vagal crest cells (which normally show parasympathetic innervation of the gut) grafted to the level of somites 18-24, differentiated into sympathetic ganglia and adrenal medulla - the normal derivatives of this region of the crest. The reverse experiment, where the adrenomedullary level neural crest was transplanted into the vagal region, gave rise to enteric ganglia containing cholinergic and peptidergic neurons. Such experiments not only established that in most cases it was the embryonic environment of the neural crest cells that determined their differentiated phenotype but it also implied that the crest cells were multipotential, at least at the population level.

There appear to be, however, some significant exceptions to the concept that neural crest cells are multipotential. The major exception is in the cephalic regions of the crest, which are unique in that they alone can give rise to the ectomesenchymal derivatives such as bone, smooth muscle, adipose tissue, meninges and endothelial cells- all crest derivatives being exclusively located in the head and upper body. In addition, there are some differences in the capacity of different regions of the crest to duplicate the normal crest region. Replacement of the mesencephalon with the trunk neural crest results in the development of an abnormal trigeminal ganglion (Noden, 1978), whereas the potential of the trunk for adrenergic differentiation is geater in the trunk than in the cephalic crest (Newgreen et al., 1980). Alternatively, when cephalic crest is transplanted to the trunk region, the neural crest cells migate into the dorsal mesentery and colonize the gut, which does not normally happen (Le Douarin & Teillett, 1973).

It is these exceptions which lead one to the view that there are indeed local differences in the composition of the crest different propensities of the various regions of the crest to be committed to different cell types. With respect to the PNS, this commitment is not extensive at least at the population level, but with respect to the mesectoderm, there is a complete restriction to the cephalic levels of the crest. The final decision, however, as to what the cells differentiate into appears to be primarily left to the environment. The identities of the environmental factors which influence these decisions are unknown and have been the object of recent study in our laboratory (see below).

In order to dissect out the events which determine neural crest development it is necessary to turn to in vitro culture techniques as described above. Under these conditions the environment of the neural crest cells can be influenced or manipulated relatively simply by adjusting the components of the medium. Thus it may be possible to determine which factors influence the development of the crest derivatives. In addition, the question of multipotentiality versus commitment can be directly asked in clonal cultures of the cells. A number of workers have begun to develop these clonal cultures and their results suggest that there is both commitment and multipotentiality in the neural crest.

Sieber-Blum and Cohen (1980) first used this approach in the clonal analysis of quail neural crest cells. They grew clones from single neural crest cells and found that some contained both catecholaminergic and pigmented cells. More recently, Sieber-Blum (1989) extended these studies using antibodies specific for the sensory lineage and found three classes of clones: clones committed to the melanogenic lineage, clones that were unpigmented and clones containing both pigmented and non-pigmented cells. The unpigmented and mixed clones all contained both catecholaminergic and sensory neurons. Thus, in this system there is evidence for tripotent cells, cells restricted to two cell lineages and fully committed cells.

Studies from the laboratory of Le Douarin also found evidence for a similarly heterogeneous range of clones (Barofflo et al., 1988; Dupin et al., 1990). In these studies, multipotent clones comprising neurons, pigmented cells and non-neuronal cells were found as well as more restricted clones of Schwann cells, satellite cells and neurons. There was also evidence of some segregation of pigment cells from neurons. In a very few cases there were clones containing cartilage, a marker for the ectomesenchymal lineage. The cartilage containing cells were not associated with neurons or pigmented cells.

These studies support the idea that both multipotentiality and commitment reside within the neural crest cells at the migratory stage. The observation of considerable heterogeneity in the clones is not necessarily a proof that there is intrinsic heterogeneity in the neural crest cells. It may be that at the time the cells are isolated they are at different stages of differentiation: some of the cells may already have gone through a number of steps down a commitment pathway and so may look to be restricted, whereas others may be a little "younger" and so may still be more multipotent. If this is the case, then the actual lineage pathways, or commitment steps, may be inferred from the segregation of the different cell types. Thus, pigment cells appear to segregate from other cell types frequently, as do non-neuronal cells from mixed clones containing both neurons and non-neuronal cells. Cartilage cells are only found in clones with non-neuronal cells including Schwann cells.

As analyses of the clones are normally undertaken after a number of weeks when there can be thousands of cells in each clone, the

microenvironment of each clone might itself vary; there might be endogenous production of different growth factors. This raises the question of which factors are required for neural crest differentiation. A possible result of this is that no clones are found which are solely neuronal. Until the conditions have been found which allow for the unrestricted growth of the neural crest derivatives, these clonal analyses will be limited.

One in vivo approach which has been used to determine the degree of multipotentiality or commitment of the neural crest cells is that of Bronner-Fraser and Fraser (1988), who microinjected single neural crest cells with a fluorescent dye prior to migration from the neural tube. The progeny of these cells were traced after two days and in some cases were found to be distributed in all of the regions to which neural crest cells normally migrate. The phenotype of these cells could not be definitively ascribed. However on the basis of morphology and antibody binding, individual clones containing sensory neurons, presumptive melanoblasts, satellite cells in dorsal root ganglia, adrenomedullary cells and neural tube cells were found. Thus, these findings support the idea that there are multipotential neural crest cells in vivo.

In mammals, very little is known about cell lineage and commitment of the neural crest. The isolation of large numbers of neural crest cells from mammals is difficult and they have a limited life span in vitro. In order to overcome these restrictions, we have developed cell lines representative of migratory neural crest cells and their progeny in order to study cell lineage associations as well as to characterize environmental factors which influence the developmental fate of neural crest cells (Murphy et al., 1991a). Previous work from our laboratory and others has shown that retrovirus mediated proto-oncogene transduction of the neural precursor cells from mouse neuroepithelium results in the production of stable neuroepithelial cell lines (Bartlett et al., 1988; Bernard et al., 1989; reviewed in Cepko, 1988, 1989). These cell lines have similar characteristics to primary neuroepithelial cells and, like their primary culture cell counterparts, they differentiate in response to fibroblast growth factor (FGF).

We have immortalized mouse neural crest cultures using retroviruses bearing the c-myc or the N-myc proto-oncogenes and have cloned a series of cell lines (Murphy et al., 1991a). In order to classify these cell lines as being linked to a particular neural crest lineage, the cell lines were examined for the expression of some lineage specific or lineage related antigenic markers which are found on neural crest derived cells. We used antibodies to glial fibrillary acidic protein (GFAP), neurofilament (NF) and the A2B5 antibody. GFAP is specific for mature glia in the central nervous system and has also been found in the glia of the enteric nervous system (Jessen and Mirsky, 1983) as well as in a subpopulation of non-myelinating Schwann cells (Yen and Fields, 1981). A2B5 reacts with a ganglioside present on neurons and probably their precursors in avian neural crest cultures (Girdlestone and Weston, 1985). It also recognizes some glial cells and their precursors in the rat central nervous system (Raff et al., 1983 and 1984).

The results of this analysis showed patterns of staining consistent with individual cell lines being at different stages of differentiation. Eleven cell lines were selected as being representative of the different types of cells which emerged from the neural crest immortalization. RNA from these 11 cell lines was analyzed for the expression of neural specific mRNAs. We examined the expression of nerve growth factor (NGF) and its receptor (NGF-R), which are expressed by cells in the peripheral nervous system, myelin basic protein (MBP) and the proteolipid (PLP) of

myelin which, in the peripheral nervous system, are specific to Schwann cells. A neuron specific gene SCG-10 (superior cervical ganglion, see Anderson and Axel, 1985) was also used in the analysis.

A major question arising from the establishment of the neural crest cell lines is whether they possess characteristic features of migrating crest cells and their differentiated progeny. Morphologically, the cell lines had a variety of appearances from flat cells resembling migrating neural crest cells through to multi-processed cells reminiscent of neurons or Schwann cells. One group of cell lines not only morphologically resembled migrating neural crest cells, but this group also was largely devoid of phenotypic markers, both antigenic and mRNA expressed by mature neural cells. These observations are consistent with the idea that some of the migrating neural crest cells are not yet committed to a single developmental pathway and probably represent stem-cells. These stem cells have presumably been frozen at this stage by the immortalization process.

Some of the cell lines also displayed a bipotential nature, a major characteristic of the neural crest. Particular cell lines expressed their bipotentiality in the expression of markers associated with two lineages. In one case a cell line, NC14.9.1, appeared to be bipotential since in a cloned population these cells expressed neurofilament as well as MBP and PLP, showing that it had characteristics of both neurons and Schwann cells. Likewise another cell line, NC14.4.9D, expressed both PLP mRNA and SCG-10 mRNA and all the cells expressed neurofilament. Similarly, multipotent neural cell lines have been isolated from newborn brain (Fredericksen et al., 1988; Ryder et al., 1990). These cell lines also share some other characteristics of our cell lines in that some of the antigenic markers examined were expressed on a small proportion of cells in particular cell lines.

The multipotential nature of the neural crest cells which were originally infected with either c-myc or N-myc containing viruses was also demonstrated by the observation that cell lines which have the same myc integration pattern, and thus must have originated from the same cell, can have quite different phenotypes. It is possible that an immortalized multipotential cell divided a number of times before differentiation of the progeny cells into the different phenotypes took place. Thus, a single crest cell can give rise to a neural crest-like line, Schwann cell progenitor and a bipotential cell line.

In addition to stem-like activities there is evidence that some of the lines also represent progenitor populations that can differentiate in culture. For example NC14.4.8 cells contained cells that differentiated after 1-2 weeks in culture, into Schwann-like cells. Further, these older cultures expressed MRNA for MBP, PLP, NGF and NGF-R. All these observations are consistent with this cell line comprising Schwann cell progenitors.

Finally, one of the cell lines appears to represent a neuronal cell line. This cell line (NC14.4.6E cells) has fine processes which contain neurofilament. In addition, the cells from this line express mRNA for the neuronal protein SCG-10, as well as for NGF, but not NGF-R or other non-neuronal markers. The cells do express vimentin, but this is not surprising since vimentin is present in neural crest cells up until neuronal differentiation.

Although cell lines representative of both neurons and Schwann cells have been characterized there is no evidence that other neural crest derived cells are represented. None of the lines contain melanin pigments or resembled melanocytes, and this is in contrast to the finding in quail

neural crest transformed by Rous sarcoma virus in which the cell lines gave rise to melanocytes (Pessac et al., 1985). However, avian and mouse neural crest appear to differ markedly in their capacity to give rise to mature melanocytes: the mouse neural crest appears to require phorbol ester (see below) to induce melanin formation in vitro; whereas melanin formation occurs spontaneously in primary avian neural crest cultures. Preliminary experiments using phorbol ester on our cell lines have shown no evidence for melanocyte differentiation. The particular viruses and oncogenes used in these experiments must also have an influence on the phenotypes of the resultant cell lines (see Cepko, 1989).

Many of the neuroepithelial cell lines responded to growth factors such as FGF (Bartlett et al., 1988) which is known to affect neural proliferation and differentiation of freshly isolated neuroepithelial cells (Murphy et al., 1990). Likewise, most of the neural crest cell lines in this study respond to FGF by proliferating and by changes in morphology, including the group 1 crest-like cells. The significance of these morphological changes in terms of differentiation is currently being investigated. In addition high cell density and foetal calf serum are often required to ensure continued growth of some of the immortalized lines. Thus, these cell lines may be very useful in assaying for factors which are important in differentiation, eg FGF, serum factors (see Ziller et al., 1983). It is possible, therefore, that the cells could be pushed down different differentiative pathway by the addition of particular factors in a similar manner to that shown for the neural crest derived sympathoadrenal cell types (see Doupe et al., 1985a and b, Anderson and Axel, 1986). In addition, mixing experiments between the cell lines and other cell types may help to uncover the importance of cell-cell interaction in neural crest development. Such experiments are effectively impossible to do with primary neural crest cultures because they rapidly become a heterogeneous population of cells.

WHAT EPIGENETIC FACTORS INFLUENCE THE DEVELOPMENT OF THE NEURAL CREST?

FGF

The observation that FGF stimulates the proliferation of most of the neural crest cell lines might be indicative of its activity on primary neural crest cells. There are a number of other indications that FGF may have an important role in neural crest development at various stages. At the migratory stage of neural crest development, FGF has been reported to have a survival role for neural crest cells (Kalcheim, 1989). If silastic membranes were inserted between the neural tube and the neural crest cells of the dorsal root ganglion anlage, there was a selective death of the neural crest cells which were distally located with respect to the silastic implants. This suggests that there are factors in the neural tube which are necessary for the survival of the migrated neural crest cells. If these silastic membranes were implanted with laminin and bFGF there was significant survival of the neural crest cells for a period of over 30 hr after grafting.

In addition to these in vivo studies, the effects of bFGF were examined in mixed cultures of avian trunk neural crest cells and somite cells or in pure cultures of neural crest cells (Kalcheim, 1989). Under the conditions of the assay, that is in a serum-free defined medium, bFGF was found to act as a survival agent for non-neuronal cells of neural crest origin (which were identified using the HNK-I antibody).

These studies were followed by a study of the location of FGF in culture and in situ at the time of neurogenesis and neural crest migration

(Kalcheim and Neufeld, 1990). In quail neural tube cells from E2, which had been cultured for one day, bFGF was found by immunocytochemistry. Staining for bFGF was also found in sensory neurons and in some non-neuronal cells in neural crest cultures. Staining was detected in spinal cord and ganglionic neurons in situ at E6 and increased towards E10. In addition bFGF was detected in mesodermal tissues dorsal to the neural tube as well as in other mesoderm-derived structures. These in situ immunohistochemical observations were supported by radio-immunoassays which showed levels of bFGF in spinal cords from as early as E3 and which increased to a maximum at E10.

The location of bFGF in situ is entirely consistent with an action for it in the development of the neural crest. Its reported action as a survival factor in vivo and in vitro on a subpopulation of non-neuronal crest cells thus probably reflects this action. However, it is not clear whether FGF may have other actions on the neural crest cells at the migratory stage, perhaps in conjunction with other growth factors, given that it has such a strong proliferative activity on the neuroepithelial cells of the neural tube, as described above, and that it stimulates the proliferation of most of the neural crest lines. We are currently investigating this possibility and have preliminary evidence that the actions of FGF may be more extensive and that it is probably acting as a proliferative agent for the majority of neural crest cells.

Perhaps the best characterized cell lineage within the neural crest is the sympathoadrenal lineage. There are three cell types in this lineage, the sympathetic neuron, the adrenal chromaffin cell and a third cell of an intermediate phenotype, the so called small, intensely fluorescent cell (SIF cell) (see Patterson, 1990; Anderson, 1989). Although progenitors of this lineage have not been isolated from neural crest cultures, they have have been isolated from embryonic adrenal medulla as well as both embryonic and neonatal sympathetic ganglia. These progenitors will differentiate into either chromaffin cells or sympathetic neurons depending on culture conditions (Doupe et al., 1985a & b; Anderson & Axel, 1986).

FGF will initiate neuronal differentiation as well as a dependency of the cells on nerve growth factor (NGF) for their survival. Glucocorticoids will stimulate the cells to differentiate into mature chromaffin cells. The evidence for the presence of FGF in the embryo around the neural tube has been presented above. In addition, FGF has been located in extracts of nervous tissue such as embryonic brain (Risau et al., 1988). The possibility that the developing sympathetic neuron precursors will find a supply of this factor at the site of ganglia is thus quite reasonable. In the adrenal medulla, on the other hand, when the precursors migrate into the adrenal gland they are probably subject to a high concentration of steroids produced in the adrenal cortex.

NGF

The role of NGF as a survival factor for the sympathetic neurons has been demonstrated over the past forty years using numerous experimental systems (see Levi-Montalcini & Angeletti, 1968). It is one of the few molecules to be shown to have a critical role in vivo for the survival of sympathetic neurons. The injection of anti-NGF antibodies into newborn mice results in the destruction of the sympathetic nervous system. Studies of the mechanism of action of NGF have resulted in it being the model factor which demonstrates the importance of target derived neurotrophic factors. In this scheme, the newly differentiated neurons sprout axons to their target fields, where there is a limited supply of a target derived survival factor. Only those neurons which have made the

right connections to the target field will obtain this factor and survive. This model provides a part of a mechanism for the control of the development of the nervous system into a three dimensional network.

A number of other factors have been implicated in the development of the sympathoadrenal lineage and in particular the development of sympathetic neurons. IGF-1 stimulates the proliferation of neurons or their precursors in cultures of rat sympathetic ganglia (DiCicco-Bloom et al., 1990). Whether this is a direct effect of IGF-1 on the proliferation of the neuronal precursor cells or whether the IGF-1 is acting principally as a survival agent and there are endogenous proliferative factors in these cultures, as described above for the neuroepithelial cells, is unclear at present. Ciliary neurotrophic factor, conversely, inhibits the proliferation of the neuroblasts and may provide a signal to initiate the differentiation of the cells (Ernsberger et al., 1989).

Other factors have been described which influence the transmitter phenotype of the sympathetic neurons. Most of the sympathetic neurons are adrenergic, except for those which innervate the sweat glands, which are cholinergic. One of the factors which may influence the switching of phenotype of these neurons to cholinergic has recently been purified and is equivalent to LIF (Yamamori et al., 1989). As discussed below, it is beginning to emerge that LIF has multiple activities within the nervous system as well as outside it.

LIF

The factors which control the development of sensory neurons from their precursor cell are not well defined. We have recently shown that leukemia inhibitory factor (LIF) a protein with multiple activities (Gearing et al., 1987; Abe et al., 1986; Yaniamori et al., 1989; Williams et al., 1988; Baumann & Wong, 1989, see above) stimulates the generation of sensory-like neurons in the mouse neural crest (Murphy et al., 1991b). This stimulation of neuron numbers in the neural crest is due either to a stimulation of differentiation of these precursor cells and/or a selective survival of the neuronal precursors.

The total number of neural crest cells in the cultures was increased in the presence of LIF. There were two to three fold more cells in the LIF treated cultures compared to controls. This suggests that LIF is acting as a survival factor for the neural crest cells, as there is no stimulation of proliferation by LIF. We further examined whether LIF may have been influencing the whole population of neural crest cells, by looking at the expression of the marker A2B5 on these cells. As stated above, A2B5 is expressed on a variety of cells in the neural lineage, in particular neural crest derived neurons and their precursors (Girdleston & Weston, 1985), although it is unclear how many of the A2B5 positive cells differentiate into neurons. In mouse neural crest cultures grown in the presence of 10% fetal calf serum alone, 5% of the cells are A2B5 positive, whereas when the cells are grown in the presence of LIF, the number of A2B5 cells increases to 40% of the whole population. This infers that LIF may be influencing a significant proportion of the whole neural crest population, perhaps down a neuronal differentiation pathway. Whether all of these cells are capable of forming neurons is another question. However this finding implies that there is still significant plasticity in the neural crest cells.

LIF does not act only on the sensory precursors, but also on maturing and mature sensory neurons as well. In cultures of dorsal root ganglia isolated at various times through sensory development up until birth, a high proportion of neurons survived in the presence of LIF. Thus

LIF is also a neuron survival factor, like NGF. Binding studies on the DRG cultures from P2 mice showed that greater than 60% of the neurons bound significant amounts of ^{125}I-LIF, which was completely inhibited by the addition of cold LIF. Furthermore, there was negligible cold-inhibitable binding of ^{125}I-LIF to non-neuronal cells in the culture. Thus, at this age, the only cells capable of responding to LIF in the DRG are the sensory neurons.

These results indicate that LIF can act throughout embryonic sensory neuron development in vitro. In neural crest cultures, it may act to stimulate neuronal differentiation and/or survival of the sensory precursors. As stated above, the effects of LIF on the bulk population of neural crest cells in the induction of the A2B5 antigen suggest that it may be acting directly to stimulate the differentiation of the neural crest cells, whether or not it is acting as a survival agent for the sensory precursors.

NGF also has a clear role in the development of sensory neurons but probably at a later stage of development. The observation that anti-NGF given via the placenta results in almost complete ablation of the sensory nervous system is proof enough of this. NGF is most likely acting as a target derived neurotrophic factor (Purves et al., 1988) in the same fashion as described for the development of the sympathetic nervous system. An alternate or additional explanation is that both NGF and LIF are required or act synergistically during development and the removal of either results in the neuronal loss. Evidence for this concept has recently been derived from in vitro experiments that indicate that endogenous NGF is required for maximum survival of embryonic sensory neurons in the presence of LIF.

One of the essential criteria to be fulfilled by a neurotrophic factor is that there appears to be a requirement for factors taken up by the nerve terminals to be retrogradely transported back to the neural perikarya. The transport of the neurotrophic factor is the signal from the target tissue to the neuron that results in neuronal survival (Hendry et al., 1974). Having demonstrated the presence of LIF receptors on sensory neurons in vitro, we investigated whether receptor mediated uptake of LIF would result in retrogade transport to the sensory neuron soma.

Adult mice were injected in the skin or muscle and in those animals injected in the skin of the foot, there was a significant accumulation of radioactivity in the sensory ganglia centered on lumbar ganglion 4 (L4). In newborn mice there was a greater accumulation of radioactivity after both leg and foot injections. The accumulation of radioactivity after skin injection again was centered on L4. Autoradiographic examination of histological sections through L4 ganglia from both adult and newborn animals injected with ^{125}I-LIF into the footpad revealed the presence of radioactive material in a subpopulation of neurons. The number of neurons with significant number of grains is between 5-10% of the population. Again there is no evidence of radioactivity associated with non-neuronal cells.

STEEL FACTOR

The melanocyte lineage is apparently determined early in development in the mouse and there is evidence that thirty-four primordial melanoblasts are lined up in pairs longitudinally during neural crest formation (Mintz, 1967). From related studies in the chick, the melanoblasts then undergo rapid proliferation and migrate laterally to the skin (Rawles, 1944; Weston, 1963) where they differentiate into mature

melanocytes. The processes which control the proliferation, migration and differentiation of these melanocyte precursors are not clearly understood. However two classes of mouse mutants point the way for the involvement of a newly characterised growth factor in this process. These are the White dominant-spotting (W) and Steel (Sl) mice. Phenotypically, mice homozygous at either of these alleles are blacked-eyed white, anaemic and sterile; some of the mutations result in lethality (reviewed in Silvers, 1979; Russel, 1979; Geissler et al., 1981).

An analysis of the mutations in these mice has revealed a complementary molecular relationship between the two alleles. Firstly, it was found that W allele coded for a growth factor receptor-like tyrosine kinase and which was identical to the proto-oncogene c-kit (Geissler et al., 1988; Chabot et al., 1988). Subsequently, the ligand for c-kit was purified and cloned and was found to be encoded by the Sl locus (Anderson et al., 1990; Williams et al., 1990; Martin et al., 1990; Zsebo et al., 1990a & b; Huang et al., 1990). Thus, this Sl factor and the c-kit receptor are strongly implicated in melanogenesis as well as germ cell production and in haemopoiesis. Because of this range of involvement the Sl factor has been variously called mast cell gowth factor, stem cell factor, and the c-kit ligand.

We tested whether Sl factor could stimulate the production of melanocytes in our neural crest cultures by adding it at the time of plating of the neural tubes. However the presence of Sl factor had no observable effect on the cultures and in particular no melanocytes arose in these cultures. Thus, it must concluded that Sl factor alone is not sufficient to stimulate the differentiation of melanocytes from their precursors in the neural crest.

In other studies, the phorbol ester drug, TPA, has been shown to influence the development of melanocytes. Human melanocytes will grow for long periods when stimulated with TPA (Eisenger & Marko, 1982; Halaban et al., 1983). Further, TPA appears to stimulate the development of melanocytes in cultures of avian dorsal root ganglia (Ciment et al., 1986). Thus, we investigated the effects of TPA on the development of melanocytes in mouse neural crest cultures after a period of two weeks. Invariably, the melanocytes appeared on the neuroepithelial sheet which grew out from the neural tube.

Given that TPA stimulates melanocyte differentiation in the neural crest cultures, it was possible that this differentiation could be influenced by Sl factor. We added Sl factor and TPA to the neural crest cultures to test this and found an approximately 10 fold increase in melanocyte numbers compared to cultures with TPA alone. Thus Sl factor is acting with TPA in the induction of melanocytes.

The synergy between TPA and Sl factor in the production of melanocytes may be a direct synergistic effect of the two factors acting on the same cell to produce melanocytes. Alternatively, Sl factor may act on the melanocyte precursors to stimulate division and/or survival but not act as a differentiating agent. These possibilities could partially be tested by pulsing the cultures first with Sl factor, then washing it out and adding TPA to separate temporally the activities of Sl factor and TPA. The results of these experiments indicate Sl factor is mainly acting on the melanocyte precursors but not as a differentiation agent. That there is a requirement for added Sl factor early in the culture period in these pulsing experiments indicates that Sl factor is acting as a survival agent for the melanoblasts. The reason that melanocytes arise in cultures containing TPA from the start might be that there is a limited amount of endogenous Sl factor in the cultures. This is quite possible as there is

expression of S1 factor in the neural tube during this time in vivo (Matsui et al., 1990).

Presumably, TPA is mimicking a function normally found in vivo at the time and place of melanocyte differentiation, which is postnatally in the skin. TPA activates the protein kinase C pathway and so may be activating any number of growth factors, hormones, cell surface molecules or other types of cell activation pathways. One possible hormone implicated in melanocyte differentiation is melanocyte stimulating hormone. However, we have found no activity of MSH in the neural crest cultures either in the presence or absence of S1 factor.

CONCLUDING REMARKS

Clearly, there must be many factors involved in the neural crest differentiation process. The factors described so far are only a few of all the factors which are required to determine this specificity. What factors influence the enteric nervous system, the parasympathetic nervous system, glial cells in the PNS, and the myriad of cells arising in the facial regions? While there are suggestions that these cell types are also influenced by soluble peptide growth factors, the identity of these factors is not known. Perhaps, some of these factors are already purified and cloned, but their roles in this system have yet to be elucidated. In addition, cell surface molecules, extracellular matrix molecules and steroids, such as retinoic acid, are also likely to contribute to this process. The other key questions which have not been addressed here are: How do these factors work? What genes are being regulated by the factors? How do they interact with genes that regulate DNA binding proteins? The search for the key regulatory genes in this process is just beginning. It is this interaction between growth factors and the transcriptional regulators that probably leads to the harmony of cell differentiation.

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