

Growth factors, glia and gliomas

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Key words: O-2A progenitor cell, glia, glioma, glioblastoma, oligodendrocyte

Summary

The abilities of growth factors to cause normal cells to express the properties associated with transformed cells is discussed in specific reference to the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell. In the O-2A lineage, it has been possible to use growth factors and other defined molecules to induce or promote in normal cells all of the main properties of tumor cells, these being continued cell division in the absence of differentiation, more subtle modulations of self-renewal probabilities, promotion of cell migration and inhibition of programmed cell death.

In addition to our studies on primary cells, our application to the growth of human tumor specimens of techniques utilized to study primary glial progenitor cells has allowed us to isolate a human glioblastoma multiforme (GBM)-derived population that expresses many properties otherwise uniquely expressed by oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Hu-O-2A/Gb1 (for *Human O-2A lineage Glioblastoma number 1*) cells responded to similar mitogens and differentiation modulators as rodent O-2A progenitors, and generated cells with features of precursor cells, oligodendrocytes and astrocytes. Moreover, ¹H-NMR analysis of amino acid composition demonstrated a striking conservation of types and quantities of free amino acids between the human tumour cells and the rodent primary cells. Hu-O-2A/Gb1 cells represent the first human glioma-derived population for which unambiguous lineage assignment has been possible. Our results thus demonstrate that the human O-2A lineage can contribute to one of the most malignant of glial tumours. Our analyses further indicate that at least two distinct glial lineages can generate glioblastomas. In addition, the highly diagnostic ¹H-NMR spectrum expressed by Hu-O-2A/Gb1 cells raises the possibility of eventual non-invasive identification of tumors of this lineage.

Cancer cells have a variety of properties that make them inappropriate inhabitants of one's body. Of these, the most important in allowing cancer cells to undergo uncontrolled growth are their ability to undergo extended self-renewal, their failure to develop into terminally-differentiated non-dividing cells and their ability to escape programmed cell death. In the oncological sciences, these traits generally are analyzed in terms of the genetic alterations that may cause them. Yet, all of these traits can also be expressed by normal cells of the body if the normal cells are exposed to appropriate growth factors or other defined molecules.

The intent of this review is twofold. First, information on normal glial cells, and their precursors, will be presented to illustrate how growth factors to which cells are likely to be exposed can influence the properties of differentiation, self-renewal and cell survival. Secondly, studies will be discussed that demonstrate the striking similarities between the best defined of glial precursor cells [the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell of the rat optic nerve] and a glioma derived from the equivalent human lineage.

The origins of the glial cells of the central nervous system (CNS)

Several different lineages are able to give rise to the glial cells of the rat CNS, the species in which CNS glial development has been most extensively characterized. On one extreme, it appears that the cells of the rat optic stalk – the embryonic anlage of the optic nerve – give rise only to a single glial population (Small et al., 1987), a glial cell-type that has been given the name of the type-1 astrocyte (Raff et al., 1983a). As this name implies, it is thought that the CNS also contains other types of astrocytes, but the true complexity of this family of cells is far from established. Some experiments conducted on spinal cord cultures, for example, have suggested the existence of as many as five distinct classes of astrocytes just in this single tissue (Miller and Szigeti, 1991). Certainly, it is clear that although the type-1 astrocytes of the optic nerve appear to be similar to astrocytes present in cultures generated from many other regions of the CNS (e.g., Raff et al., 1983a, 1988; Noble and Murray, 1984), they are not the only source of this category of glia. For example, far on the other extreme from the putatively unipotent cells of the optic nerve are totipotent cells able to give rise to both neuronal and glial lineages, producing a variety of neurons as well as oligodendrocytes and astrocytes (Williams et al., 1991; Reynolds and Weiss, 1992; Vescovi et al., 1993; Davis and Temple, 1994). Several such precursor populations have been described in the CNS of embryonic, perinatal and adult rats, and the relationship between the cells studied in different laboratories is not yet clear.

Still other lineages exist in an intermediate territory, giving rise to a restricted number of different cell types. Of the lineages with restricted developmental potential, the most extensively characterized is called the oligodendrocyte-type-2 astrocyte (O-2A) lineage. The precursor cells of this lineage were first identified in cultures derived from perinatal rat optic nerves (Raff et al., 1983b), where they were found to give rise to two mature glial cell populations: oligodendrocytes and type-2 astrocytes. Oligodendrocytic differentiation of these O-2A progenitor cells occurred when progenitors

were grown in chemically-defined medium and progress along this differentiation pathway did not require the presence of inducing factors. In contrast, astrocytic differentiation required the presence of appropriate inducing factors, such as the still unidentified factor(s) present in fetal sera of a number of different species (Raff et al., 1983 a,b,c). Fortunately for the early development of research on these cells, it is possible to obtain useful numbers of O-2A progenitors simply by dissociating the optic nerves of the 7-day old rats (Raff et al., 1983b, Noble and Murray, 1984). In such cultures, grown in chemically-defined medium, the percentage of O-2A lineage cells in the starting culture can be as high as 50%, and only a minority of these cells will have differentiated into oligodendrocytes *in vivo* at the time of cell isolation.

Oligodendrocytes, the cells that produce myelin in the central nervous system, are a well-characterized cell-type *in vivo*. Type-2 astrocytes, in contrast, remain a problem for the developmental neurobiologist, for it is not yet clear when and where – and some would say even whether – such cells might occur *in vivo* (Fulton et al., 1991). Some believe that the O-2A progenitor cell should simply be called an oligodendrocyte precursor cell (e.g., Skoff and Knapp, 1991) while others utilize the O-2A lineage terminology. Being believers that differentiation pathways that occur *in vitro* also are utilized *in vivo*, we utilize the latter terminology. That this decision may be warranted is supported by the results of a variety of experiments in which O-2A progenitor cell lines have been implanted in demyelinating lesions of the rat spinal cord, and have been found to produce both oligodendrocytes and astrocytes *in vivo* (reviewed in Franklin et al., 1995). Regardless of the nomenclature preferred by a particular reader or author, it is important to recognize that the rules of Shakespeare often also apply to lineage nomenclature ('What's in a name? That which we call a rose/By any other name would smell as sweet') and that thus far it appears that laboratories utilizing these different nomenclatures are studying one and the same precursor cell population.

Biological studies on the O-2A progenitor cell

Having identified a precursor cell for oligodendrocytes, it was next necessary to identify the cellular and molecular signals that controlled division of such cells. Initial studies on generation of oligodendrocytes from O-2A progenitor cells isolated from optic nerves of perinatal rats presented the paradox that the cells we were studying were isolated at a time of maximal division of this lineage *in vivo* (Skoff et al., 1976a,b), yet cells did not divide in tissue culture. Resolution of this paradox began with the discovery that cortical astrocytes promoted O-2A progenitor division *in vitro* (Noble and Murray, 1984). The astrocytes used in these studies expressed a phenotype like that of type-1 astrocytes of the optic nerve, the first identifiable glial cells to appear in the nerve (Miller et al., 1985). The similarity of these two populations led us to suggest that the type-1 astrocytes of the optic nerve were responsible for supplying the mitogen(s) required to keep O-2A progenitors in division. Moreover, populations of O-2A progenitors grown in the presence of purified cortical astrocytes were capable of undergoing extended division while also continuing to generate more oligodendrocytes (Noble and Murray, 1984), a pattern of behavior like that occurring *in vivo*. Thus, the failure of O-2A progenitors to divide in our initial *in vitro* studies was due to the lack of necessary mitogens, which appeared to be supplied by another glial cell type.

O-2A lineage mitogens

The effects of purified cortical astrocytes, and of type-1 astrocytes from the optic nerve, on O-2A progenitor division *in vitro* appear to be mediated by platelet-derived growth factor (PDGF; Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988). O-2A progenitors exposed to either PDGF or astrocyte-conditioned medium exhibited a bipolar morphology, migrated extensively (with an average migration rate of $21.4 \pm 1.6 \mu\text{m/hr}$) and divided with an average cell cycle length of 18 h (Noble et al., 1988). Moreover, antibodies to PDGF blocked the mitogenic effect of type-1 astrocytes on embryonic

O-2A progenitor cells, causing these cells to cease division and to differentiate prematurely even when growing in medium conditioned by type-1 astrocytes (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988). Thus, this single mitogen was able to elicit a complex behavioral phenotype from O-2A progenitors which included normal functioning of the cellular mechanisms involved in the measurement of elapsed time. Further studies have indicated that neurons, which also promote division of O-2A progenitors *in vitro* (Gard and Pfeiffer, 1990; Hunter and Bottenstein, 1991), may also be a source of PDGF. Indeed, the results of *in situ* labeling of sections of normal adult CNS with PDGF cDNA probes suggests that neurons may be the major producers of this mitogen in adult tissue (Sasahara et al., 1991; Yeh et al., 1991). However, the specific contributions of either neuronal or astrocytic production of PDGF to the development of the O-2A lineage *in vivo* is not yet known.

O-2A progenitors can also express a variety of developmental programs other than the one elicited by exposure to PDGF (e.g., Bögler et al., 1990; McKinnon et al., 1990; Mayer et al., 1992), and it is these other programs that are germane to understanding the control of precursor cell self-renewal. For example, O-2A progenitors induced to divide by basic fibroblast growth factor (bFGF) were multipolar and showed little migratory behaviour (Bögler et al., 1990). In addition, cells induced to divide by bFGF had a cell-cycle length of 45 ± 12 hr, in contrast with the 18 ± 4 hr cell cycle length elicited by exposure to PDGF. These results indicate that PDGF and bFGF function in the O-2A lineage as modulators of differentiation as well as functioning as promoters of cell division. PDGF and bFGF also differ in their effects on oligodendrocytes themselves, in that only bFGF is able to promote division of these cells (Eccleston and Silberberg, 1985; Sane-to and deVellis, 1985; Bögler et al., 1990). Most importantly, for the concerns of this review, bFGF can inhibit the differentiation of purified O-2A progenitors (McKinnon et al., 1990; Mayer et al. 1992), an inhibition which can be over-ridden by a factor (or factors) secreted by astrocytes (Mayer et al., 1992), and also by ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (Mayer et al., 1994).

Precursor cell expansion by co-operating growth factors:

The most intriguing result of our studies with PDGF and bFGF was the discovery that O-2A progenitors exposed simultaneously to these two mitogens continued to divide without differentiating into oligodendrocytes (Bögler et al., 1990). For example, cultures of optic nerves of 19d old rat embryos began to generate oligodendrocytes after 2 days when established in the presence of PDGF alone (Raff et al., 1988), yet the generation of oligodendrocytes was effectively suppressed for 10 days when cells were grown in the presence of PDGF + bFGF (Bögler et al., 1990). Further experimentation demonstrated that O-2A progenitors can be continually grown for at least many weeks *in vitro* so long as cells are continuously exposed to both of these mitogens. Cell populations expanded in this manner are functionally normal, and can be utilized to repair experimental demyelinated lesions by cell injection (Groves et al., 1993).

The ability of PDGF and bFGF to have the cooperative effect of suppressing differentiation is of interest in light of our current understanding of principles underlying cooperation between oncogenes. One of the most important clues to understanding the molecular mechanisms underlying the development of cancer was the discovery (Land et al., 1983a,b; Ruley, 1983) that transformation, at least *in vitro*, requires cooperation between an immortalizing gene (i.e., a gene that suppresses terminal differentiation) and a growth control gene (i.e., a gene that is involved in the signal transduction pathways essential to the stimulation of cell division). Although it is clear that the pathway to neoplastic transformation is far more complex than simply the cooperation between two oncogenic mutations, it remains the case that this phenomenon of oncogene cooperation remains one of the foundation stones in our understanding of cancer.

Our observations on the effects of exposing a precursor cell simultaneously to PDGF and bFGF suggest that expression of an immortalizing gene may not even be necessary in the generation of the transformed phenotype. The possibility should perhaps also be considered that cooperation between ap-

propriate members of the growth control gene family might itself be sufficient to create cells able to override normal limitations on mitotic lifespan. As malignant gliomas tend to express a variety of growth factors capable (at least theoretically) of acting in an autocrine manner, such cooperation may have a direct outcome not only on the growth of these tumors but on their failure to undergo terminal differentiation into non-dividing cells.

The ability of appropriate combinations of growth factors to promote extended precursor cell division may represent a general phenomenon in precursor cell biology, as indicated for example by the importance of growth factor cooperation in promoting the extended division *in vitro* of haematopoietic stem cells (Cross and Dexter, 1991) and primordial germ cells (Matsui et al., 1992). In addition, it also appears that bFGF, or other members of the FGF family, are able with some frequency to cooperate with other growth factors to effectively suppress precursor cell differentiation. It seems intriguing in this regard that malignant tumors express at least one member of the FGF family with high frequency. Such FGF expression has been generally thought to be associated with the progression of tumor cells to an angiogenic phenotype, thus allowing them to recruit the blood vessels and blood supply required if the tumor is to grow beyond 1mm in size. In light of the ability of bFGF to cooperate with other growth factors to suppress cell differentiation, one also wonders whether autocrine expression of this growth factor would help to suppress terminal differentiation, thus enhancing tumor cell self-renewal.

The timing of differentiation and the probability of precursor cell self-renewal

The control of precursor cell self-renewal is far more complex than the simple question of whether a clonally related family of cells (whether of normal cells or tumor cells) undergoes self-renewal or differentiation as all-or-none phenomena. Current studies on the control of differentiation in O-2A progenitor cells suggest instead the existence of a finely tuned rheostat capable of setting self-renewal

probabilities at any value between one and zero depending upon the exogenous signals to which a precursor cell is exposed.

On a general level, it is clear that the timing of differentiation can be controlled both by cell intrinsic mechanisms and by environmental signals. An example of a cell-intrinsic timing mechanism is offered by the switching from production of fetal to adult haemoglobin in the erythrocytes produced by fetal or adult-derived haematopoietic stem cells. The timing at which adult hemoglobin is produced following transplantation of fetal haematopoietic stem cells to adult animals is determined by the age of the donor embryo, strongly indicating the regulator of this timing to be a cell-intrinsic biological clock residing within the haematopoietic stem cell (Bunch et al., 1981; Wood et al., 1985; Melis et al., 1987; reviewed in Groves et al., 1991). An example of the latter process, wherein the timing of differentiation is initiated by signalling molecules produced exogenously to the differentiating cell, is offered by the dramatic effects of thyroid hormone on modulating the timing of differentiation in amphibian species (e.g., Wang and Brown, 1993; Tata, 1994; Brown et al., 1995). As will be discussed next, an example of a timing mechanism that may be controlled by both cell-intrinsic mechanisms and the action of exogenous signalling molecules is the timely generation of oligodendrocytes.

The first experimental insights into the control of the timing with which a dividing O-2A progenitor cell would differentiate came from studies demonstrating that purified cortical astrocytes could also promote the correctly timed differentiation *in vitro* of O-2A progenitors isolated from optic nerves of embryonic rats (Raff et al., 1985). In these experiments, O-2A progenitor cultures were prepared from optic nerves of embryos of various ages and grown on astrocyte monolayers. The number of days that elapsed before the first appearance of oligodendrocytes in these cultures was precisely correlated with the embryonic age from which the cells were isolated, such that cells from younger animals went through a longer period of cell division before generating their first oligodendrocytes. Moreover, regardless of the age from which the cells were isolated, the first oligodendrocytes appeared *in vitro* at

a time corresponding to the time when they would have appeared *in vivo* (i.e., the day of birth of the rat).

The observation that clonal families of O-2A progenitor cells dividing under the influence of type-1 astrocytes or PDGF frequently undergo synchronous and symmetrical differentiation throughout a cell family led to the suggestion that the timed generation of oligodendrocytes is regulated by a cell-intrinsic clock that resides within the O-2A progenitor cell (Temple and Raff, 1986). It is important to note, however, that similarly symmetric and synchronous differentiation has not been observed in studies on O-2A progenitor cells from other CNS regions (Vaysse and Goldman, 1990; Lubetzki, et al., 1992; Zhang and Miller, 1995). Moreover, no previously published data appears to have directly addressed the question of whether the first appearance of oligodendrocytes from dividing embryonic progenitor cells occurs through symmetric clonal differentiation. Nonetheless, the intrinsic clock model originally proposed by Temple and Raff (1986) remains the dominant experimental model for the analysis of timed differentiation in this lineage (Temple and Raff, 1986; Raff et al., 1988; Hart et al., 1989a,b; Noble, 1991; Wren et al., 1992; McKinnon et al., 1993; Barres et al., 1994a,b; French-Constant, 1994).

Studies on the generation of oligodendrocytes by O-2A progenitor cells grown in chemically-defined medium (Bottenstein & Sato, 1979) in the presence of both PDGF and basic fibroblast growth factor (bFGF) indicate that it is possible to distinguish between the measurement of time by these precursor cells and the ability of the measuring process to induce differentiation. As discussed above, the combined application of PDGF + bFGF induces O-2A progenitor cells to undergo repeated division in the absence of differentiation (Bögler et al., 1990) and thus extends the mitotic lifespan of these cells beyond that observed when cells are exposed only to PDGF. Nonetheless, it is also clear that cells grown for progressively longer periods of time in the presence of both mitogens become increasingly unresponsive to stimulation by PDGF alone (Bögler and Noble, 1991, 1994). Thus, cells prevented from differentiation by exposure to this combination of

mitogens behave as though the biological clock that limits their mitotic lifespan has continued to function and has brought the cells to the brink of differentiation, yet the enactment of this progression is inhibited so long as both growth factors are present.

The model of differentiation control suggested by our studies on O-2A progenitor cells grown in the presence of PDGF + bFGF – in which differentiation controlled by an internal clock can be inhibited by exogenous signals – is mirrored by a model suggested by studies of Barres et al (1994b) on the actions of thyroid hormone on dividing O-2A progenitor cells. The results of these studies were interpreted to suggest that dividing O-2A progenitor cells have an absolute requirement for the presence of a hydrophobic signalling molecule (such as thyroid hormone [T3], retinoic acid, or dexamethasone) in order to be able to generate oligodendrocytes. Thus, according to this model, the clock that measures the mitotic lifespan of O-2A progenitor cells must work in positive co-operation with an exogenous signalling molecule in order to induce differentiation. This hypothesis is attractive due to the many observations suggesting that signalling molecules such as thyroid hormone play crucial roles in the timing of development. Such a model also would be consistent with observations that hypothyroid animals show a retarded time course of myelin generation in vivo (e.g., Balazs et al., 1969; Patel et al. 1979; Legrand, 1986; Dussault and Ruel, 1987; Rodriguez-Pena et al., 1993). This hypothesis, is not consistent, however, with contrasting demonstrations that retinoic acid suppresses the generation of oligodendrocytes in cultures of embryonic rat CNS (Noll and Miller, 1994; Laeng et al., 1994), thus working in precisely the opposite manner from that proposed by Barres et al. (1994b). Moreover, the experiments conducted by Barres et al. on the role of thyroid hormone on oligodendrocyte generation were carried out using cells derived from postnatal rats, and thus did not address the hypothesis that thyroid hormone – or some other hydrophobic signalling molecule – plays an important role in the appropriately timed first appearance of oligodendrocytes.

The need for a revised model for the control of the timely generation of oligodendrocytes

Several unexpected findings emerging from our own recent studies (Ibarrola et al., 1996) on the generation of oligodendrocytes by dividing O-2A progenitor cells suggest that the mechanisms regulating the timing of the initial appearance of oligodendrocytes are more complex than has been suggested previously by ourselves and others (e.g., Temple and Raff, 1986; Raff et al., 1988; Hart et al., 1989a,b; Noble, 1991; Wren et al., 1992; McKinnon et al., 1993; Barres et al., 1994a,b; French-Constant, 1994). In particular, we have found that it is possible to distinguish experimentally between the probability that a clone of dividing O-2A progenitor cells will generate at least one oligodendrocyte at an appropriate time in vitro and the actual extent of oligodendrocyte generation in that clone. In other words, it appears to be necessary to distinguish between the propensity of a clone of cells to initiate differentiation and the subsequent control of self-renewal probabilities.

The model for timed differentiation emerging from our most recent studies on oligodendrocyte development is illustrated in Figure 1. This model has the following features:

(i) The initial generation of oligodendrocytes generally occurs in association with asymmetric division and differentiation within clonal families of dividing O-2A progenitor cells. At present, it appears that the mechanism inducing this initial generation of oligodendrocytes is cell-intrinsic.

(ii) Once clones have begun to generate oligodendrocytes, the probability of progenitor self-renewal can be modulated by a variety of cell-signalling molecules. Thus, this regulation is cell-extrinsic. It appears that cells dividing in the presence of PDGF have an intrinsic (if as yet unknown) self-renewal probability. Factors such as NT-3 and bFGF increase this self-renewal probability, while factors such as CNTF and T3 reduce the self-renewal probability. The latter factors also appear to enhance the probability that entire clones of cells will differentiate in a synchronous manner. Whether this effect is stochastic or reflects activation of a true symmetry mechanism is not yet known.

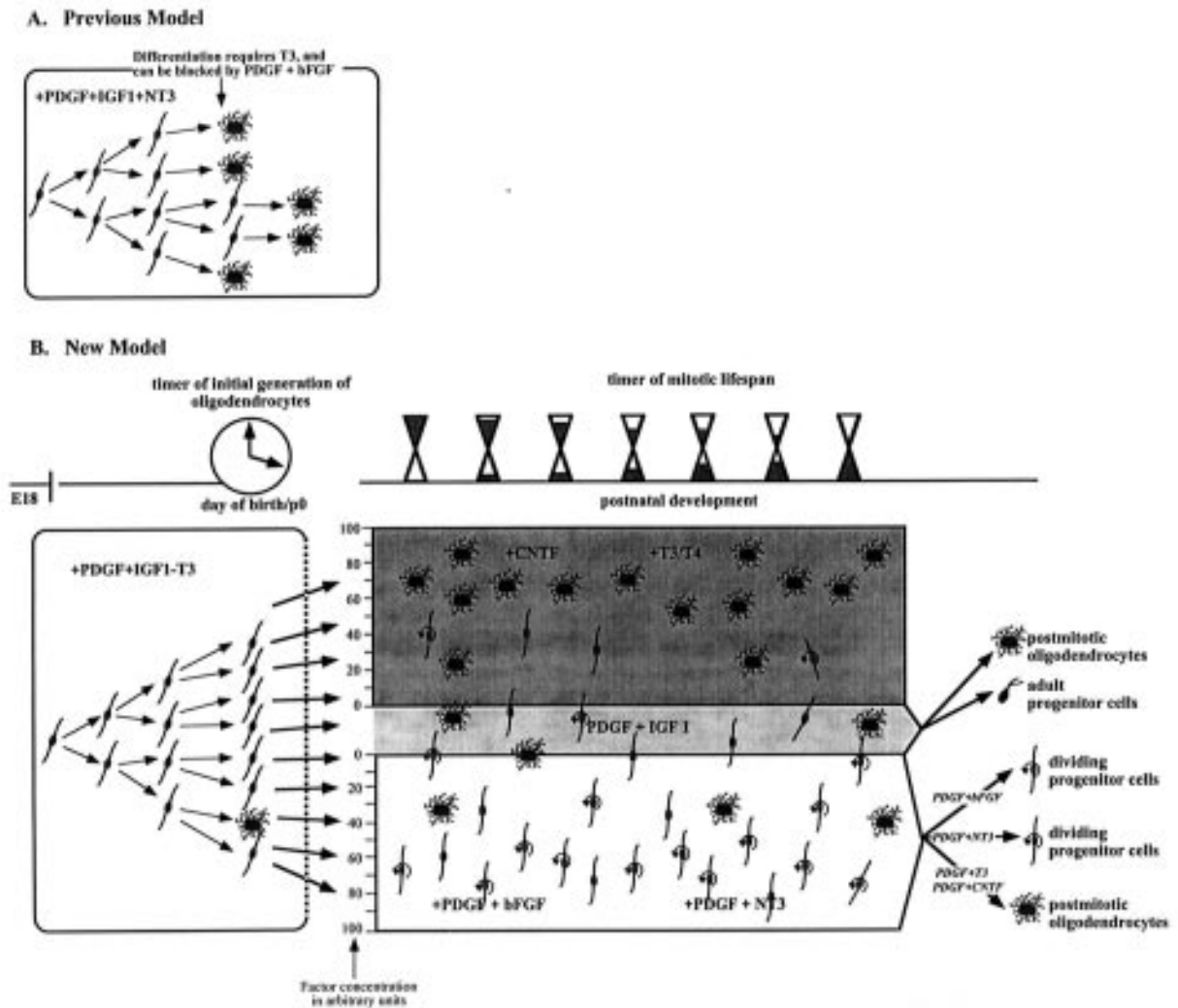


Figure 1. (A) The previous model of the control of the timely generation of oligodendrocytes. Dividing O-2A progenitor cell clones undergo symmetric division and synchronous differentiation within a limited number of cell divisions. (B) A revised model of the control of the timely generation of oligodendrocytes: O-2A progenitor cells from the embryonic rat brain divide and generate more progenitors in vitro up until a time equivalent to the rat's day of birth. At this point oligodendrocytes are generated within single clones of O-2A progenitors in association with asymmetric division and differentiation. After this point, progenitors can either undergo self-renewing divisions (ϕ) or can generate oligodendrocytes (\otimes), with the probability of either event occurring being modulated by the factors present in the extracellular environment. CNTF and T3 increase the probability of oligodendrocyte generation, while NT-3 and FGF increase the probability of self-renewing division occurring. The mitotic lifespan of dividing progenitors is limited by a functionally distinct biological clock (∇). The ability of this second biological clock to induce oligodendrocyte generation and cessation of division can be over-ridden by the combination of PDGF + FGF or, if cells are grown in the absence of T3, by the combination of PDGF + NT-3. In conditions where O-2A progenitor cells are enabled to continue dividing beyond their normal mitotic lifespan, this second clock continues to measure elapsed time and brings cells to a stage in which cells become refractory to the induction of cell division by PDGF alone and rapidly cease division and undergo differentiation if FGF is removed (for the former condition) or T3 is added (for the latter condition).

Although it is not known whether the mechanism (s) involved in regulating the extent of oligodendrocyte generation within individual clones is distinct from the one that initiates oligodendrocyte generation, these mechanisms are distinct in their biological function. Moreover, the ability of thyroid hormone to modulate self-renewal probability but not the initial generation of oligodendrocytes suggests that these two mechanisms are distinct at least in part.

(iii) Dividing perinatal progenitor cells contain a cell-intrinsic biological clock that measures a limited mitotic lifespan. Although it is not known whether this clock is mechanistically distinct from the one that initiates oligodendrocyte generation, these two clocks are distinct in their biological function.

(iv) Even in conditions where progenitor self-renewal is favored, the clock controlling the limited mitotic lifespan of dividing O-2A progenitor cells continues to function.

(v) Eventually the clock that limits mitotic lifespan reaches the end of its measuring period. Providing environmental conditions permit, most or all cells will become oligodendrocytes (but see point vi)

(vi) A second differentiation pathway that may be available to dividing perinatal progenitor cells is the generation of O-2A^{adult} progenitors, which differ from their perinatal counterparts in morphology, cell cycle length, migratory characteristics apparent ability to undergo prolonged self-renewal when stimulated to divide by astrocytes (Wolswijk and Noble, 1989, 1992; Wolswijk et al., 1990, 1991; Wren et al., 1992; Engel and Wolswijk, 1996). It is not yet established whether this transition is also controlled by a cell-intrinsic biological clock, although existing data is consistent with such a possibility (Wren et al., 1992; Noble et al., 1992)

(vii) If perinatal progenitor cells are grown in conditions where the generation of oligodendrocytes is prevented, progenitor cells are able to keep dividing even after their normal mitotic lifespan has elapsed. Such continued division can be promoted by signals that inhibit the generation of oligodendrocytes (i.e., PDGF + bFGF) or by the absence of signals (such as T3) that promote the generation of oligodendrocytes.

(viii) If progenitor cells are maintained in a dividing state for a length of time that exceeds their normal mitotic lifespan and then switched to conditions permissive for oligodendrocyte generation, then progenitors rapidly cease division and differentiate into oligodendrocytes. It is not known if such progenitors also generate O-2A^{adult} progenitor cells.

The possibility that there might exist more than one mechanism for ensuring timely development of a differentiated cell type is perhaps not surprising in light of the tendency of biological systems to develop multiple means of ensuring the occurrence of necessary events. For example, by utilizing clonal differentiation and limitation of mitotic lifespan as a secondary, rather than a primary, timing system it would theoretically be possible to allow maximally flexible responsiveness to the extracellular environment early on. The mechanism ensuring the eventual differentiation of entire clones of cells could then help to insure the removal of the widely-distributed and rapidly dividing O-2A progenitor cells from the maturing CNS by forcing clones to differentiate into oligodendrocytes and possibly also into slowly-dividing O-2A^{adult} progenitor cells (Wolswijk and Noble, 1989; Wolswijk et al., 1990, 1991; Wren et al., 1992; Noble et al., 1992).

In light of our recent findings that human O-2A progenitor cells can give rise to glioblastoma multiforme (see below; Noble et al., 1995), the most malignant of human brain tumours, understanding precisely how these different control points function to limit the mitotic lifespan of O-2A progenitor cells may have a relevance that extends beyond analysis of the mechanisms underlying normal tissue development. In addition, the contribution of growth factors to modulating the self-renewal probabilities of cancer cells needs to be closely examined.

Differences between O-2A progenitor populations of the perinatal and adult CNS

In the course of our attempts to gain insights into the cellular mechanisms underlying repair of demyelinated lesions in the adult animal, we also initi-

ated studies on O-2A progenitors of the adult CNS. In our initial studies, which were again focused on the rat optic nerve, we found that these O-2A^{adult} progenitor cells differed from their perinatal counterparts in several ways. The biology of these cells has been discussed in detail in several different articles and reviews (Wolswijk and Noble, 1989, 1992; Wolswijk et al., 1990, 1991; Wren et al., 1992; Noble et al. 1992; Noble et al., 1995), to which the interested reader is referred for detailed information.

A brief summary of the differences between O-2A^{adult} and O-2A^{perinatal} progenitors is as follows: O-2A^{adult} progenitors have a unipolar morphology *in vitro* (Wolswijk and Noble, 1989), whereas O-2A^{perinatal} progenitors are usually bipolar (Small et al., 1987; Wolswijk and Noble, 1989). In addition, O-2A^{adult} progenitors have a longer average cell-cycle time *in vitro* than O-2A^{perinatal} progenitors (65h v. 18h; refs Noble et al., 1988; Wolswijk and Noble, 1989), migrate more slowly (4 $\mu\text{m}/\text{h}$ v. 21 $\mu\text{m}/\text{hr}$; Small et al., 1987; Wolswijk and Noble, 1989) and take longer to differentiate (5 days v. 2 days for 50% differentiation; Wolsd Noble, 1989). Furthermore, O-2A^{adult} progenitors stimulated to divide by type-1 astrocytes are vimentin⁻O4⁺ while dividing O-2A^{perinatal} progenitors are vimentin⁺O4⁻ (Raff et al., 1984; Wolswijk and Noble, 1989).

Our experimental data indicate that the most likely ancestors of O-2A^{adult} progenitors are a sub-population of the O-2A^{perinatal} progenitor cells (Wren et al., 1992). It is also interesting in this regard that exposure of O-2A^{adult} progenitor cells to the combination of PDGF + bFGF causes these cells to re-express – at least transiently – the properties of O-2A^{perinatal} progenitor cells and to also inhibit their differentiation (Wolswijk and Noble, 1992). This transition offers still another demonstration of the manner in which simple combinations of growth factors can cause cells to undergo alterations in cell cycle length and self-renewal probability.

In the context of gliomas, the observations that also might be relevant to our concerns with the differences between perinatal and adult precursor cells are that it is clear that there are differences between childhood and adulthood gliomas. A particularly striking example of such a difference is found in the pilocytic astrocytomas that occur predomi-

antly in childhood and adolescence, and which have both a different molecular phenotype and a different prognosis than the astrocytomas found in adult patients (Russell and Rubinstein, 1989; von Deimling et al., 1993). Is it possible that the differences between the tumours occurring in children and the tumours occurring in adults reflect different lineage origins of tumours in people in different age groups?

The modulation of glial cell survival

One of the other effects of growth factors is to promote cell survival and prevent cells from undergoing programmed cell death. Within the O-2A lineage, there have been a number of recent studies on proteins that modulate the survival of oligodendrocytes, and the reader is referred to such papers as (Barres, et al., 1992, 1993; Barres and Raff, 1994; Louis et al., 1993; Mayer and Noble, 1994; Mayer et al., 1994).

Cell death can also be modulated by small molecules with likely physiological relevance. Thus far, the small molecules of the greatest interest in the O-2A lineage have been antioxidants, and most particularly compounds capable of altering the cellular levels of glutathione, the major intracellular protector against damage from reactive oxidative intermediates. Of these the compound of greatest experimental utility has been N-acetyl-L-cysteine (NAC). This is one of several compounds that can be used to augment intracellular levels of glutathione, the major scavenger of reactive oxidative intermediates (ROI) present in all eukaryotic forms of life (Smilkstein, et al., 1988; Aruoma, et al., 1989; Burgunder, et al., 1989; Anker and Smilkstein, 1994). Glutathione is generally required to protect cells against damage by oxidants, and is able to reduce and thereby detoxify these potentially damaging chemical species. NAC enters cells readily and replenishes the intracellular cysteine required to produce glutathione, thus leading to an increase in glutathione levels. NAC also reacts directly with ROI, thus protecting cells against these toxic compounds.

There are at least two reasons why the effects of

glutathione - and possibly of other antioxidants - on cell growth may be of interest in analyzing the biology of cancer cells. First, such cell survival proteins as bcl-2 may work through antioxidant pathways. (Hockenbery et al., 1993; Kane et al., 1993). In addition, an extensive body of experimentation indicates that levels of cellular glutathione, or of glutathione-S-transferase activity, are correlated with resistance to a variety of chemotherapeutic compounds (Mistry, et al., 1991; Lewis, et al., 1992; Ripple, et al. 1993; Perry, et al., 1993; Kodera, et al., 1994; Ngo and Nutter; 1994; Pendyala, et. al, 1995; Pratesi, et al.; 1995). While it is clear that a great deal of experimentation is still required to understand the complexities of this relationship (which does not hold true for all tumor populations or for all compounds used in chemotherapy), it is equally clear that understanding this relationship will provide information of relevance to the understanding of chemosensitivity and chemoresistance.

Our experimental concerns have thus far been focused on the role of glutathione not just in keeping cells alive but also in modulating the responsiveness of cells to growth factors. In the context of this review, then, we are concerned with the possibility that the same mechanisms that may be involved in conferring chemoresistance may also play more fundamental roles in enhancing tumor cell division and survival.

Our own studies have revealed that compounds such as NAC can not only protect cells against cell death induced by such cytotoxic stimuli as tumor necrosis factor- α (TNF- α), but also can enhance dramatically the activity of growth factors working through stimulation of receptor tyrosine kinase pathways (Mayer and Noble, 1994). Exposure of oligodendrocytes to NAC also markedly enhanced the extent of survival obtained with suboptimal quantities of known trophic factors, although NAC was not by itself sufficient to rescue cells from death associated with growth factor deprivation (Mayer and Noble, 1994). Cultures of pure oligodendrocytes treated with any dose of CNTF examined, and most doses of IGF-1, contained significantly more live oligodendrocytes if cultures were also exposed to 1mM NAC. Of particular interest, the presence of NAC in cultures exposed to

doses of CNTF or IGF-1 that by themselves had little or no effect on cell survival was now associated with the presence of significant numbers of live oligodendrocytes. We also observed (Mayer and Noble, 1994) dramatic effects of NAC on cell survival in cultures of spinal ganglion neurones, derived from day 16 rat embryos and exposed to sub-optimal doses of NGF. Cultures exposed to NAC plus either 1 or 10 ng/ml NGF contained 300-1000% more neurones than those exposed to NGF alone.

To determine whether other compounds with anti-oxidant activity could also co-operate with known trophic factors to synergistically promote cell survival, we examined the effects of 0.5ng/ml CNTF on oligodendrocyte survival when applied together with Vitamin C or Trolox. Although neither of these antioxidants had any effect when applied by themselves, the addition of either compound together with 0.5ng/ml of CNTF now resulted in significant levels of oligodendrocyte survival.

The spectrum of protective activity offered by NAC makes this molecule stand out as being of particular interest for continued investigation. In future research, it will be important to determine the mechanism(s) by which NAC causes the effects we observed. At present, there are two major known modes of action for this compound, both of them related to antioxidant activity. NAC is an effective scavenger of free radicals, and also is one of several compounds that can be used to augment intracellular levels of glutathione (Meister et al., 1986; Aruoma et al., 1989; Burgunder et al., 1989; Taniguchi et al., 1989; Staal et al., 1990). Both of these activities may contribute to the ability of NAC to protect oligodendrocytes against death induced by TNF- α or glutamate exposure.

In contrast to the possible mode of action of NAC in protecting oligodendrocytes against death induced by exposure to toxic stimuli, the role of NAC in promoting cell survival in conditions of trophic factor deprivation is considerably more enigmatic. It is also intriguing to speculate whether stimulation of cells by growth factors alters the function of similar metabolic pathways as those modulated by NAC, Vitamin C, Trolox or progesterone. For example, the observation that CNTF and NAC both rescued oligodendrocytes from TNF- α induced cell

death raised the possibility that these compounds exerted their effects through similar mechanisms. If this were the case, then CNTF should also have been able to confer protection against glutamate-induced killing of oligodendrocytes. The mechanism(s) by which CNTF protected oligodendrocytes from death in these experiments must have been at least in some manner different from NAC, however, since CNTF did not protect these cells against glutamate-induced death. It is extremely intriguing, however, that NGF has recently been found to induce increases in activity of gamma-glutamylcysteine synthetase activity (the rate limiting enzyme for glutathione synthesis), and also induces increases in catalase and glutathione peroxidase levels in PC12 cells (Pan and Perez-Polo, 1994; Jackson, 1994; Sampath, 1994). Thus, there is good reason to consider the possibility that trophic factors do have as part of their action the regulation of cellular glutathione levels and cellular redox state.

The mesenchymal paradox of glioma biology

Our work on rodent cells would be of little ultimate interest in the context of human disease if the principles elucidated through their study could not be applied to the study of human development and the treatment of human clinical problems. Confidence in the potential relevance of our work on rodent tissues to the analysis of human oligodendrocytes and their precursors has been recently increased by the results of experiments on cells derived from human gliomas.

Like a number of laboratories interested in human gliomas, we have for a long time been generating cell lines that we have hoped would prove useful both in the study of these human tumours and in the study of human lineages of the central nervous system. And, like other laboratories pursuing these interests, we have run up against the problem that a very large proportion – up to 90% – of human glioma specimens grown in standard tissue culture conditions give rise within several passages to populations that have no glial characteristics and instead resemble mesenchymal cells (Bigner et al., 1981; Shapiro and Shapiro, 1984; Kennedy et al., 1987;

Westphal et al., 1989). These mesenchyme-like cells can be derived from tumours of every category and every grade of malignancy. To make matters even more confusing, it also is clear that it is possible to derive such fibronectin-positive and GFAP-negative populations simply by cloning of GFAP-positive parental glioma lines (Westphal et al., 1988) and that such populations can also be derived from rat astrocytes immortalized with Simian Virus 40 Large T antigen (Geller and Dubois-Dalcq, 1988).

The present state of knowledge about the mesenchymal-like derivatives of gliomas is limited. Although some of the mesenchyme-like cells that arise from growth of gliomas in standard tissue culture conditions may be derived from meningeal cells rather than from the glioma cells themselves, it also is clear that in other cases these are bona fide tumour cells that display the molecular abnormalities characteristic of gliomas (Bigner et al., 1981; Shapiro and Shapiro, 1984; Davenport and McKeever, 1987a,b). It also appears that these mesenchymal-like cells can contribute to the tumour mass in the patient (Kennedy et al., 1987).

Among the many unanswered questions about the mesenchymal-like cells that can be derived from gliomas, the following strike us as being of particular importance:

(1) *What is the molecular basis for this transition?* One would very much like to know how it is that the glial phenotype is supplanted by a mesenchymal phenotype. It is also important to determine whether this transition is a one-way pathway, and whether there are conditions in which the mesenchymal-like cells derived from GFAP⁺ gliomas can be reconverted to a glial-like phenotype.

(2) *Is this transition associated with an alteration in the biological properties of the resultant cells?* Our own preliminary examination of the mesenchymal-like cells suggest that they may express different surface molecules than those expressed by their GFAP⁺ counterparts. To our knowledge, however, no studies have been reported in which the mesenchymal derivatives of a GFAP⁺ glioma have been compared with their glial ancestors in respect to response to growth factors, expression of different cell adhesion molecules or capacity for invasion of neural tissues.

(3) *Does this transition represent an aberrant pathway that becomes accessible to neural tumours or does it instead reflect the developmental potential of the cell of origin of the tumour?* At present, there is insufficient information about CNS development to speculate usefully about the answer to this question.

(4) *What is the frequency of this transition in vivo?*

(5) *Does this transition have any relevance to response to therapy?* These last two questions are closely connected to each other. If this transition does not occur in vivo, then it is simply a phenomenon of the tissue culture dish and of biological but not therapeutic interest. At the moment, the weight given to such a possibility will be based on the extent to which the reader believes in the "flying potato hypothesis of tissue culture," (which states that cells put into tissue culture behave abnormally and turn into flying potatoes; Noble et al., 1995). If one believes that experiments in tissue culture provide a valid insight into what may occur in vivo, then there will be more concern about the relevance of this transition to the patient's prognosis and treatment. If this transition frequently does occur in vivo, then one wants very much to know whether the two resulting populations differ in their growth and survival requirements, migratory behavior, and sensitivity to radiation and chemotherapy. As increasing numbers of attempts are made to develop therapeutic strategies that involve stimulation of the immune system to kill glioma cells, one also very much wants to know whether there would be common antigens that would allow both populations to be the target of a reaction by the immune system, or whether it would be necessary to provoke immune reactions against two separate biological entities. Finally, to the extent that *ex vivo* genetic modification is used to make cells immunogenic prior to re-implantation in the patient, it is critical to be able to grow cells in such a manner that the population put back into the patient closely resembles the population taken out in the first place.

In respect to the concern of this review with growth factors, it is important to consider the possibility that the glial to mesenchymal transition described above is mediated by specific agents present in serum. That this may be so is indicated by the results that will be described next.

A human glioma of the O-2A lineage

Regardless of the extent to which the mesenchymal derivatives of gliomas are of profound or of trivial importance, it is clear that the frequency with which these populations arise has greatly impeded the development of a lineage-based analysis of gliomas that relies on the tools of cellular biology rather than on the tools of histopathology. In order to attempt to generate more useful glioma-derived populations for our purposes we recently have changed our approach to the growth of these cells *in vitro* and have abandoned traditional tissue culture techniques.

In an attempt to break through the impasse created by the results described above, we initiated a program of research in which glioma-derived cells were grown utilizing the same methods that had enabled us, over a decade ago, to generate populations of O-2A progenitor cells that would grow in a tissue culture dish much in the manner they might grow in vivo. At that time we realized that re-creating the micro-environment in which precursor cells would find themselves in vivo might enable us to conduct meaningful biological experimentation with such cells in vitro, an approach that has turned out to be useful not just for the O-2A lineage but for many biological systems.

As it has turned out, the simplest growth conditions that we established now have provided us with the one possible tumour population for which we knew enough to ask a variety of questions. For the reasons described below, we believe that we have now isolated a *bona fide* tumour of the O-2A lineage, and thus we have named this cell population Hu-O-2A/Gb-1 (for *Human O-2A lineage G*loblastoma number 1). To grow these cells, tumour specimens were grown in chemically-defined medium that first was conditioned by purified rat cortical astrocytes, precisely as we described a decade ago for the growth of rat O-2A progenitor cells (Noble and Murray, 1984).

Hu-O-2A/Gb-1 cells express antigens characteristic of the O-2A lineage of the rat (and are HNK-1⁺, A2B5⁺ and GD3⁺) and can generate both more progenitor-like cells and O4⁺GalC⁺ oligodendrocytes when grown in chemically-defined medium condi-

tioned by purified rat cortical astrocytes. When these cells are plated in medium containing fetal calf serum, the oligodendrocyte pathway is suppressed and differentiation along the astrocyte pathway is enhanced, as also occurs for rodent O-2A progenitor cells. Moreover, the Hu-O-2A/Gb-1 cells are stimulated to divide by both PDGF and bFGF, and simultaneous exposure to both of these mitogens suppresses differentiation along the oligodendrocyte pathway, another characteristic shared with their rodent counterparts. All of these properties have been maintained through at least 20 passages of these cells *in vitro*.

Even more striking are our observations that Hu-O-2A/Gb1 cells express a $^1\text{H-NMR}$ spectrum which is essentially identical to that otherwise uniquely expressed by O-2A^{perinatal} progenitors derived from rat tissues (see Urenjak 1992, 1993 for $^1\text{H-NMR}$ characterization of normal glial cells). In these experiments a perchloric acid extraction is made from cells of interest, and this extract is studied in such a manner as to yield information about the kinds and quantities of free amino acids and other small metabolites contained within the population. We initially found that all of the major cell types of the CNS can be unambiguously distinguished from each other by their $^1\text{H-NMR}$ profiles (Urenjak, 1993). In our current studies we have found that of 10 glioblastoma populations analyzed by $^1\text{H-NMR}$ spectroscopy, only the Hu-O-2A/Gb-1 cells possess a metabolite composition like that of rodent O-2A^{perinatal} progenitor cells. The spectra obtained from the other glioblastoma populations, which do not appear on biological grounds to be of the O-2A lineage, are very different from that of either the human or rat O-2A lineage cells.

As indicated by the name given to this cell population, our human O-2A lineage cells were derived from a patient with glioblastoma multiforme. It is perhaps intriguing in this regard that some of the characteristics of glioblastoma multiforme are that the cells of this tumour are highly invasive, contain many primitive looking cells, and can generate cells of a variety of morphological phenotypes. These terms would also describe accurately the O-2A progenitor cell itself, and possibly any other early precursor cell of the CNS. Thus, it may be that some of

the most characteristic features of the malignant tumours of the CNS, such as their capacity to invade the brain and spinal cord, are reflections of the properties of the precursor cells from which these tumours might be derived.

We would like to suggest that our current findings might be of interest to researchers interested in development and neoplasia of the human CNS for several reasons.

(1) One of the important conclusions that may be drawn from our studies is that the analysis of cells of the rat CNS appears to have considerable potential utility for our attempts to study cells of the human CNS. This is of course a matter of considerable relief.

(3) Critically, we now have firm ground from which to create a biologically-based lineage analysis of human gliomas. While this is not going to be a rapid process, it is now a relatively straightforward one. We hope that the identification of a single glioma for which the lineage of origin is unambiguously known may be analogous with the importance of the discovery of GFAP as a specific marker of astrocytes (Bignami et al., 1972): once this marker existed, and one cell population existed about whose identity one could be certain, subsequent antigenic markers could be defined much more easily. In the years ahead it will be necessary to define markers of O-2A lineage tumours that allow all such gliomas to be identified easily. The defining of this family of gliomas will then make it very much easier to identify the next biological family of tumours.

We also would suggest that our $^1\text{H-NMR}$ spectroscopic analysis is consistent with the existence of a minimum of two separate lineage contributions to human glioblastoma multiforme. We have thus far seen a surprising consistency between the small metabolite profiles expressed by primary cells of the rat CNS and tumours of the human CNS. For example, our analyses of rodent meningeal cells and human meningioma cells (Florian et al., 1995) have provided findings qualitatively similar to our comparisons between rodent O-2A progenitor cells and Hu-O-2A/Gb-1 cells. The two questions we must now rapidly address are (i) whether tumours of the O-2A lineage occur frequently or, on the other extreme, whether tumours of the sort we have now

isolated are extremely rare events and (ii) how many other lineages contribute to the generation of human gliomas.

One of the tools for detecting tumours of the O-2A lineage that may prove most rapidly useful is ¹H-NMR spectroscopy of living patients. One of the most important identifying peaks for these cells is N-acetyl-aspartate (NAA), an amino acid readily detected in whole brain scanning procedures. Thus far, our data suggests that any adult patient with a tumour mass containing abundant NAA would be most likely to have a tumour of the O-2A lineage.

(6) It will be of interest to determine how generally useful the simple alterations we have introduced to our tissue culture techniques prove in the isolation of other gliomas with informative phenotypes. This modification in our strategies for isolating useful cell lines was critical in allowing the isolation of the Hu-O-2A/Gb-1 cells, for had the original tumour biopsy specimen been grown in medium containing fetal sera there would have been no oligodendrocytes to discover in the cultures. In addition, when cells are grown in the presence of fetal serum, the NAA peak also disappears. Thus, the two most important indicators that we were working with a tumour of the O-2A lineage would have been lost to us had these cells been grown using the tissue culture techniques utilized in most laboratories. We therefore hope that other laboratories will benefit from these findings, and that the next generation of glioma cell lines that are generated by the many scientists interested in these tumours will be of considerable usefulness in our attempts to understand human neural development and neoplasia.

Acknowledgements

Support for the work discussed in this review has come from many sources over the past decade, including the Multiple Sclerosis Society of Great Britain, the Medical Research Council, the Wellcome Trust (NG), the Cancer Research Campaign, Action Research, the Ludwig Institute for Cancer Research and the Carrie Rudolph Trust. Particular recognition, however, is due to the Preuss Foundation, and in particular to its director Peter Preuss

and its scientific advisor, Dr. Lorraine Marin, who supported and encouraged this program of research from its very earliest stages.

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