GROWTH FACTOR EXPANDED-GLIAL RESTRICTED PRECURSOR CELLS: SUPPORT OF NEURONS BY DERIVED ASTROCYTES

by

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FINAL READING APPROVAL

To the Graduate Council of the University of Utah:

I have read the thesis of Jill Kristin Howard in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative material including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the supervisory committee and is ready for submission to The Graduate School.

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ABSTRACT

Growth factor expanded precursor cells may be useful for therapeutic strategies in central nervous system tissue repair and regeneration. The ability to maintain precursor cells in a state of self-renewal allows for the production of large numbers of cells. The use of glial-restricted precursor cells (GRP) is therefore of great interest in the use of derived astrocytes for reparative strategies, such as on synthetic devices for guiding nerve growth. GRP cells could be a critical improvement because of the decrease in neuron supporting properties that differentiated astrocytes display with time in culture. In accordance to what has been observed for differentiated cells, precursor cells may also continue to measure elapsed time despite their state of self-renewal. It has not been addressed whether these internal timing mechanisms result in altered properties of the derived cells. This study investigated the changes in neurite outgrowth and cell adhesive properties of astrocytes derived from growth factor expanded GRP cells over various periods of time in culture. Astrocyte differentiation was induced after GRP cells were expanded in culture for up to seven weeks. Cerebellar granule neuron adhesion and neurite outgrowth were measured on monolayers of GRP-derived astrocytes at weekly intervals. Extended self-renewal of GRP cells in culture did not pause the functional changes associated with astrocyte maturation and astrocytes derived from expanded progenitors increased their surface area in correspondence with the

length of time in culture. In addition to the flattened morphologies, prolonged proliferation also resulted in decreased chondroitin sulfate proteoglycan expression. These changes in astrocyte morphology and proteoglycan expression correlated with decreased neurite outgrowth and cell adhesion, and may correspond to the capability of GRP cells to intrinsically time the progress of development despite their state of self-renewal. An important implication of these findings is the insight that growth factor expansion of precursor cells in culture resulted in cells with a progressive decrease in neuron supporting properties.

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INTRODUCTION

Interactions Between Neurons and Astrocytes

Astrocytes are the most abundant cell type of the brain and essential for the development, maintenance, repair, and maturation of the central nervous system (CNS). Glial structures are closely associated with neuronal migration during development and axon guidance, which plays a major role in generating the structure and circuitry of the CNS. During early development, astrocytes and their progenitors guide the migration and outgrowth of neurons.

Axon Guidance by Astrocytes

Evidence from in vitro and in vivo experiments suggests that astrocytes can guide neurite pathfinding, and patterning in the CNS (Steindler et al., 1988; Smith et al., 1990; reviewed in Sajin and Steindler, 1994). Astrocytes and their progenitors provide guidance to growing axons during embryogenesis, not only by providing a structural scaffold but also by providing the directional cues that either repel or attract growing axons. Singer et al. (1979) observed that during development axons followed the channels made by glial cells. Since then, others have observed that the scaffold formed by astrocytes directs and supports neuronal migration and axonal elongation during development (Rakic, 1971, 1988; Gasser, 1990). This is accomplished by providing an adhesive substrate for neuronal outgrowth, as well as by forming restrictive boundaries to limit axon

pathfinding. In the developing CNS, boundaries are formed by glia to guide axons to the appropriate targets in the cerebral commissures, anterior commissure, corpus callosum, internal capsule, developing forebrain (Silver et al., 1982, 1993) diencephalic/telencephalic junction (Silver, 1984), the somatosensory barrel fields of the cortex (Cooper and Steindler, 1986) and the optic chiasm (Navascues et al., 1987; Silver et al., 1987; Sretavan, 1990; Godement et at., 1990).

Consistent with in vivo studies (Smith et al., 1986; Smith et al., 1988), astrocytes are a preferred substrate for the outgrowth of neurons in vitro (Noble et al, 1984; Fallon, 1985; Smith et al., 1990). Neurons have been shown to prefer growth on astrocytes over other cell substrates. When axons are given a choice between different cell types, astrocytes are also preferred over other neurites (Noble et al., 1984).

Astrocytes provide an attractive surface to neurons by expressing a number of adhesion molecules that can support and influence neurite outgrowth, including NCAM (Noble et al., 1985), N-cadherin (Tomaselli et al., 1988; Neugebauer et al., 1988) and laminin. The latter has been shown to be a powerful neurite outgrowth promoting molecule (Liesi et al., 1983, 1984, 1988). The importance of these molecules has been demonstrated using function-blocking antibodies targeted to the ligands or their receptors. These assays confirmed that astrocytes mediate neurite adhesion and outgrowth through adhesive molecules on their surfaces (Tomaselli et al., 1988; Smith et. al, 1990; Neugebauer et al., 1988). Astrocytes also produce and secrete glycoproteins and proteoglycans. These extracellular matrix (ECM) molecules have been suggested

to influence axonal outgrowth by either being permissive or inhibitory (reviewed by Powell et al., 1997; Fitch and Silver, 1997; Margolis and Margolis, 1997). The combined influence of cell-surface and ECM molecules contributes to the influence of astrocytes on axon pathfinding in development.

Neuronal Maintenance by Astrocytes

Astrocytes not only support neurons in their growth and pathfinding during development, but they are also intimately involved in the maintenance and survival of neurons in the CNS. Astrocytes produce a wide variety of growth factors which singly or in combination selectively regulate the morphology, proliferation, differentiation, and survival of neurons in vivo and in vitro (Yoshida and Gage, 1992). The role of neuronal growth factors produced by astrocytes has been demonstrated in vitro by showing a higher survival rate, as well as increased neurite extension for neurons when grown on astrocyte monolayers or in the presence of astrocyte conditioned media (Noble et al., 1984; Fallon, 1985; Rousselet, 1988).

Astrocytes also play a critical role in neuronal maintenance by regulating the extracellular environment of the CNS (Walz, 1989; Montgomery, 1994). Astrocytes are closely related to the electrically active sites of neurons, and contribute to synaptic transmission by controlling extracellular potassium levels and by removing certain classes of neurotransmitters. Astrocytes protect neurons by their ability to take up the excitatory neurotransmitter glutamate, which is a key mediator of excitotoxicity (Choi, 1988; Swanson et al., 1994), and by reducing the damaging influences of free radicals and nitric oxide (Eddleston and Mucke,

1993; Volterra et al., 1994; Montgomery, 1994). Astrocytes protect neurons by buffering the extracellular environment as well as by detoxifying the CNS. These protective astrocyte functions complement the growth supporting role of astrocytes.

Due to the unique relationship between astrocytes and neurons, many new strategies for CNS repair and regeneration have incorporated astrocytes into their experimental designs. Astrocytes have been transplanted into lesion sites of the CNS (Smith et al., 1986; Emmett et al., 1988; Smith and Miller, 1991), genetically engineered to over-express outgrowth promoting molecules (Mohajeri et al., 1996; Yazaki et al., 1996), and utilized to provide pathways for nerve guidance and regeneration. All these strategies take advantage of the growth promoting and supportive properties of astrocytes. However, in certain instances astrocytes can become inhibitory to the outgrowth and regeneration of neurons.

Astrocytes in Response to Injury

In addition to the supportive role of astrocytes, they can also become reactive following injury to the CNS, which inhibits the growth of neurons. There are many components of the CNS healing response following injury, but the most unique and prominent is the astrocyte response to injury. Astrocytes from the adult CNS become reactive in response to injury and form a glial scar. Reactive astrocytes undergo morphological changes including the up-regulation of the intermediate filament glial fibrillary acidic protein (GFAP), this phenomenon is known as gliosis (McLoon, 1986; Frank and Wolburg, 1996; Stichel and Müller, 1994).

GFAP expression by astrocytes, which is also commonly used as a standard astrocyte marker, is the most sensitive indicator of brain injury or damage. In addition to the physical barrier astrocytes create in response to injury, they also respond by increases in proliferation, migration, and hypertrophy. Taken together, these responses ultimately result in the formation of an extensive basal lamina as well as physiological changes in the wound environment (Latov et al., 1979; Mathewson et al., 1985; Reier et al., 1986).

Reactive astrocytes up-regulate the secretion of various ECM molecules, which include cellular fibronectin (CFN), chondroitin sulfate proteoglycans (CSPG), as well as glycoproteins laminin, fibronectin, and tenascin C (Stichel and Müller, 1998). Several of these molecules have been found to be inhibitory to the regeneration of neurons, in particular CSPGs and tenascin C (Stichel and Müller, 1998). Interestingly, many of these molecules are expressed during normal development and are down-regulated after the generation of the CNS is complete (Brodkey et al., 1993; Aubert et al. 1995).

Astrocytes are an important component of the CNS wound response. Reactive astrocytes result in the formation of a physical and molecular barrier for regenerating axons at the site of injury. This scar formation and its inhibition on axonal regeneration was first described by Ramón y Cajal (1928) and continues to be a primary focus in efforts to overcome the regeneration barrier created by reactive astrocytes following CNS injury. The inhibitory response of reactive astrocytes to regeneration is in sharp contrast to their positive influences on neurite elongation during development or the injured neonatal brain. In fact, the

response of reactive astrocytes to injury has been shown to vary with age, where the injured neonatal brain allows for regeneration, and the adult brain fails to support regeneration processes (Reier and Houle, 1988). This suggests that astrocytes may change their functional properties with age as well as after injury.

Functional Changes of Astrocytes with Age

In the CNS, the properties of astrocytes appear to change during maturation, and maturation of the CNS is accompanied by a decreased ability to recover from injury. It is clear that astrocytes promote neurite outgrowth during development (Silver, 1984) and in vitro (Noble et al., 1984; Fallon, 1985). Astrocytes regulate axon elongation and provide a cellular substrate on which axons can grow for long distances to their specific targets. However, in the adult CNS there is little axon growth.

Another aspect that demonstrates this functional difference between immature and mature astrocytes are their responses to injury. In analogy to the different functional roles of astrocytes in the young and mature CNS, it is well established that astrocytes cause regenerative failure in the mature CNS following injury (Reier and Houle, 1988), whereas injury to the neonatal CNS during development results in minimal glial scarring. In addition to these age-related structural differences, lesions in embryonic or early postnatal brains are less functionally detrimental than those in adults (Devor, 1975, 1976). It becomes clear that after injury to an aged brain, neurons are unable to regenerate. This failure of neuronal regeneration in mature animals cannot be attributed to the neuronal cell, but rather a changes in the supportive properties of astrocytes.

Transplantation techniques have shown that neurons still maintain their ability for regrowth, but are inhibited by the surrounding glial cell populations (Kromer et al., 1981, 1985). Consequently, these observations suggest that neurons do not lose their ability to regenerate in the adult CNS, but that it is the astrocytes that change their ability to support axon growth with age (Geisert and Stewart, 1991).

Evidence to support the hypothesis that astrocyte maturation results in changes in their ability to support neurite outgrowth comes from studies performed by Smith and colleagues (1986) who used a nitrocellulose implantation paradigm in the corpus callosum as a model for regeneration. The corpus callosi of neonatal mice were lesioned prior to the completion of the pathway. A piece of Millipore filter paper was then placed into the lesioned cavity and it was assessed whether the development of the corpus callosum would continue. They observed that GFAP-positive astrocytes covered the implant. Immature astrocytes were capable of greatly supporting neurite outgrowth despite the fact that they were reactive. However, implants that were placed into older animals were covered with reactive GFAP-positive astrocytes that did not support neurite outgrowth. This observation was further explored by culturing purified cortical astrocytes on the implants prior to implantation. They found that astrocytes from the neonatal cortex were able to support neurite outgrowth when maintained in culture for less than one week. In contrast, those astrocytes that remained in culture for longer periods were unable to support neurite outgrowth (Smith et al., 1986). These data show that immature astrocytes are able to promote robust neurite outgrowth when transplanted directly into the mature mouse CNS, even though regeneration

is typically inhibited by this environment. Unlike immature astrocytes, adult astrocytes produced a dense scar when transplanted that inhibited axon growth (Smith et al., 1986). Therefore, axonal regeneration in the mature CNS may be stimulated by reintroducing immature astrocytes at the lesion site, but this ability is lost after astrocyte maturation has reached a particular critical period. Clearly, maturational changes of astrocytes influence their ability to support neurite outgrowth.

The observation that the ability to support neuronal outgrowth decreases after astrocytes have aged beyond a particular critical period was further explored in vitro. Millipore implants were placed into the cerebral cortex of neonatal and adult animals and then removed at varying time points. They were placed in culture to serve as a substrate for embryonic hippocampal neurons, and these neurite outgrowth assays showed that neurites grew significantly longer on neonatal astrocytes as opposed to adult astrocytes (Rudge and Silver, 1990). These results provide additional evidence for a change in outgrowth promoting properties due to astrocyte age. Using a similar approach, Smith and Silver (1988) demonstrated that astrocytes from newborn rodents could suppress scar formation and promote axon growth when introduced into the adult CNS after being maintained in tissue culture for a short time. However, astrocytes lost this ability during the fourth week of culture, which showed that astrocytes age in vitro at a similar rate as in vivo.

Taken together, these studies show that astrocyte maturation both in vivo and in vitro change the functional ability of astrocytes to support neurite

outgrowth. The evidence from in vitro and in vivo transplantation studies using cultured astrocytes suggest that there is a functional difference between immature and mature astrocytes in supporting neurite outgrowth. However, the mechanisms responsible for these inherent changes remain unclear. The inability of mature astrocytes to support axon outgrowth may be a result of intrinsic changes taking place during astrocyte maturation, a reduction in growth promoting molecules, or an increased expression of outgrowth-inhibiting molecules.

To test for these possibilities, astrocytes were allowed to age for different periods and the extent and molecular basis of neurite outgrowth on these astrocyte cultures of different ages was compared (Smith et al., 1990). As mentioned previously, NCAM, N-cadherin, and laminin (which is a ligand for the integrin β1 receptor) play a substantial role in neurite outgrowth when expressed on the surface of immature astrocytes (Noble et al., 1985; Grumet and Edelman, 1988; Tomaselli et. al., 1988; Liesi et al., 1983, 1988). Smith et al. (1990) investigated neurite outgrowth on astrocyte monolayers aged in culture, and also assessed outgrowth in the presence or absence of antibodies to these adhesion molecules or their receptors. Antibodies to NCAM significantly reduced neurite outgrowth on immature, but not mature astrocytes, while antibodies to the integrin β1 receptor (which primarily binds laminin as a ligand) reduced neurite outgrowth on immature and, to a lesser extent, on mature astrocytes. These data suggest that cell surface molecules that mediate neurite outgrowth change with astrocyte

age. A virtual loss of NCAM is observed in addition to reduced laminin-mediated interactions.

These data suggest that astrocytes mature in culture due to intrinsic changes that reduce their capacity to support neurite outgrowth. Astrocyte maturation appears similar in vivo and in vitro. Brain derived astrocytes support outgrowth in vivo until around the third postnatal week (Smith et al., 1986) and newborn astrocytes decline in their outgrowth promoting properties after 4 weeks in culture (Smith and Silver, 1988; Schreyer and Jones, 1987).

Verifying the results in astrocyte monolayer cultures, an age effect has also been shown using three-dimensional astrocyte cultures. Again, astrocytes that had been in culture for a short time were more permissive for neurite outgrowth compared to aged cells (Fawcett et al., 1989). These results demonstrate that astrocytes mature in vivo and in vitro at a similar time schedule and indicate that the functional changes during astrocyte maturation are intrinsically regulated. Astrocytes in culture are therefore functionally similar to maturing cells in vivo. These findings are of great importance for the therapeutic use of astrocytes with regenerative strategies and for answering basic questions of cell biology and timing.

The unique relationship between neurons and astrocytes makes the latter an ideal cell type for reparative strategies such as on devices designed to provide pathways during nerve guidance and regeneration. However, the therapeutic application of astrocytes for such devices has been limited because of the problems associated with the maturation of astrocytes in culture (Smith et al.,

1988; Schreyer and Jones, 1987), as well as the limited supply. Possible strategies to avoid the decline in outgrowth promoting properties that occur when astrocytes age in culture include the use of even younger astrocytes or astrocytes derived from expanded astrocyte precursor cells. Younger astrocytes would constitute a primary cell population with greater outgrowth promoting properties, and could be kept in culture longer before showing a decline in their properties. Even more promising is the use of growth factor expanded progenitor cells. Such cells can be kept proliferating in culture and can be differentiated at the appropriate time. The use of such progenitors may potentially avoid many unwanted effects of astrocyte maturation in vitro.

Astrocyte Progenitors

Radial Glia

It has generally been accepted that radial glial cells are the first subtype of glia to appear in the brain (Misson et al., 1991), and it has been recognized that they provide a scaffold for neuronal pathfinding during development. Many observations have been made that describe radial glial and their involvement in the patterning of the CNS (Rakic, 1972; Rakic and Nowakowski, 1981; Misson et al., 1991). Radial glia are recognized by their distinct phenotype, highly branched processes that extend radially into the mantle layer, as well as expression of the RC2 antigen. They provide a scaffold for the migration of newly generated neurons en route from the mantle layer ventricular zones to final destinations.

Ramón y Cajal (1955) first hypothesized that radial glia are the precursors to astrocytes. He based this hypothesis on Golgi stains of glia at various early

stages of development. More recent studies have confirmed his original hypothesis. By using more direct tracing methods, it has been shown that radial glia can transform into astrocytes in the cerebrum and in the spinal cord (Voigt, 1989; Culican et al. 1990; Mission et al., 1991). Others have presented evidence suggesting that radial glia can also give rise to oligodendrocytes (Hirano and Goldman, 1988). One such study showed that the presence of radial glia is necessary for the migration of oligodendrocyte precursors out of the ventral half of the cord (Warf et al., 1991).

Considerable progress has been made in understanding the time of origin of the radial glial cell, its role in neuronal migration , as well as the ability of radial glia to form astrocytes and oligodendrocytes. However, research has been restricted to in vivo phenotypic observations. Much work is still needed in defining the developmental potential of radial glia and their relationship to other identified glial precursor cells.

Glial Restricted Precursor Cell

The sites of origin for neurons and glia in the CNS are the ventricular zones. The ventricular zone forms part of the columnar neuroepithelium and is composed of a pseudostratified, single layer of progenitor cells. These cells proliferate and migrate outward to populate the subventricular zone as well as the intermediate and marginal zones. It has been generally assumed that the ventricular zone is composed of progenitor cells that are capable of giving rise to all varieties of neurons and glia in the CNS.

The neuroepithelium of the ventricular zones progresses from a single layer of mitotically active progenitor cells to a more complex structure that is composed of multiple cell layers. This neuroepithelium is composed of neuroepithelial stem cells (NEPs), which are multipotent stem cells that have the capacity to undergo self-renewal and proliferation, as well as differentiation into neurons, oligodendrocytes, and astrocytes (Davis and Temple, 1994; Gritti et al., 1995). These multipotent stem cells are believed to give rise to other precursor cells that are further restricted in their differentiation potential. A precursor cell has been identified that emerges from NEP cells with a differentiation potential restricted to glial lineages (Rao and Mayer-Pröschel, 1997; Rao et al., 1998).

The glial restricted precursor cell (GRP) is believed to be the earliest identified glial precursor. The early appearance of GRP cells during spinal cord development is consistent with this hypothesis. Through clonal analysis it was shown that NEP cells from E10.5 rat neuroepithelium are multipotent stem cells that give rise to neuronal restricted precursor cells (NRPs), as well as glial restricted precursors (GRPs; Kalyani et al., 1997; Mayer-Pröschel et al., 1997). NEP cells that lack A2B5 immunoreactivity soon thereafter give rise to $A2B5^+$ cells that can be isolated around E13.5 in the spinal cord. At this time, $A2B5^+$ cells lack the expression of either oligodendrocyte or astrocyte differentiation markers (Mayer-Pröschel et al., 1997) and can differentiate into two distinct astrocyte populations as well as into oligodendrocytes. However, these precursor cells do not have the ability to differentiate into neurons even when kept in culture

conditions that have been shown to induce a neuronal phenotype (Rao and Mayer-Pröschel, 1997).

GRP cells are capable of extensive self-renewal in vitro. The differentiation potential of the GRP cell has been investigated in culture. GRP cells can be expanded throughout multiple passages without losing their multipotentiality and continue to proliferate when grown in the presence of platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). GRP cells that have proliferated in culture for an extended period of time possess the same ability to undergo tripotential differentiation that is typical for primary clones after being recloned (Rao et al., 1998). Thus, despite continued proliferation, GRP cells still possess the ability to differentiate into either astrocytes or oligodendrocytes when given the appropriate cell culture conditions.

The fate of GRP cells can be regulated in vitro by adding different supplements of growth factors. For example, GRP cells grown in 10% fetal calf serum (FCS) yield astrocytes primarily of the $A2B5^-/GFAP^+$ phenotype, whereas addition of ciliary neurotrophic factor (CNTF) and bFGF primarily yields $A2B5^+/GFAP^+$ astrocytes. These above mentioned astrocyte phenotypes can also be induced from GRP cells by adding bone morphogenetic proteins (BMP) 2,4 and 7.

Five astrocyte populations have been observed in embryonic spinal cord (Miller and Szigeti, 1991). Without available markers to distinguish between all classes it can not be determined clearly whether GRP cells can differentiate into the full complement of astrocytes when grown in various culture conditions. In

addition to astrocytes, GRP cells can also give rise to oligodendrocytes with the addition of CNTF and PDGF or thyroid hormone (T3; see Figure 1).

Although it has been established that these different types of astrocytes can emerge from GRP cells, it has yet to be determined what the relationship is between GRP cells and radial glia. It is not clear whether GRP cells can differentiate into radial glia, which are an embryonic precursor to astrocytes, or whether they correspond to radial glia in vivo. Transplantation experiments of GRP cells into the CNS of rats have verified that the differentiation potential of GRP cells is not restricted to astrocytes and oligodendrocytes, but also includes derived cells that phenotypically resemble radial glia (Herrera, Mayer-Pröchel, and Tresco unpublished data; Yang and Luskin, unpublished observation).

An important goal in understanding the relationship among the diverse classes of CNS glia has been reached with the identification of the GRP cell as the earliest glial precursor cell and as a direct descendant from the multipotent NEP stem cell. However, the relationship between GRP cells and other glial progenitors is unclear. For instance, it is not known whether the GRP cell gives rise to all other glial precursors, or whether it is among other precursors derived from NEP cells. However, it is clearly know that GRP cells are the earliest cell restricted to the glial cell fate, and their use can be of great value to understanding glial development and may allow for new approaches to CNS repair. To make use of GRP cells for CNS repair, it needs to be clarified whether a precursor cell can be expanded in culture without changes in the properties of the derived cell types.

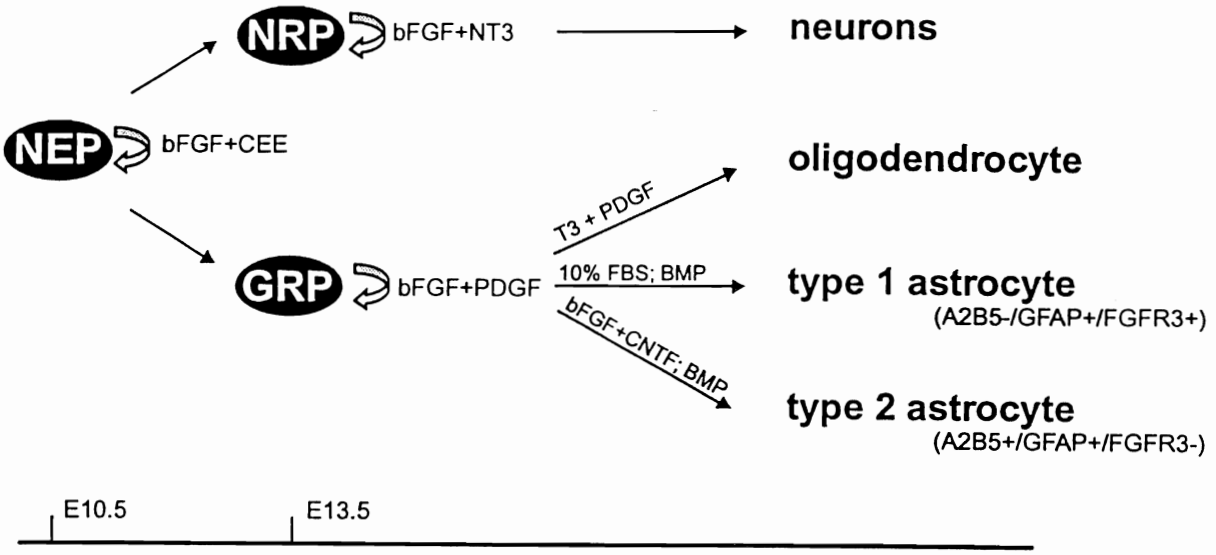

Figure 1. Generation of glial cells from multipotent stem cells

Neuroepithelial stem cells (NEP) can be expanded in the presence of basic fibroblast growth factor (bFGF) and chicken embryo extract (CEE). They are multipotent stem cells that have the potential to give rise to neuronal-restricted precursor cells (NRP) and glial-restricted precursor cells (GRP), both of with can be expanded in the presence of bFGF and an appropriate mitogen. The glial restricted precursor cell is tripotential and can give rise to oligodendrocytes after the addition of platelet-derived growth factor (PDGF) and thyroid hormone (T3). Two antigenically distinct astrocytes can also emerge from GRP cells by the addition of either 10 % fetal bovine serum (FBS) or ciliary neurotrophic factor (CNTF).

Therapeutic Approaches

The availability of GRP cells has opened new avenues for investigating the developmental biology and clinical applications of glial cells. Importantly, the potential clinical use of these cells includes the treatment of regenerative failure in the CNS following injury, especially since the first CNS progenitor cell discovered in vitro has shown great promise. The oligodendrocyte-type-2 astrocyte (O-2A; Raff et al., 1983), which is the progenitor cell of the myelin-forming oligodendrocyte, has already shown remarkable capabilities in remyelinating experimental demyelinating lesions (Groves et al., 1993; Warrington et al., 1993). Precursor cells have therefore become a new focus in CNS reconstruction therapy (for reviews see Blakemore and Franklin, 1991; Fisher, 1997; Brüstle and McKay, 1996; and references therein) and have already been tested as treatments for demyelinating diseases and Parkinson's disease. It seems likely that GRP cells may also be an invaluable tool for therapeutic interventions that result in CNS tissue repair, for example, through transplantation experiments as well as by providing substrate pathways for neuronal regrowth. Astrocytes that are derived directly from GRP cells may provide excellent substrates for promoting axonal regrowth and regeneration following injury to the adult CNS, that is, if astrocytes derived from GRP cells behaved like precritical aged cells.

One of the most appealing properties of precursor cells is their ability to self-renew. The addition of particular growth factors to the culture medium can halt differentiation and trap cells in a state of self-renewal for long periods of time,

where cells continue to proliferate as progenitors. This is of enormous potential value in the therapeutic use of precursor cells, which requires the use of large populations of precursor cells. Expanding the cell population in vitro can result in greater numbers of cells for experimental use.

Growth factor expanded-precursor cells are in wide use today. However, there is a remarkable void when it comes to published studies that investigate the properties of cells originally derived from expanded precursor populations. It is not known whether the functional properties of a given cell type that was derived from a growth factor-expanded precursor cell changes with time of continued proliferation. This question prompted us to revisit the two main factors that control cell fate, i.e., cell extrinsic cues from the environment and intrinsic properties of the cell. The interaction between these cues in the development of a precursor cell has previously been described for developmental timing of the O-2A progenitor (Temple and Raff, 1986; Noble and Murray, 1984; Bögler and Noble 1991, 1994).

Cell Intrinsic Timing

The differentiation of the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell in the rat optic nerve is a very clear example of how the interplay between cell intrinsic timing mechanisms and extracellular signals is thought to play a role in development. O-2A progenitors are the precursors of oligodendrocytes. The development of oligodendrocytes in the rodent CNS commences on the day of birth and continues for 2 weeks. It appears to be regulated by an internal clock. This was suggested by the regular timing of

oligodendrocyte differentiation from O-2A progenitor cells. In vitro, the same concise timing was observed from clones of O-2A progenitor cells. When O-2A progenitor cells from the same clone were separated into separate dishes, they differentiated into oligodendrocytes at the same time (Temple and Raff, 1986). Even more impressive, different groups of O-2A precursor populations within the same culture dish divided and differentiated with separate time courses from one another, but according to the timing of their own cell population (Temple and Raff, 1986). These observations provides strong evidence that it is not extrinsic factors, but rather intrinsic mechanisms that are regulating the temporal pattern of cell divisions and differentiation for the O-2A progenitor cell.

It was also discovered that extrinsic factors can influence or override the intrinsic timing mechanism of oligodendrocyte differentiation. O-2A progenitors will prematurely divide into oligodendrocytes in culture unless they are induced to divide by a particular astrocyte-derived mitogen, PDGF (Noble and Murray, 1984). With the addition of PDGF, the temporal pattern of oligodendrocyte division observed in vivo is replicated in vitro. The addition of extracellular signaling molecules, such as bFGF, can halt the terminal differentiation of the O-2A cell and keep them in a state of self-renewal for prolonged periods (Bögler et al., 1990). Withdrawal of bFGF, in media containing PDGF and T3, will stop cell division and initiate differentiation by releasing a proliferating precursor from the cell cycle (Noble et al., 1988). These observations suggest that progenitor cells can be expanded for experimental applications, and that a cell and its functional development can be suspended until the cells are induced to differentiate by the

addition of growth factors. However, this conclusion could not be reached if the cell continues to measure elapsed time despite the presence of external cues that arrest the cell cycle. It was observed that the measurement of time by O-2A progenitor cells can still proceed even when the apparent differentiation is repressed (Bögler and Noble, 1991; Bögler and Noble, 1994). When populations of O-2A progenitors are grown in the presence of PDGF and bFGF for longer periods, they differentiate more rapidly into oligodendrocytes after bFGF is removed. This behavior indicates that the internal mechanisms involved in cell timing are still measuring time even when they are unable to stop dividing at the appropriate time.

Similar temporal regulation as in O-2A progenitor populations during development is seen in fibroblast populations in vitro. Fibroblasts can be grown in culture for a finite number of cell divisions, until they reach a point at which cell division can no longer be induced (i.e., senescence). Fibroblasts grown in different conditions or from different animals undergo the same number of cell divisions before reaching senescence. This consistency suggests an intrinsic cell timing mechanism (reviewed by Goldstein, 1990). It was shown that cell timing could be overcome by the introduction of an oncogene, which allows fibroblasts to divide indefinitely (Groves et al., 1991). When the oncogene was inactivated, the cells rapidly stopped dividing and entered a state of senescence (Jat and Sharp, 1989). These observations suggest that, despite the arrest of cell differentiation by extra-cellular signaling molecules or the activation of an oncogene, the cells continue to behave as though their internal clock is measuring elapsed time. This

brings the cells to a threshold of differentiation irrespective of whether they are capable of cell division. The continued measurement of time by growth-factor expanded precursor cells that were expanded in culture raises the possibility that the properties of derived cell types change with time. Clearly, the evaluation of the properties of growth factor-expanded precursor cells is of importance with respect to their potential as a source for CNS repair and regeneration.

Effects of Continued Proliferation on Glial-Restricted Precursor Cells

Due to the current efforts for using growth factor expanded-precursor cells for CNS tissue repair and regeneration, and the available data indicating that progenitors age in culture when proliferating, it is of importance to understand whether cell types derived from expanded precursor cells have changed their inherent cell properties. It would be extremely attractive to be able to expand large numbers of cells for clinical use and to maintain these cell populations in culture for extended periods of time. If cells derived from freshly isolated precursor cells behave similar to those cells derived from progenitors expanded in culture for several weeks, then the use of precursor cells becomes practical. However, if precursor cells expanded in culture produce offspring with changed properties according to the age of their progenitors then this finding would have wide implications on the therapeutic use of these cells. This result would indicate that internal mechanisms involved in cell timing are still measuring elapsed time while the precursor cell population is expanded. The age of the precursor cell would therefore determine the properties of the derived cell type. In the effort to

utilize the unique properties of astrocytes as substrates for neuronal regeneration and guidance, we must be able to avoid the loss of these unique properties when astrocytes age in culture. A potential solution could be to derive astrocytes from pools of expanded glial-restricted precursor cells. However, this approach must be evaluated by first investigating the behavior of astrocytes derived from expanded GRP cells after they have been expanded as GRP cells for prolonged periods.

To determine the effects of age on growth factor expanded-precursor cells and the changes within their progeny with time, we have expanded glial restricted precursor cells in culture and induced the differentiation of astrocytes during successive weeks. Properties of these GRP-derived astrocytes were analyzed to determine whether they change in relation to the progenitor age in culture or in relation to their age with respect to the initial differentiation from precursor cells. Changes in neurite outgrowth, neural adhesion, surface area, and CSPG expression were assessed for astrocytes that were derived from GRP cells at different ages in culture.

METHODS

Substrate Preparation

Glass coverslips (12 mm; Fisher Scientific) were flamed and placed in 24-well plates (Costar), covered in 500 µg/ml of poly-L-lysine (PLL; Sigma) prepared in dH_20, and incubated overnight. Coverslips were rinsed with dH_20 and then covered with a laminin/fibronectin solution. Laminin, used at a concentration of 20 µg/ml (Biomedical Technologies Inc,) was added to DPBS (Gibco) along with fibronectin, diluted to a concentration of 25 µg/ml (Sigma). The laminin/fibronectin solution was allowed to incubate for 5 hr, 37°C. Coverslips were then rinsed with DPBS and allowed to dry.

Preparation of Rat Granule Neurons

Cerebella from 7 day old rats were removed, dissected free of meninges, minced, incubated for 12 min at 37°C in DMEM (Gibco) containing 1% trypsin and 0.1% Dnase. Following digestion, soybean trypsin inhibitor (SBTI, 0.52 mg/mL; Sigma) was added and the tissue centrifuged at 600g for 5 min, then resuspended in DMEM containing 0.1% Dnase. Fire-polished pasteur pipettes in decreasing diameter were used to triturate the tissue, and the remaining cell suspension was allowed to sit at room temperature for 2 min to allow undisrupted chunks of tissue to settle to the bottom. The supernatant was collected, centrifuged at 1000 g for 3 min, followed by 3000 g for 1 min, and resuspended in

DMEM. The cell suspension was incubated at 37°C for 30 min in a tissue-culture-grade dish coated with 500 µg/ml of poly-L-lysine (PLL; Sigma). This panning step binds contaminating cell types such as astrocytes and menigeal cells leaves a supernatant composed of granule neurons. The supernatant was harvested and centrifuged at 1000 g for 3 min, followed by 3000 g for 1 min, resuspended in DMEM-FBS (DMEM with 10% fetal bovine serum, 2 m*M* of glutamine, and 25 µg/ml of gentamycin), and counted in a hemocytometer. Hippocampal neurons used in additional experiments were also prepared as described for cerebellar granule cells, with the exception that hippocampi where dissected from postnatal day 1 rat pups.

For neurite outgrowth assays, approximately 5000 cells were placed in each well, either on confluent astrocyte monolayers or glass coverslips coated with PLL, fibronectin and laminin. Neurite outgrowth on coated glass coverslips were measured for each granule neuron preparation. Laminin and fibronectin from the same lot were used throughout experiments to keep their purity constant. Changes in the robustness of neurite outgrowth on coverslips result from inherent differences among animals. The results were therefore normalized for between-animal differences to account for differences between neuronal preparations. Samples were normalized by dividing the outgrowth (in microns) of each neuron of a particular sample, by the mean outgrowth (in microns) of the neuronal preparation on the coated glass coverslip of the same sample.

Preparation of Rat Cortical Astrocytes

Astrocytes were isolated from cerebral cortices on postnatal day 1, 7, and 13 rats as described previously (Noble and Murray, 1984), but with slight modifications. Briefly, cerebral cortices were removed, placed in L15 medium (Gibco), dissected free of meninges, and minced using scalpels. Tissue was then incubated at 37°C for 30 min in a digestion mixture made of 0.35 % collagenase (Sigma) in DMEM. Following addition of SBTI, the tissue was centrifuged at 1000 g for 5min then 3000 g for 1 min, and resuspended in Hank's Balanced Salt Solution (HBSS) containing 0.25 % trypsin and 0.025 % EDTA (Sigma). The tissue was then incubated at 37°C for 15 min after which SBTI (0.52 mg/ml) was added. The solution was centrifuged at 1000 g for 5 min, and 3000 g for 1 min. The cells were resuspended in DMEM-FBS, triturated using a 1-cc syringe with needles of decreasing diameter, plated in tissue-culture flasks coated with laminin (0.25 µg/ml; Biomedical Technologies Inc.), and maintained in culture. When cells became confluent, the flasks were sealed and placed in a 37°C shaker overnight. The flasks were allowed to shake at the maximum speed without causing the media to froth. The supernatant was removed on the next day and replaced with DMEM-FBS and 20 µ*M* of cytosine arabonoside (Ara-C, Sigma) for 2 days, after which the media was replaced with fresh DMEM-FBS. This procedure routinely produces cultures of > 95 % astrocyte purity, which was verified for each substrate by staining for glial fibrillary acidic protein. Following this procedure, astrocytes were never passaged during the aging process but were maintained in culture with DMEM-FBS, which was replenished every 3 days

until harvested at varying time points. At each time point cells were removed from flasks and incubated in HBSS containing 0.025 % EDTA and 0.25% trypsin for 5 min at room temperature. The cells were centrifuged at 1000 g for 5 min, resuspended in DMEM-FBS, and counted on a hemacytometer.

Approximately 50,000 cells from the cell suspension were plated onto the center of coated glass coverslips and incubated at 37°C until they formed a confluent monolayer. At this time granule neurons or hippocampal neurons, freshly prepared as described before, were added to the astrocyte monolayer and co-cultured at 37°C for exactly 24 hrs. The coverslips were then fixated with 4 % paraformaldehyde for 15 min, followed by 0.5 % triton for 5 min. In order to maintain consistency throughout the entire experiment, all media conditions were kept constant by using new, well-buffered medium and fetal calf serum from the same lot.

Preparation of Glial-Restricted Precursor Cells

Glial-restricted precursor (GRP) cells were isolated from the spinal cords of E13.5 Sprague-Dawley rats by using procedures described previously (Rao and Mayer-Pröschel, 1997). A2B5+ immunopurified GRP cells were plated in tissue culture flasks coated with PLL, laminin, and fibronectin, and then grown in chemically defined medium consisting of DMEM-F12 (Gibco) with additives described by Bottenstein and Sato (1979). To sustain proliferation, the prepared media were also supplemented with bFGF (20 ng/ml) prepared as described previously (Stemple and Anderson, 1992). By necessity, GRP cells were passaged as needed to prevent differentiation due to high density conditions, and

placed in fresh media every other day. To generate GRP-derived astrocyte monolayers for neuronal co-culture experiments GRP cells were induced to differentiate by replacing the media with DMEM-FBS. Serum has previously been shown to cause progenitors to differentiate into type-1 astrocytes (Mayer-Pröschel et al., 1997). After 2 days the flasks were sealed and placed in a 37°C shaker in order to remove excess progenitors. The GRP-derived astrocytes were then plated at a density of 50,000 cells per coverslip and the experiments were continued as described for cortical astrocyte preparations. GRP-derived astrocyte experiments were done in duplicate, with two separate cell dissections that were maintained in parallel to one another.

Immunocytochemistry

Standard staining procedures were as described (Mayer et al., 1994), but with slight modifications. Anti-glial fibrillary acidic protein antibodies were used as an astrocyte-specific marker (Dako), anti-beta-III-tubulin antibodies as a neuron specific marker (Sigma), and antibodies for anti-chrondroitin sulfate proteoglycan (Sigma). All secondary antibodies were purchased from Southern Biotechnology Associates and conjugated with Alexa dyes according to a standard protocol (Molecular Probes).

All antibodies were applied for 1 hr at a working dilution of 1:1000, except cell surface antibodies (anti-chondroitin sulfate proteoglycan) which were used at 1:500. Following fixation in 4 % paraformaldehyde for 15 min, cells were further treated in 0.5 % triton for 5 min prior to addition of primary antibodies. Following wash steps secondary antibodies were added at a working dilution of 1:200.

Double and triple labeling experiments were performed by incubating all cells at once with the appropriate combinations of primary antibodies, followed by non-cross-reactive secondary antibodies. The coverslips were then washed in PBS and distilled water and mounted cell-side down using anti-fade to slow fluorescent decay. Slides were viewed with epifluorescent illumination on a Nikon microscope and also examined using an Olympus confocal microscope.

Several coverslips from each type of astrocyte monolayer did not receive neurons and were stained with astrocyte and neuron specific antibodies (GFAP, beta-III-tubulin) as well as the nuclear stain DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; Molecular Probes) at 1:1000 in distilled H_2O for 5 min. The cells were then counted to determine the level of endogenous neuronal contamination. Coverslips of only neurons were taken from each neuronal preparation and stained identical to the astrocyte prep to determine the amount of astrocyte contamination within the granule neuron preparation. Only astrocyte monolayers with a purity of greater than 90 % pure were used in addition to neuronal preparations with of than 10 % cell contamination.

Neuronal Cell Adhesion and Outgrowth Assays

Neuronal cell adhesion was assessed by counting the number of adhering neurons on four entire coverslips for each substrate and time point using epifluorescent illumination. Neurite outgrowth was determined by digitally capturing random fields using a flourescense microscope (Nikon) and a digital camera. The length of the longest neurite from each neuronal cell body was measured using ImagePro Plus digital software (Media Cybernetics). In this way,

200-250 neurites were quantified per substrate and time point. Astrocyte surface area was assessed by randomly capturing fields, and surface area was measured with in each field. For both assays, the means from four samples were pooled for each time point. Experiments involving GRP-derived astrocytes were done in duplicate. All data are reported as the mean (± the standard error of the mean). Statistical differences between cell populations grown on each substrate were analyzed using a two-tailed Student's *t* test assuming unequal variance. Comparisons of separate substrates were performed using an Anova, and differences between time points within a particular substrate were compared using Benferroni post hoc tests. P values of less than 0.05 are reported as significant.

RESULTS

Neurite Outgrowth and Cell Adhesion on Astrocytes

Astrocytes were maintained in culture for up to 7 weeks to determine whether primary embryonic astrocytes decreased their inherent neurite outgrowth promoting properties with time in culture. Each week, portions of each primary astrocyte population were allowed to form a confluent monolayer. The astrocyte monolayers were used as substrates to co-culture cerebellar granule neurons for 24 hrs. We measured the relative changes in neurite outgrowth and neuronal attachment on embryonic astrocytes as well as astrocytes from neonatal animals of increasing age (P1, P7, P13).

By using neonatal astrocytes of different ages, we compared embryonic outgrowth promoting properties with those already established for neonatal astrocytes (Smith et al., 1988; Schreyer and Jones,1987; Fawcett et al, 1989). We found that primary embryonic astrocytes initially supported higher levels of neurite outgrowth and cell adhesion in comparison to neonatal astrocytes. However, decreased outgrowth promoting properties that have been described for neonatal astrocytes were also found after embryonic astrocyte monolayers had been maintained in culture (Figure 2a, 2b).

To quantify the difference in neurite outgrowth on embryonic compared to neonatal astrocytes, the length of the 200 longest neurites was compared on

each substrate at each time point. After 1 week, neurites growing on embryonic astrocytes were approximately 2.0 times longer than those on neonatal astrocytes. However, this difference decreased to approximately 1.3 times after 3 weeks in culture. The same pattern was observed when cell attachment was assessed. There were approximately 91 % more attached granule neurons on primary embryonic astrocyte monolayers compared to neonatal astrocytes, and the difference decreased to approximately 28 % within 3 weeks (Figure 2b, 2c). The results obtained with our experimental design confirmed earlier findings with neonatal astrocytes (Smith et al., 1988; Schreyer and Jones,1987; Fawcett et al., 1989). In addition, we observed that embryonic astrocytes decreased their cell adhesion and outgrowth promoting properties with increased time in culture, similar to neonatal astrocytes.

Neurite Outgrowth and Adhesion on GRP-Derived Astrocytes

To determine whether astrocytes changed their properties after their precursors had proliferated in culture, we induced astrocyte differentiation after GRP cells were expanded in culture for up to 7 weeks.

To also assess the differences between astrocytes derived from growth factor expanded-GRP cells and age-matched astrocytes grown in serum, we collected GRP cells from rat E13.5 spinal cord (from a single dissection) and separated them into two groups. Half of the population was kept proliferating with the addition of bFGF. Each week, a portion of the expanded GRP cells was taken from the same initial founding population, treated with serum that induced differentiation into astrocytes of the $A2B5^-/GFAP^+$ phenotype, and allowed to form

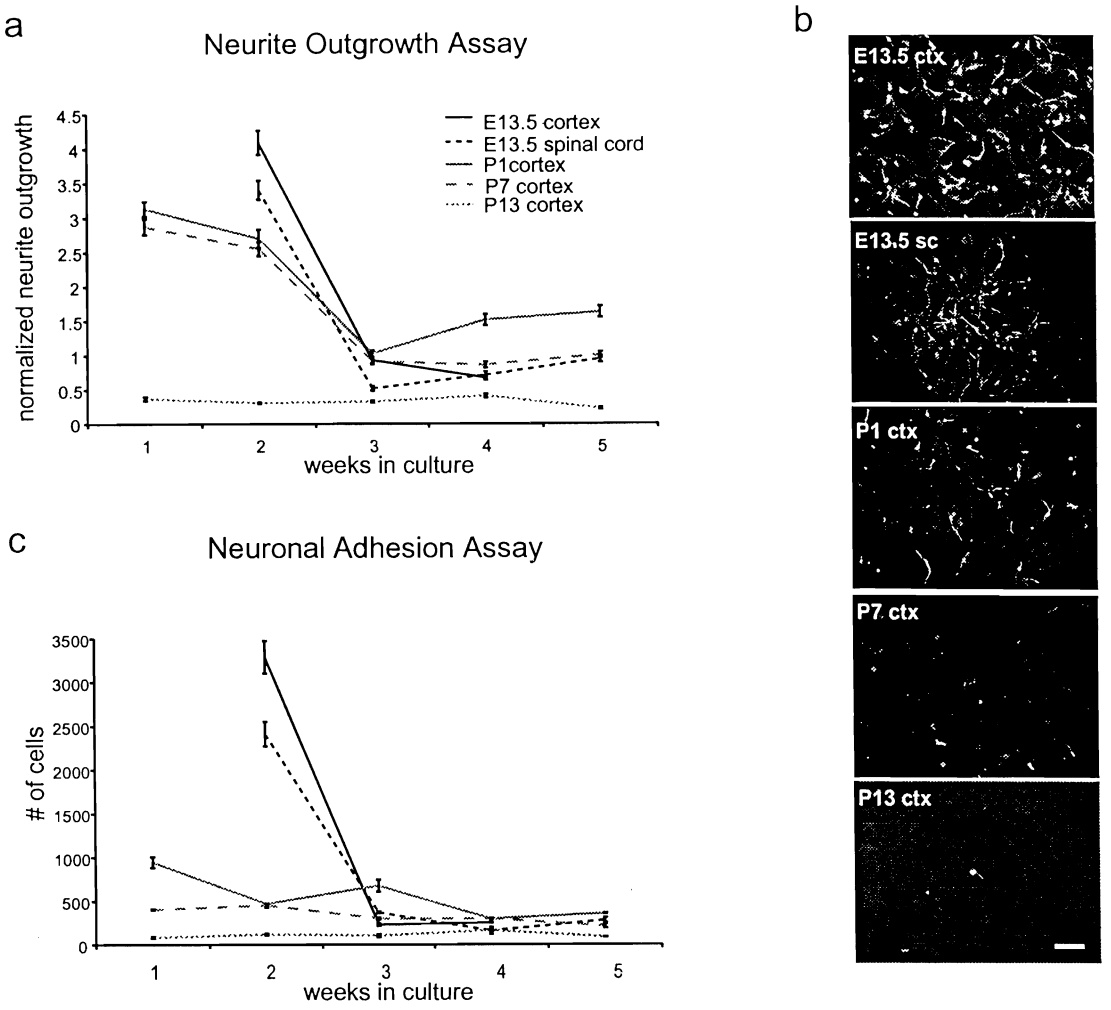

Figure 2. Comparison of Cerebellar Granule Neurite Outgrowth and Adhesion on Embryonic and Neonatal Astrocyte Monolayers

(a) Neurite outgrowth of cerebellar granule neurons on either embryonic or neonatal astrocyte monolayers was compared. β-III-tubulin positive cerebellar granule neurons were grown on each of these layers for 24 hrs. Embryonic astrocytes initially supported higher levels of neurite outgrowth, but their outgrowth promoting properties decreased with time in culture, as previously described for neonatal astrocytes.

(b) Comparison of cell adhesion on embryonic and neonatal astrocytes. Embryonic astrocytes initially greater initial cell adhesion properties than neonatal astrocytes, but this property also decreases with age in culture.

(c) Representative images of primary astrocyte cultures viewed using epifluorescent illumination. Labels indicate the age and region of origin for each astrocyte monolayer [i.e., (E) embryonic, (P) postnatal, (sc) spinal cord, and (ctx) cortical]. Scale bar = 100 μM.

confluent monolayers. The other half was immediately placed in serum conditions and maintained as astrocytes in culture. The outgrowth promoting properties of both substrates were sampled weekly, from 2 to 7 weeks, by adding cerebellar granule neurons to the GRP-derived astrocyte monolayers and co-culturing for 24 hrs. The comparison of these two groups is critical for determining the effects of growth factor expansion on the growth promoting properties of GRP-derived astrocytes, because both groups are composed of astrocytes taken from the same dissection but had aged as astrocytes or as self-renewing precursors. Comparisons of neurite outgrowth between these groups showed statistical differences ($p < 0.001$). However, the Anova did not indicate a significant interaction, meaning that the neurite outgrowth promoting properties for both astrocyte populations showed a similar decline with time (Figure 3a). Astrocytes derived from growth factor expanded GRP cells showed a decrease in their outgrowth promoting properties, indicating that deriving astrocytes from proliferating GRP cells does not fully overcome the problems of decreased neurite outgrowth with age in culture.

In order to verify that this phenomenon is consistent we also used GRP-derived astrocytes from a separate dissection and replicated the experiment. Measurements of neurite outgrowth and cell adhesion were taken from neurons plated on each GRP-derived astrocyte population. The same pattern of outgrowth and adhesion was found for all GRP-derived astrocyte monolayers at each time point irrespective of the GRP population. The more complete series of

measurements was used for further comparisons with astrocytes derived from other sources.

In order to determine if there are regional astrocyte differences that could influence initial neurite outgrowth in our comparisons, we compared astrocytes form two different CNS regions. Outgrowth promoting properties of E13.5 spinal cord astrocytes were compared to E13.5 cortical astrocytes. Cortices were taken from E13.5 rat embryos at the same time as GRP spinal cord cells were isolated. The astrocytes that were purified from cortices were used as an additional substrate in the neurite outgrowth assays. Statistical comparisons between E13.5 spinal cord astrocytes and E 13.5 cortical astrocytes showed no statistical differences in outgrowth promoting properties between the astrocyte monolayers from separate regions. Therefore, regional differences cannot account for possible differences in the pattern of growth promoting properties of astrocytes.

The properties of neuronal cell adhesion and outgrowth are related, and the changes in neuronal adhesion were also analyzed in addition to outgrowth. There is an approximate 93 % decrease of adherent cells on GRP-derived astrocytes between week 2 and the final week 7. This is similar to an approximate 86 % decrease for age-matched astrocytes matured in culture (Figure 3b). These results indicate that neuronal cell adhesion decreases with time in culture for both astrocyte populations. In analogy with neurite outgrowth, there appears to be partial preservation of the cell adhesion to astrocytes derived from growth factor expanded-precursors.

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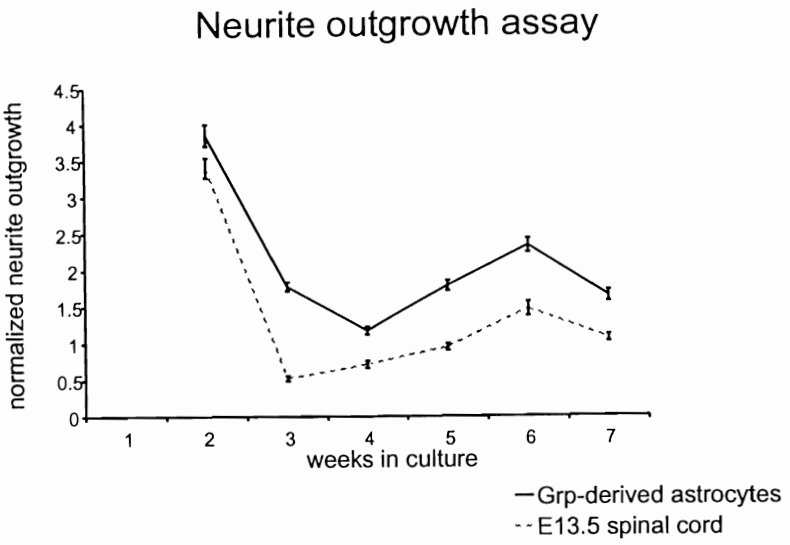

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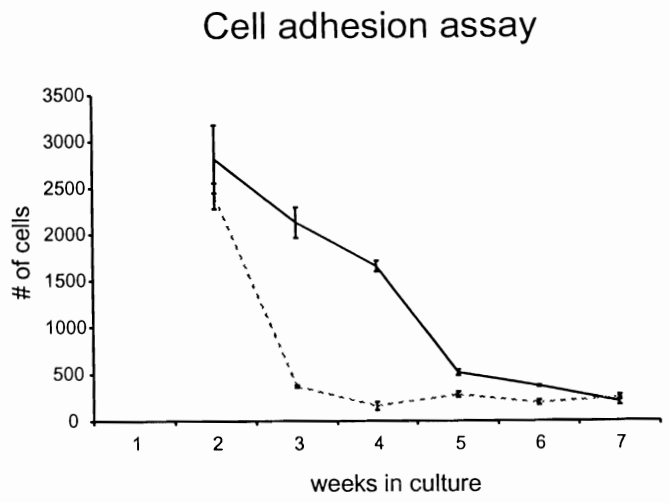

Figure 3. Neurite Outgrowth and Cell Adhesion on Glial Restricted Precursor-Derived Astrocytes

(a) Neurite outgrowth on astrocytes derived from expanded glial restricted precursors (GRP-derived astrocytes) to cells that were taken from the same population and aged in culture as differentiated astrocytes (E13.5 spinal cord). Neurite outgrowth compared between the two astrocyte substrates was statistically different ($P < 0.001$). However an Anova did not show significant interactions indicating that both populations have a similar decline in neurite outgrowth with time. Post hoc tests confirmed that the progressive decrease in neurite outgrowth was statistically significant ($P < 0.001$).

(b) Changes in neuronal cell adhesion for each type of astrocyte monolayer. Neuronal cell adhesion decreased for both astrocyte populations. However, there appeared to be a partial preservation of cell adhesion for the GRP-derived astrocyte monolayers in comparison to age-matched cells grown in serum.

It has also been shown that the neurite outgrowth and adhesion properties of neonatal astrocytes decrease after maturation in culture, and that this change is a direct result of intrinsic cellular changes (Smith et al., 1988; Schreyer and Jones,1987; Fawcett et al, 1989; Smith et al., 1990). Taken together the present results indicated that a similar decrease was also observed for astrocytes derived from expanded precursor cells after the precursor cells had been expanded in culture. The decrease of neurite outgrowth and cell adhesion followed a similar pattern irrespective of the source of the astrocytes, but there was a statistically significant preservation of outgrowth promoting properties for astrocytes derived from expanded precursor cells.

Neurite Outgrowth and Adhesion of Hippocampal Neurons

To assess whether regional differences among neuronal populations interact with influences that GRP-derived astrocytes have on neurite outgrowth, we also measured outgrowth of hippocampal neurons on GRP-derived astrocytes and compared these data to the data from cerebellar granule neuron assays. Comparisons of cerebellar and hippocampal neurite outgrowth on GRP-derived astrocyte monolayers revealed that both neuronal populations showed decreased neurite outgrowth and cell attachment with the increased age of progenitor populations (Figure 4). The analysis reflected that both neuron populations expand their processes according to their expected morphological differences ($p < 0.001$, for each time point), but the age of the astrocyte population influenced neurite growth to a similar extent (no significant interaction).

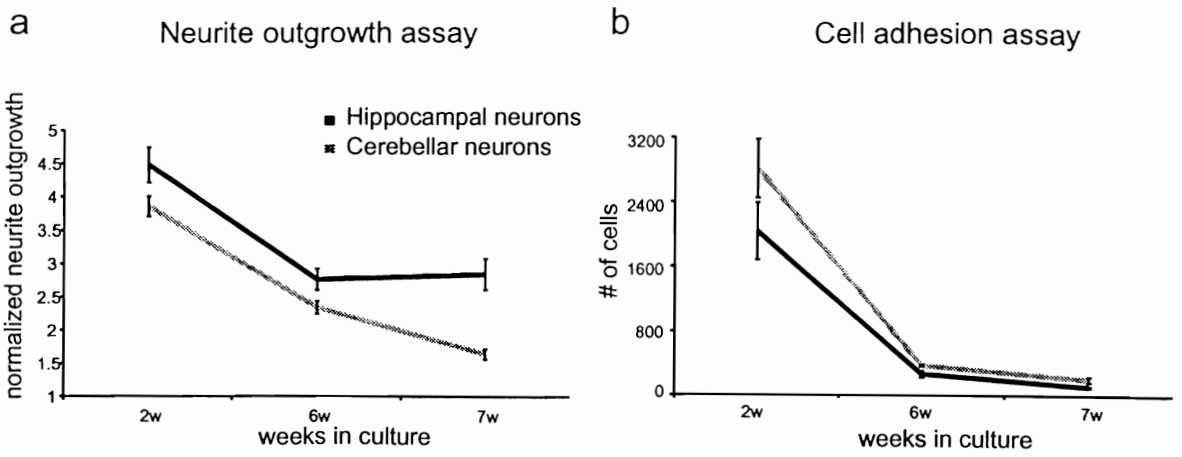

Figure 4. Comparison of Cerebellar and Hippocampal Neurite Outgrowth on GRP-Derived astrocyte Monolayers

(a) Neurite outgrowth was measured for both cerebellar granule neurons and hippocampal neurons. Hippocampal neuron populations include granule neurons, pyramidal neurons, and interneurons. GRP-derived astrocytes have the same effect on both neuronal populations (see text for a detailed description)

(b) The adhesive properties of astrocytes derived from expanded GRP cells decreased when precursors were expanded for longer periods. This effect was independent of regional differences between neuronal populations.

In order to conclusively determine that neurons are directly modified by astrocyte surfaces, we needed to confirm that neurons were growing on top of the astrocyte monolayer. Using confocal microscopy, the physical relationship between neurons and astrocytes was closely examined. At high magnification (60X objective), images were captured through the depth of a field in 0.3 μm increments, and the immunoflouresence of the stained cell types was examined in relation to one another. Confocal microscopy confirmed that neurons were on top of the astrocyte monolayers (Figure 5).

a

b

Figure 5. Structural Relation Between Astrocyte Monolayers and Neurons

(a) Representative confocal image capturing the physical relationship between GFAP positive GRP-derived astrocytes (green) and β-III-tubulin positive cerebellar granule neurons (red). The horizontal line indicates the area of the z axis were the depth of the section was captured in (b). Scale bar = 20 μm.

(b) Z section captured in 0.3 μm increments through the depth of the co-culture. Arrows indicate corresponding cell bodies in (a) and (b). The opposition of the immunoflouresence of both cell-types indicates that the neuron (red) is located directly on top of the astrocyte monolayer (green). Scale bar = 20 μm.

Changes in GRP-Derived Astrocyte Morphology

Among the changes seen in the maturation of neonatal astrocytes is an increase in surface area and a change from a stellate to a more flattened morphology. These changes in astrocyte surface area accompany the maturation of neonatal astrocytes and correlate with a decrease in neurite outgrowth promoting properties (Smith et al., 1986). In order to investigate the changes that go along with the decrease in neurite outgrowth promoting properties by GRP-derived astrocytes, changes in surface area and morphology were examined. The surface area of GRP-derived astrocytes was measured for each time point. The average surface area increased in parallel with the time precursors were expanded in culture (Figure 6a). The increase in surface area was observed even though the precursors had been cultured as astrocytes for equal time periods ($p < 0.001$). Post hoc tests confirmed that surface area after 5 weeks to 7 weeks of expansion was significantly greater then the initial area (all p values < 0.001).

In order to determine whether changes in astrocyte morphology are associated with the increase in surface area, digital images were captured for each astrocyte monolayer derived from increasing progenitor age and the relative morphologies were compared between time points. These images clearly demonstrate that there are differences in astrocyte morphology that are associated with the expansion of progenitors in culture for prolonged periods. Astrocytes derived from GRP cells that had been expanded for 2 weeks appeared stellate with long cytoplasmic extensions, whereas astrocytes derived from GRP cells that had proliferated in culture for longer periods had an

increasingly flatter morphology (Figure 6b). These findings indicate that GRP cells from the same population differentiate into astrocytes with different morphologies after continued expansion in culture. The change in morphology appears to progress from a stellate to flattened astrocyte. The flat morphology of GRP-derived astrocytes appeared to correlate with a decreased support of neuronal outgrowth and adhesion.

The above changes in GRP-derived astrocyte morphology could correspond to the generation of different astrocyte cell types from GRP cells at different time points or to the differentiation into the same cell type with different morphologies. To examine whether GRP cells that have proliferated for extended periods in culture differentiate into different astrocyte cell types, we used antigenic immunoreactive markers that are specific for certain astrocyte phenotypes. For example, type-1 astrocytes are typically generated from GRP cells after the addition of serum, and are characterized by $A2B5^-/GFAP^+/FGFR3^+$ immunoreactivity. Conversely, type-2 astrocytes are characterized by $A2B5^+/GFAP^+/FGFR3^-$ immunoreactivity. Differentiation of GRP cells into either type-1 or type-2 astrocytes depends on the culture conditions (Rao et al., 1997). In the current study, all astrocyte monolayers demonstrated immunoreactivity of the $A2B5^-/GFAP^+/FGFR3^+$ type independent of the self renewal time period of their precursor cells (Figure 7). According to our ability to distinguish astrocyte types antigenically, the change in morphology is not due to the presence of different astrocyte cell types, but rather a consequence of a progressive change of one antigenically-distinct astrocyte cell type.

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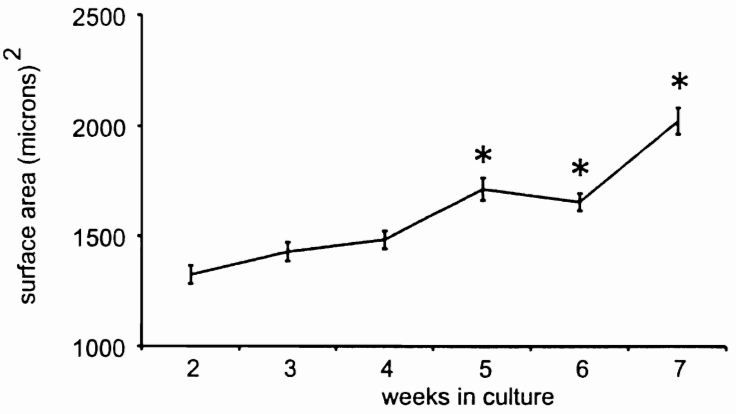

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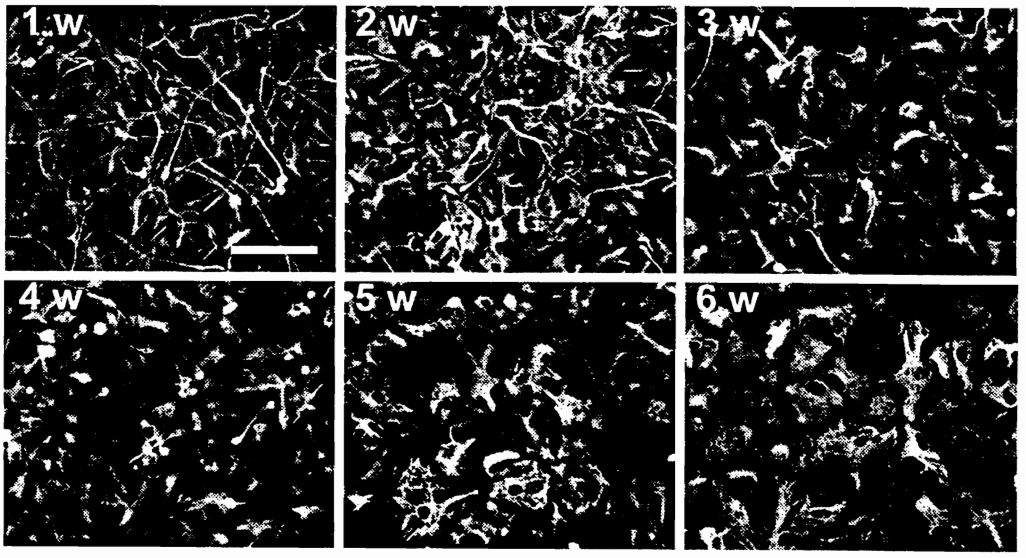

Figure 6. Changes in Surface Area and Morphology of Astrocytes Derived from Expanded GRP Cells.

(a) GRP cells were proliferated in culture and, each week, a portion of the GRP population was differentiated into astrocytes to form confluent monolayers. The surface area of GFAP positive astrocytes was measured for each time point. Astrocyte surface area increased in parallel with the time precursors were expanded. Post hoc tests confirmed that the surface area in weeks 5,6, and 7 was significantly larger than in week 2 (all p values < 0.001).

(b) Images of GFAP positive astrocyte monolayers. The length of time progenitors were expanded in culture before differentiating into astrocytes is indicated in the upper left corner (w = weeks). Astrocytes derived from progenitors expanded in culture for short periods are stellate with long cytoplasmic extensions, whereas those from later time points have an increasingly flatter morphology. Scale bar = 100 μm.

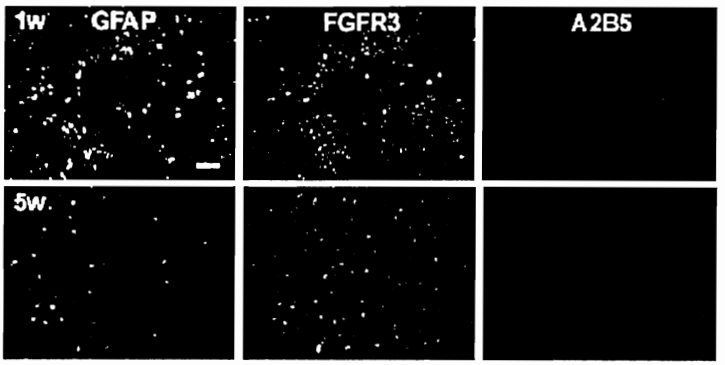

Figure 7. Phenotypic Characterization of Astrocytes Derived from Expanded GRP Cells

Astrocyte cell types derived from expanded GRP cells were determined antigenically. Astrocytes from each time point showed $A2B5^-/GFAP^+/FGFR3^+$ immunoreactivity, which indicates a type-1 astrocyte phenotype. Representative time points show astrocytes derived from precursors expanded for 1 week and 5 weeks. Scale bar = 100 μm.

Changes in the Expression of Extracellular Matrix Molecules

The decrease in the ability of mature astrocytes to promote neurite outgrowth in vitro has been shown to be due, at least in part, to the increased expression of extracellular CSPGs, which are inhibitory to neurite outgrowth (Snow et al., 1990; Lochter et al., 1993). In order to determine whether the decrease in neurite outgrowth and attachment to GRP-derived astrocytes is also due to an increase in CSPG expression, immunocytochemistry was performed on the various aged GRP-derived astrocyte substrates using an anti-chondroitin sulfate proteoglycan antibody. Surprisingly, there was a decrease in the relative levels of CSPG expression with the increased time of GRP cell expansion in culture (Figure 8a). CSPG expression is down-regulated in astrocytes derived from aged GRP cells and changes in CSPG expression did not seem to be linked to the growth inhibiting properties of astrocytes derived from expanded GRPs.

This result is in stark contrast to the maturation of neonatal astrocytes and the discrepancy was further investigated by comparing GRP-derived astrocytes with primary astrocytes.

CSPG levels are known to be high in the adult CNS due to the increased CSGP expression by mature astrocytes (reviewed in Aubert et al., 1995). The fact that the adult CNS lacks the capability to promote neurite outgrowth prompted us to measure changes in astrocyte CSPG expression with continued maturation in culture. Astrocytes from animals of increasing age (E13.5, P1, P7, P13) were isolated and the pattern of CSPG expression was compared. CSPG expression in primary astrocyte monolayers cultured from animals of increasing age demonstrated that CSPG levels are high in embryonic astrocytes, decreased below the detection level on post natal day 1, and then increased again in P13 astrocytes (Figure 8b). These primary astrocytes were maintained in culture and changes in CSPG expression were assessed each week similarly to what was performed for GRP-derived astrocytes. Embryonic astrocytes decreased their CSPG expression with age in culture. In contrast, postnatal astrocytes increased CSPG expression with age in culture (Figure 8b). These data suggest that astrocytes derived from different ages of expanded GRP cells decrease CSPG expression when they are maintained in culture, and the occurrence of the same behavior was also verified for embryonic astrocytes in culture. In conclusion, there appears to be no correlation between increased levels of CSPG expression and the decreased neurite outgrowth promoting properties of GRP-derived and embryonic astrocytes that are maintained in culture.

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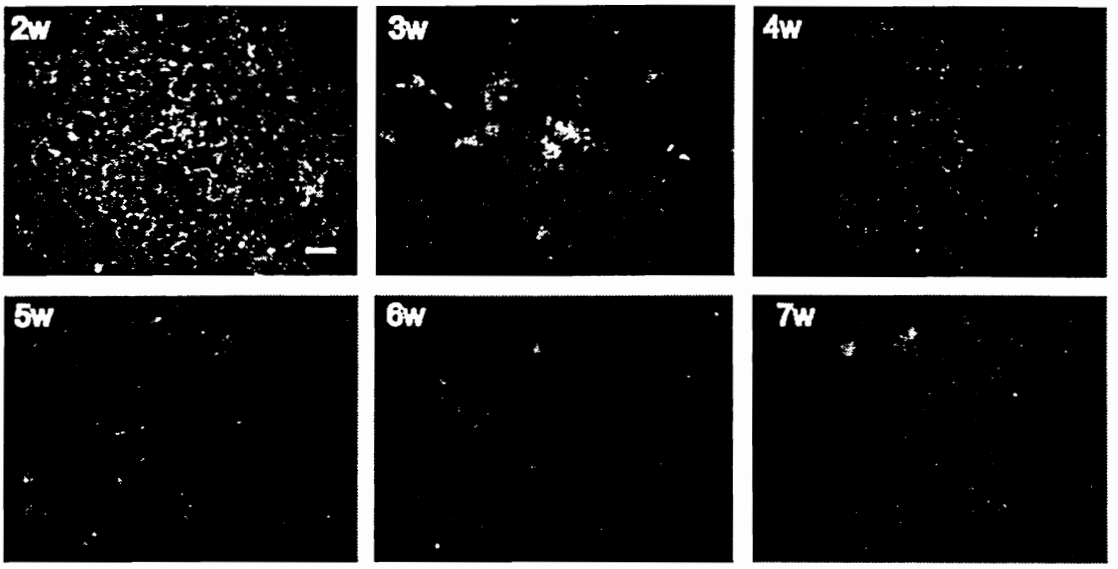

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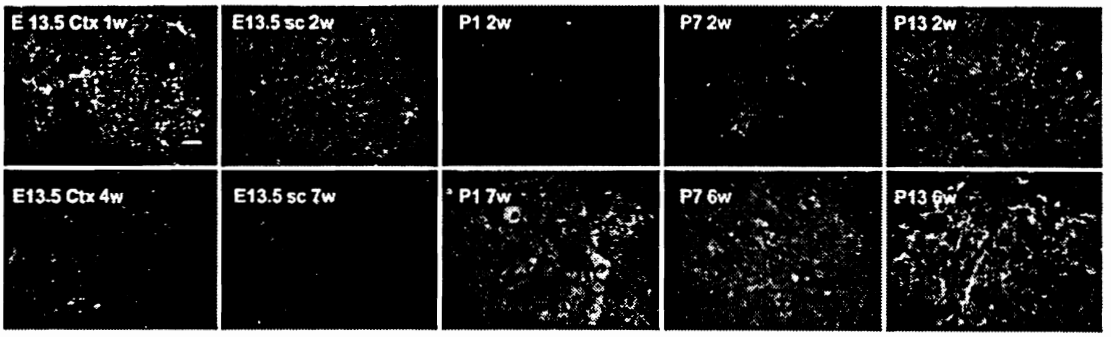

Figure 8. Decreased CSPG Expression by Astrocytes Derived from Expanded GRP Cells.

(a) Monolayers of astrocytes derived from GRP cells expanded in culture. Left corner indicates weeks precursors were expanded in culture before differentiation. Substrates were stained for chondroitin sulfate proteoglycans (CSPG), which appear to be down-regulated by astrocytes derived from older progenitors. Scale bar = 100 μm.

(b) CSPG expression by embryonic and neonatal astrocytes. The top row is composed of primary astrocyte monolayers, and the bottom row displays astrocyte monolayers from the same primary preparation, aged in culture. Anti-CSPG antibodies reveal the trend in CSPG expression during development; an initial embryonic decrease in CSPG expression is seen, which increases again in neonatal animals. Neonatal astrocytes maintained in culture increase CSPG expression, whereas embryonic astrocytes decrease CSPG expression. Scale bar = 100 μm.

DISCUSSION

Due to the unique relation between astrocytes and neurons, many new strategies for CNS repair and regeneration have incorporated astrocytes into their design. The neurite outgrowth supporting properties of astrocytes make them an attractive cell type for use on devices designed for nerve guidance and regeneration. However, the therapeutic use of astrocytes on such devices has been limited by the insight that astrocyte maturation in culture is accompanied by a decrease in their inherent capacity to support neurite outgrowth (Smith et al., 1988; Schreyer and Jones,1987; Fawcett et al., 1989). Therefore, the use of astrocytes from earlier stages of development, or astrocytes derived from expanded precursor pools, could be an improvement and could help researchers avoid unwanted properties that change by the maintenance of astrocytes in culture.

The experiments in this report investigated the changes in neurite outgrowth and cell adhesion in association with astrocytes derived from growth-factor expanded glial restricted precursor cells after these precursors had been expanded in culture for prolonged time periods. Results can determine whether the use of growth factor expanded precursors cells is practical for strategies involving CNS tissue repair and regeneration and can also answer fundamental questions about developmental biology. For comparisons with the findings for

expanded GRP-derived astrocytes, the aging of embryonic astrocytes in culture was also assessed. The trends for the maturation of GRP-derived and embryonic astrocytes were further compared with the established results for neonatal astrocyte maturation in culture (Smith et al., 1988).

We found that primary embryonic astrocytes provide a better substrate for cell adhesion and neurite outgrowth in comparison with primary neonatal astrocytes, but also showed that these properties decreased with the maturation of embryonic astrocytes in culture. Our findings indicate that embryonic astrocyte maturation in culture results in a significant, but not complete, reduction in their ability to promote neurite outgrowth. This is consistent with the maturation of neonatal astrocytes in culture (see Figure 2).

Monolayers of both embryonic and neonatal astrocytes lose their outgrowth promoting properties after 3 weeks in culture. These observations suggest that there appears to be an intrinsic timing mechanism that changes the functional capacity of astrocytes of all ages to support neurite outgrowth and adhesion in culture. This is similar to the phenomenon that others have described for neonatal astrocyte maturation in vitro (Smith et al., 1988; Schreyer and Jones, 1987; Fawcett, 1989). The timing for the in vitro decline of neonatal astrocyte growth promoting properties has been found to coincide with in vivo changes (Smith et al., 1986, Smith and Miller, 1991). These results suggest that intrinsically regulated functional changes take place during astrocyte maturation, which cause growth promoting properties to decrease with time.

We also investigated the cell adhesion and neurite outgrowth promoting properties of astrocytes derived from GRP cells that were expanded in culture. The fact that the onset of differentiation from a GRP cell into an astrocyte can be suspended by the addition of a particular astrocyte mitogen, PDGF, and the extracellular signaling molecule, bFGF (Rao et al., 1998), has led to the hypothesis that astrocytes derived from GRP cells could overcome the problems that are associated with astrocyte maturation in culture. Expansion of GRP cells could provide researchers with large numbers of cells that are capable of differentiating into astrocytes with substantial neurite outgrowth promoting properties. We have provided evidence that astrocytes derived from glial restricted precursors show a decline in neurite outgrowth promoting and cell adhesion properties in relation to the length of time their precursors expanded in culture. We also showed that GRP cells taken from the same primary population but aged as astrocytes in culture show a similar decrease in outgrowth promoting properties (Figure 3). Despite their continued proliferation as GRP cells, astrocytes derived from GRP cells at increasing time points have functional properties similar to age-matched astrocytes that were grown in serum as differentiated cells. Astrocytes derived from GRP cells are therefore not free from the effects of increased time in culture. This result is critical for the therapeutic use of growth factor expanded GRP cells and for the potential use of other progenitors. Prolonged expansion of precursor cells may in general result in the differentiation of cells with decreased functional properties, as observed here for the maturation of astrocytes in culture.

The decrease in cell adhesion and neurite outgrowth promoting properties of GRP-derived astrocytes was verified by using a second GRP population. This replication confirms that this functional loss is a robust phenomenon when astrocytes are derived from GRPs that were expanded in culture. We also verified that the loss in outgrowth promoting properties is independent from regional differences among astrocyte populations and independent of regional differences between neuronal populations (Figure 4). These data are consistent with previous findings that have demonstrated that the initiation of neurite outgrowth is independent of regional astrocyte and neuron differences within the first 24 hrs in culture (Le Roux and Reh, 1995; Dijkstra et al., 1999). The present result can therefore be attributed to the maturation of GRP cells in culture. Extended self-renewal of the GRP cell through the addition of an extracellular signaling molecule in culture did not halt the functional aging. Astrocytes derived from aged GRP cells in culture showed a similar decrease in inherent functional properties as astrocytes that aged as differentiated cells.

The finding that cell adhesion and outgrowth promoting properties decrease regardless of whether astrocytes are derived from aged progenitors or aged as differentiated cells raises the possibility that intrinsically regulated timing mechanisms exist for growth factor expanded GRP cells. This timing mechanism could result in functional changes in accordance with the length of proliferation in culture. Previous studies have shown that another glial progenitor cell, the O-2A precursor cell, continues to measure elapsed time despite the addition of particular mitogens that hold a precursor cell in a state of self-renewal (Bögler

and Noble, 1991). These findings could also apply to GRP cells, which may time the progress of development, and result in maturational changes inherent to derived astrocytes despite their expansion as GRP cells. Our results are consistent with this mechanism and suggest that the GRP cell is capable of measuring elapsed time during self-renewal as indicated by the differentiation of astrocytes with different functional properties.

In order to understand the interaction between functional changes and intrinsic timing mechanisms, we looked at markers that had been observed to change during neonatal astrocyte maturation such as a change in the shape and appearance of mature astrocytes (Smith et al., 1986), alterations in the cell surface molecules (Smith et al, 1990), as well as an increased expression of inhibitory ECM molecules (Snow et al., 1990; Lochter et al., 1993). We investigated whether and to what extent these changes occur in astrocytes derived from expanded precursor cells.

Previous studies have shown that a change in astrocyte morphology from a stellate shape to a more flattened morphology is associated with decreased efficiency in promoting neurite elongation (Smith et al., 1986). Such a change in astrocyte morphology has been observed during the maturation of neonatal astrocytes, young astrocytes are stellate and become flattened with age (Smith et al., 1986). We show that astrocytes derived from progenitors expanded in culture, increase in surface area in correlation with the length of the time period during which a progenitor had been expanded in culture (Figure 6a). Using fluorescence microscopy, we also showed that GRP-derived astrocyte monolayers

demonstrated a distinct difference in morphology during earlier compared to later time points. Astrocytes derived from GRP cells that had been expanded for 1 week in culture have a stellate shape with longer cytoplasmic extensions, astrocytes derived from older progenitors have a flat appearance with a reduction of cytoplasmic processes (Figure 6b). These morphological differences may be involved in the decreased ability of the GRP-derived astrocytes to promote neurite outgrowth. Flattened cells may not provide the proper scaffolding due to their reduced volume of cytoplasmic processes, which provide a geometrically less promoting cell substrate (Freed et al., 1985). In vitro studies have shown that a flattened morphology decreases the adhesive properties of fibroblasts and epithelial cells (Grinnel, 1976), and in vivo studies have verified these results for astrocytes (Smith et al., 1986). Therefore it is conceivable that a decrease in cell adhesion and neurite outgrowth on GRP-derived astrocytes is at least partially due to the differentiation of GRP cells into astrocytes with different morphologies.

These morphological differences may be explained by either the differentiation of GRP cells into different types of astrocytes after a longer time in culture, or the differentiation of GRP cells into a single astrocyte cell type with an age dependent morphology. We attempted to distinguish between these possibilities by using antigenic immunoreactive markers that are specific for certain astrocyte phenotypes. All astrocyte monolayers demonstrated immunoreactivity of the $A2B5^{-}/GFAP^{+}/FGFR3^{+}$ type, which is the expected astrocyte phenotype after serum induction, independent of the length of time their precursor cells were held in a state of self renewal. These results indicate that all

GRP-derived astrocytes were type 1 astrocytes according to antigenic markers (Figure 7). It therefore suggests that astrocytes of the same phenotype with different morphologies emerge from expanded GRP populations after they have been expanded for different time periods in culture. These changes are related to internal changes that take place in expanded GRP cells with time in culture.

Another change that is associated with the maturation of neonatal astrocytes and that reduces their ability to support neurite outgrowth and adhesion is the increase in the production of inhibitory ECM molecules, in particular inhibitory CSPGs (Snow et al., 1990; Lochter et al., 1993). In order to assess whether increased expression of inhibitory CSPGs is involved in the decreased neurite outgrowth promoting properties of GRP-derived astrocytes, immunocytochemistry was performed on the various aged GRP-derived astrocyte substrates using an anti-chondroitin sulfate proteoglycan antibody. We have shown that the relative levels of CSPG expression decrease with the increased time GRP cells were expanded in culture (Figure 8a), and we also showed that this same decrease in CSPG expression is seen in E13.5 cortical and spinal cord embryonic astrocytes that were aged in serum (Figure 8b). These data indicate that CSPG expression is down-regulated in astrocytes derived from aged GRP cells, as well as age-matched astrocytes. This is in sharp contrast to the response of neonatal astrocytes in culture, which show up-regulated CSPGs with increased time in culture (Figure 8b).

It seems apparent that an increase in inhibitory CSPG expression is not responsible for the decrease in neurite outgrowth on embryonic astrocytes as has

been shown for neonatal astrocytes. However, the decrease in neurite outgrowth of GRP-derived astrocytes and embryonic astrocytes would be consistent with the interpretation that neurite promoting CSPGs are lost as observed in other astrocyte populations. For example, promoting CSPG mediated interactions has been described in the embryonic neocortex (Emerling and Lauder, 1996). In these studies, CSPGs were shown to be responsible for cell attachment and neurite outgrowth within the neocortex; these interactions were abolished by the enzymatic removal of chondroitin sulfate. Embryonic neocortex expresses either stimulatory or inhibitory CSPGs that are localized in different regions. An example of a growth promoting CSPG that is found on the surface of embryonic astrocytes is the chondroitin sulfate proteoglycan dermatan sulfate dependent-1 proteoglycan (DSD-1-PG; Faissner et al., 1994). Perhaps the decline in neurite outgrowth promoting properties of astrocytes derived from aged progenitors, as well as aged embryonic astrocytes, is due to the down-regulation of growth promoting CSPGs, which have been shown to be expressed by embryonic astrocytes. This theory would fit well with the general pattern of CSPG expression seen in primary astrocyte monolayers taken from animals of increasing age (i.e., E13.5, P1, P7, P13).

Changes in CSPG expression from primary astrocyte monolayers of animals of various ages are seen. High CSPG levels are observed in embryonic astrocytes. They decreased below the detection level on postnatal day 1 and then increased again in P13 astrocytes (Figure 8b). These changes seem correlated with the functional roles of CSPGs in the developing CNS. CSPGs expressed by

embryonic astrocytes have been described to be intimately involved in the directing and guiding of neurons during CNS development by providing the directional cues that either repel or attract growing axons (reviewed in Fitch and Silver, 1997). Later in development, the inability of the CNS to regenerate following injury has been attributed to the up-regulation of astrocyte CSPGs in the adult CNS, which inhibits neurite outgrowth (reviewed in Powell et al., 1997). Therefore, the role of the different subtypes of CSPGs in development coincides with the levels of CSPGs detected by immunocytochemistry in our experiments. By considering the overall developmental pattern of CSPG expression, decreased expression in embryonic and GRP-derived astrocytes in culture is not surprising. CSPGs have been found to have various functions depending on location and time of expression. Future experiments are needed to verify whether the observed CSPG trend is consistent with the presence of particular types of CSPG molecules, which are involved in each stage of astrocyte development.

Taken together, our results suggest that similar changes are observed during the maturation of neonatal astrocytes and the maturation of embryonic and GRP-derived astrocytes aged in culture, and that these changes go along with their decreased capability of supporting cell adhesion and neurite outgrowth. One intriguing finding of our study is that the prolonged expansion of precursor cells results in the generation of astrocytes with decreased neurite supporting properties. A possible reason for these changes is the capability of GRP cells to intrinsically time the progress of development despite their induced state of self-renewal.

Possible mechanisms that could be involved in timing the progress of development in the presence of extrinsic molecules that block differentiation could include the following. (1) An intracellular timing mechanism measures elapsed time by sequentially activating a series of genes encoding regulatory proteins, with each in turn activate the next gene in sequence, until a final gene product triggers a development event. The final event might be blocked by external signals, but the series of gene activation still continues. (2) Another explanation could be an accumulation of a particular factor within precursor cells as they proliferate. The relative levels of this factor during differentiation may influence the resultant properties of the derived cell. Such a mechanism has been described for proliferating O-2A progenitor cells.

The cdk cell-cycle inhibitor p27/kip1 accumulates in the O-2A progenitor cell during proliferation. Cdk proteins regulate phases of the cell cycle at the right time and place. Cdk inhibitor p27/kip1 arrests the cell cycle in the G_1 phase and accumulates with the continued proliferation of the O-2A precursor cell in culture. Increased levels of p27 have also been observed in differentiated oligodendrocytes derived from O-2As (reviewed in Durand and Raff, 2000). A similar mechanism could operate during the continued expansion of GRP cells in culture. An increased accumulation of a particular factor would result in the differentiation of astrocytes with various inherent levels of this factor. The emerging astrocytes would then show that behaviors resemble those at a particular developmental stage. This could explain why astrocytes derived from expanded precursor cells exhibit the same neurite supporting behaviors as age-

matched astrocytes allowed to develop in culture. The same rational would also apply if a different mechanism for measuring time is functional.

In conclusion, we have shown that GRP cells expanded in culture produce astrocytes with decreased cell adhesion and neurite outgrowth promoting properties in accordance to the age of their progenitors. These findings have wide implications for the use of progenitor cells for possible therapeutic applications. One must realize that the prolonged expansion could result in the differentiation of cell types with functional properties that correspond to the progenitor cell's ability to measure elapsed time in culture. We have made these initial observations, but much work is needed to further determine the mechanisms involved. The interplay between intracellular timers and extracellular signals is very complex and current standard culture conditions may lack the appropriate mitogen that is able to completely suspend the internal timing mechanism. However, at the present time growth factor expansion of precursor cells in culture results in altered derived cell types, with different functional capabilities.

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