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REGULATION OF NEURAL PRECURSOR DIFFERENTIATION IN THE EMBRYONIC AND ADULT FOREBRAIN

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SUMMARY

1. Precursors form the neuroepithelium of the developing cortex and also from the adult sub-ventricular zone, can be cloned *in vitro* after stimulation with fibroblast growth factor (FGF)-2 and have the potential to give rise to both neurons and glia. The generation of neurons from these clones can be stimulated by either a factor derived from an astrocyte-precursor line, Ast-1, or FGF-1.

2. Neuronal differentiation stimulated by FGF-1 can be inhibited by diacylglycerol-lipase inhibitor and mimicked by arachidonic acid, suggesting that the neuronal differentiation is signalled through the PCLγ pathway.

3. The sequential expression of FGF-2 and FGF-1 within the developing forebrain neuroepithelium fits with the different functions the two FGF play in precursor regulation.

4. We have shown that the precursor response to FGF-1 is regulated by a heparan sulphate proteoglycan (HSPG) expressed within the developing neuroepithelium. Precursors restricted to the astrocyte cell lineage can be stimulated by epidermal growth factor or FGF-2; however, the differentiation into GFAP positive astrocytes appears to require a cytokine acting through the leukaemia inhibitory factor β receptor.

Key words: arachidonic acid, clones of precursors, epidermal growth factor, fibroblast growth factors, heparin sulphate, multi-potential precursors, neuronal differentiation, neuronal precursors, precursor commitment, proteoglycans, sub-ventricular zone.

INTRODUCTION

The cerebral cortex is the product of precursor cells contained within the neuroepithelium of the dorsal aspect of the telencephalon. The precursor cells proliferate rapidly and generate the first neurons which migrate to the pial surface to form the pre-plate which later becomes the sub-plate. The preplate neurons have recently assumed increased importance since it now appears that they may retain positional information which formally resided in their neuroepithelial precursors and which is required for targeting the appropriate thalamic input (Ghosh *et al.* 1990). In contrast to these first formed neurons, some of the later neuronal progeny of the neuroepithelium (also called the ventricular zone) which migrate primarily in association with radial glia (Rakic 1978) and penetrate the sub-plate to form the cortical plate, have been shown to be geographically unaligned with their parent precursor and probably lack positional information (for review see O'Leary *et al.* 1994). Once the neuronal populations have been generated the majority of cell proliferation occurs within the sub-ventricular zone, the progeny of which appear to be predominantly glial cells.

In order to understand the foundation for the structural and cellular development of the cerebral cortex, we need to define the genetic and epigenetic events which regulate neuronogenesis and gliogenesis and also to characterize the lineage potential of precursors within the germinal zone. Previous studies using retroviral markers have suggested that the majority of precursors within the developing cerebral cortex are restricted to either the glial cell or the neuronal lineage (for review see Luskin 1993); this interpretation, however, is hampered by: (i) an inability to label cell precursors at the onset of neurogenesis; (ii) inadequate phenotyping of all the progeny of a single precursor (clone); and (iii) not being able to monitor progeny of a single clone over an extended period of time. Our studies have centred on sampling the precursor population at various times during neurogenesis. Using defined growth conditions and clonal analysis *in vitro* we have been able to examine both the lineage repertoire of individual precursors and gain some insight into the epigenetic factors which influence lineage determination of precursor cells.

LINEAGE POTENTIAL OF FOREBRAIN PRECURSORS

Cloning of precursors from the earliest neuroepithelium

In the mouse, embryonic day 9 (E9) or E10 is the earliest that neuroepithelial cells can be successfully isolated from the developing telencephalon: at this stage less than 1% of the cells have any neuronal markers (Drago *et al.* 1991). Previous studies have shown that neuroepithelial cells isolated at this stage can be stimulated to proliferate with fibroblast growth factor-2 (FGF-2; Murphy *et al.* 1990), a factor that is expressed by the neuroepithelium at this time of development (Drago *et al.* 1991; Nurcombe *et al.* 1993), and so this factor was chosen to stimulate precursor plated out at one cell per well. Clones arose at the frequency of about one in 20 cells plated (Kilpatrick

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& Bartlett 1993) and consisted of flat epithelioid cells. The clones continued to proliferate over 2–3 weeks but contained few if any neurofilament-positive neurons; after 10 days many of the clones contained significant numbers of astrocytes (GFAP-positive). The observation that many of the clones did contain a low number of neurons as well as glia confirmed our previous studies using immortalized cell lines from E10 that some of the precursors were in fact multi-potential (Bartlett *et al.* 1988). However, the major finding was that the majority of clonal progeny failed to differentiate, an observation confirmed by the high re-cloning rate of the clonal progeny (>85%; Kilpatrick & Bartlett 1993). At this time we had identified a factor contained within medium conditioned by an astrocyte-precursor cell line, Ast-1, which stimulated neuron differentiation in E10 neuroepithelium (Kilpatrick *et al.* 1993); by applying CM to the clones we were able to induce significant neuronal production within 1–2 days in a significant proportion of clones (Kilpatrick & Bartlett 1993). This provided the first evidence that a discrete second signal was required for neuronal differentiation. Recent evidence suggests that Ast-1 is a tumour growth factor (TGF)-β-like molecule (Murphy 1994, unpubl. data), however, its precise nature and expression pattern *in vivo* is not yet known. It now appears, however, that a more potent signal for initiating neuronal differentiation is likely to be FGF-1 (as described later).

Evidence for early commitment of neuronal precursor within the developing neuroepithelium

Although the *in vitro* clonal analysis indicates that many of the precursors with significant proliferative potential are bipotential, several studies using retroviral markers have indicated that there are cells with restricted lineage capabilities. Recently we have been able to identify a cell-surface marker which is associated with commitment of precursor cells to the neuronal lineage, and have been able to demonstrate that as early as E10 many of the neuroepithelial cells are committed to the neuronal lineage. Unlike glia and virtually all other cell types in the body, neurons have an inability to express MHC class I molecules on their surface in response to interferon-γ (IFN-γ; Bartlett *et al.* 1989), thus we decided to examine the stage at which unresponsiveness occurs. At E9, all the neuroepithelial cells can express MHC molecules, as development of the cortex proceeds; however, an increasing percentage of the precursor cells lose their ability to be induced (Bailey *et al.* 1994). We have selected the non-inducible cells by cell sorting and have demonstrated that on replating under a variety of conditions these cells have the potential to give rise exclusively to neurons (Bailey *et al.* 1994). Thus, it appears as if these precursors are committed to the neuronal lineage. Although the neuronal precursors have the ability to proliferate, this is much more restricted than the uncommitted precursors; an observation that may explain the limited size of neuron-only clones seen *in situ* in the forebrain labelled by retroviral markers (Walsh & Cepko 1992).

Evidence for lineage committed precursors later in cortical development

To examine the potential of precursors later in development, we have cloned cells from E17 forebrain and have found that FGF-2 stimulates three types of precursors: one that is restricted to producing astrocytes; one that produces predominantly neurons and one that is similar to the E10 precursor and generates both astrocytes and neurons (Kilpatrick & Bartlett 1995). It is of note that the differentiation of these clones does not require an exogenous second signal as do E10 clones. This can be interpreted as indicating that these precursors already have received the appropriate signals *in situ* and, in the case of the multipotential clone, it may reflect the presence of factors produced by other cells within the clone, such as astrocytes. This result confirms the earlier studies with retrovirus tracers but again indicates that there are still numerous precursor cells capable of generating neurons present, even at this time nearing the end of neuronal production in the mouse cortex. It was also found that the glial committed precursor in the E17 forebrain could be selectively stimulated by epidermal growth factor (EGF; Kilpatrick & Bartlett 1995).

Presence of multipotential precursors in the adult mouse forebrain

Richards *et al.* (1992) and Reynolds and Weiss (1992) described the presence of neuronal precursors within the adult brain. We have now managed to localize these precursors to the subventricular zone (SVZ) of the lateral ventricle, the remnant of the germinative layer which originally gave rise to the cells of the cerebral cortex. Using the same procedures as for the E10 cells, we have been able to generate epithelioid clones from cells of the SVZ by stimulating them with FGF-2. Like the E10 clones, the SVZ clones generated with FGF-2 contain GFAP positive cells but no neurons. Unlike E10 precursors, however, approximately 50–60% of the SVZ clones, when stimulated with FGF-1, contained greater than 50 neurons which stained positively for the neuronal marker microtubule associated protein-2 (MAP-2).

FGF-1 STIMULATES NEURONAL DIFFERENTIATION

FGF-1 and FGF-2 stimulate the same precursor

The observation that FGF-1 stimulated SVZ clones to produce neurons whereas FGF-2 stimulated clones only produced glia, could be explained in two ways: either they stimulated different precursors, or FGF-1 induced neuronal differentiation in addition to proliferation. To test this we first generated clones with FGF-2 and after 5 days switched them to FGF-1. We found that approximately 55% of the switched clones produced neurons of identical phenotype to that observed in clones initiated with FGF-1; indicating that the precursor population stimulated with both factors was probably identical.

Requirement for heparan sulphate proteoglycan for FGF-1 action

The concept that FGF-1 may be the primary stimulator of neuronal differentiation was supported by our previous observations that FGF-1 expression in the neuroepithelium of the telencephalon coincided with the time neuronogenesis commenced approximately E11 (Nurcombe *et al.* 1993). FGF-1, however, failed to stimulate neuron production in E10 clones.

This discrepancy may in part be explained by our observation that the action of FGF-1 required the presence of a brain-derived heparin sulphate proteoglycan (HSPG). We found that at the onset of FGF-1 expression in the neuroepithelium, there was concomitant expression of a HSPG (Ford *et al.* 1994) which preferentially bound FGF-1 and was required for neuroepithelial stimulation (Nurcombe *et al.* 1993). When E10 clones were generated in the presence of FGF-1 and the appropriate HSPG, MAP-2-positive neurons were generated in approximately 60% of clones. The requirement for HSPG appears to be due to its ability to bind and present FGF-1 to its receptor; in addition, the direct binding of the HSPG to the FGF receptor may also be involved in signal transduction. The corollary of this result is that the adult precursors must constitutively express the appropriate HSPG on its cell surface. Although we have not definitively shown this, neuronal differentiation of adult precursors can be prevented by the addition of another FGF-1 presenting molecule, heparin. This demonstrates that it is the context in which FGF-1 is presented to its receptor that determines the signalling pathway.

FGF-1 stimulates neuronal differentiation through a second messenger pathway distinct from the proliferative pathway

The binding of FGF to its receptors (of which there are four well characterized members) leads to receptor dimerization and trans-autophosphorylation on certain tyrosine residues. This in turn creates binding sites for molecules with SH2 domains, including *src* and PLCγ, and activates several signalling cascades which may be independent or synergistic (Schlessinger & Ullrich 1992). It has been shown that the proliferative signal initiated by FGF is not dependent on PLCγ activation (Peters *et al.* 1992), but recent evidence suggests that activation of this molecule is required for neuronal functions such as neurite outgrowth (Williams *et al.* 1994). We have investigated whether this pathway, which proceeds from PLCγ activation to produce diacylglycerol (DAG); to the conversion of DAG to arachidonic acid (AA) by DAG lipase; and finally to calcium influx, was involved in stimulating neuronal differentiation. We examined the effect of two components, DAG lipase inhibitor and AA, in this pathway. The addition of DAG lipase inhibitor in the dose range of 5–10 μg/mL to FGF-1 stimulated clones resulted in total inhibition of neuron formation whereas the total number of clones was not substantially reduced, supporting the notion that the neuronal differentiation pathway required the production of AA. In addition, >90% of the clones which could be generated with AA acid at 100 μg/mL were of the neuronal type, again supporting the idea that signalling through this pathway predominantly promotes neuron differentiation. The addition of AA to FGF-2 stimulated clones also induced neuronal differentiation similar to that induced with FGF-1.

EGF stimulates non-neuronal precursors

As mentioned previously, EGF stimulated precursors appeared to only give rise to clones containing astrocytes. This was also true of the adult precursors. In order to further examine whether this reflected a different precursor population or merely a failure to produce neurons, we have added both AA and FGF-1 to these cultures but none of the clones has produced neurons. Clones generated with EGF under serum free conditions, called neurospheres, also failed to generate neurons under these conditions which was in contrast to that previously reported by Reynolds and Weiss (1992). A possible explanation for this is that previous studies have not been carried out at the clonal level and since EGF has been shown to support the survival of mature neurons *in vitro*, it may suggest that these surviving neurons have been incorporated into the neurospheres. It is also possible that the conditions we have used are not conducive to the neuronal differentiation of EGF stimulated precursors.

ASTROCYTE DIFFERENTIATION REQUIRES A FACTOR ACTING THROUGH THE LIFβ RECEPTOR

From the studies described it appears that the production of astrocytes from their precursors does not require discrete epigenetic signalling apart from that required to stimulate cell division. Another explanation, however, could be that astrocyte production (as measured by GFAP expression) which does not occur until 7–8 days after plating in an E10 (Abney *et al.* 1988) is due to the production of endogenous factors within the clone. Recent experiments tend to support this latter contention, as E10 neuroepithelial cells cultured *in vitro* can be prevented from differentiating into astrocytes by antibodies which bind to, and block ligand binding to the leukaemia inhibitory factor (LIF)β receptor (Richards *et al.* 1994, unpubl. data). In addition, mice bearing a mutation in this receptor appear to have an astrocyte deficiency (Ware *et al.* 1994, unpubl. data) and we have recently shown that E10 neuroepithelial cells obtained from these mice fail to differentiate into astrocytes when plated *in vitro* (Koblar *et al.* 1994, unpubl. data). LIF and ciliary neurotrophic factor (CNTF), which both act through this receptor, have been shown to influence astrocyte production *in vitro*; however, animals with mutations in either of the genes coding for these ligands appear to have normal astrocyte production. Thus, other members of the family, like Oncostatin M, or an as yet unidentified ligand, may be responsible.

In conclusion, the results of *in vitro* clonal analysis have demonstrated that the precursor cells which give rise to the cerebral cortex require a number of growth factors to regulate their proliferation and differentiation. It now appears that FGF-1, in association with a specific HSPG, stimulates neuronal differentiation through the PLCγ pathway and a molecule acting through the LIFβ receptor regulates glial differentiation. There is a multipotential precursor which persists throughout development, presumably by self-renewal, which is also present in the adult SVZ, in addition to precursors which appear to be committed to the neuronal or glial pathway. EGF appears to be predominantly involved in stimulating precursors which are committed to the glial pathway. By using clonal analysis and defined growth factors it should now be possible to examine the mechanisms regulating precursor differentiation into the different sub-types of neurons found in the cortex. Ultimately it should also facilitate an understanding of how epigenetic factors and genetic factors interact to regulate early cortical development.

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