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Cell number control and timing in animal development: the oligodendrocyte cell lineage

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ABSTRACT Our studies of oligodendrocyte development in the rodent optic nerve provide clues as to how cell numbers and the timing of differentiation may be controlled during mammalian development. Both cell number and the timing of differentiation depend on intracellular programs and extracellular signals, which together control cell survival and cell division. As the cells seem to compete for limiting amounts of both survival signals and mitogens, the levels of these extracellular signals must be tightly regulated, but it is not known how this is achieved. The timing of cell-cycle exit, and therefore the onset of differentiation, seems to depend in part on the progressive accumulation of the intracellular Cdk inhibitor p27/Kip1, but it is still unclear how the level of this protein is controlled over time in the dividing cells. The timing of cell-cycle exit is also regulated by thyroid hormone, which, along with other hormones, seems to coordinate the timing of development in various organs, much as the timing of the multiple changes in metamorphosis in both vertebrates and invertebrates is coordinated by hormones. In this sense, one might think of mammalian development as a prolonged metamorphosis.

KEY WORDS: *optic nerve, clock, thyroid hormone, p27/Kip1*

"The most obvious differences between different animals are differences in size, but for some reason the zoologists have paid singularly little attention to them."

J.B.S. Haldane, On Being the Right Size, 1927.

Introduction

Even today, biologists generally pay little attention to how the size of an animal or an organ is determined. Although growth can occur by cell enlargement and the accumulation of extracellular matrix, it mainly occurs by cell division, so that size generally reflects cell numbers. We are larger than mice mainly because we contain more cells than a mouse. How, then, are cell numbers determined during animal development?

Cell number depends on both cell division and cell death, both of which depend on intracellular control systems that are regulated by extracellular signals. The core of the intracellular system that controls the cell division cycle consists of cyclin-dependent protein kinases (Cdks), which are cyclically activated to trigger the different phases of the cell cycle. The Cdks are themselves regulated by a variety of intracellular proteins, including cyclins, which activate them, Cdk inhibitors, which suppress them, and kinases and phosphatases, which either inhibit or activate them (Morgan, 1995). By contrast, the core of the cell death program consists of a family of cysteine proteases (caspases), which act in a proteolytic cascade, cleaving one another and key intracellular proteins, to kill the cell in a controlled way (Martin and Green, 1995). The activation of these death proteases is regulated by members of the Bcl-2 family of proteins, which either promote or inhibit activation by an unknown mechanism (Korsmeyer, 1995).

The extracellular signals that regulate cell division or cell death can either activate or inhibit the intracellular mechanisms; they can operate locally or systemically and can be soluble, bound to cell surfaces, or associated with the extracellular matrix. The challenge is to understand how the intracellular mechanisms and the large number of extracellular signals interact to ensure that each organ contains the right numbers of each cell type.

Controls on cell division

Except for the initial cleavages of the zygote and blastomeres, which are apparently cell-autonomous, most animal cell divisions are thought to depend on extracellular growth factors made by other cells. If a cell in culture, for example, is deprived of such factors the cell cycle arrests, usually at a checkpoint in G1 called

Abbreviations used in this paper: Cdk, cyclin-dependent protein kinase; PCD, programmed cell death; PDGF, platelet-derived growth factor; p27, p27/Kip1; TH, thyroid hormone.

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the restriction (R) point, and the cell enters a modified G1 state (G0), in which the cell-cycle control system is partly dismantled, in that some of the Cdks and cyclins disappear (Zetterberg, 1995). Such "social controls" on cell division presumably help to ensure that a cell divides only when another cell is needed. Even when continuously stimulated by other cells, however, a cell will not carry on dividing forever: during development, for example, most precursor cells divide a limited number of times before they stop and terminally differentiate into cells that will not divide again; even cells that have the potential to divide throughout life, will eventually stop if stimulated to proliferate continuously in culture -a phenomenon called cell senescence. (Hayflick, 1965).

Much effort has been devoted to identifying the extracellular signaling molecules that stimulate cell division, as well as the intracellular signaling pathways that they activate. By contrast, until recently, relatively little effort has been devoted to determining the mechanisms responsible for stopping cell division at the appropriate time during development. The stopping mechanisms are important, as they influence both cell numbers and the timing of terminal differentiation.

Controls on cell survival and cell death

Just as animal cells need signals from other cells to divide, so most also need signals from other cells to survive (Raff, 1992). If deprived of such survival signals, at least in culture, the cells activate an intrinsic death program and kill themselves –a process called programed cell death (PCD). Such social controls on cell survival may help ensure that a cell survives only where and when it is needed (Raff, 1992).

In mammals, extensive PCD begins at the blastocyst stage of development and continues throughout life. PCD serves many functions in development, and one of these is to regulate cell numbers (Jacobson *et al.*, 1997). Cell death can be just as important in controlling cell numbers as cell division. In the developing vertebrate nervous system, for instance, many types of neurons are overproduced and then compete with one another for limiting amounts of survival signals (neurotrophic factors) secreted by the target cells they innervate: only about half get enough to survive, while the others undergo PCD. In this way the numbers of neurons are matched to the numbers of target cells they innervate (Oppenheim, 1991). It seems likely that similar mechanisms operate in many developing organs to help match the numbers of different cell types.

Oligodendrocytes are an example where this type of control is crucial (Barres *et al.*, 1992). In the rest of this paper we review our studies on oligodendrocyte development in the rodent optic nerve, which illustrate how intracellular and extracellular controls combine to regulate cell numbers and the timing of differentiation.

Oligodendrocyte development in the rat optic nerve

The rodent optic nerve contains two main types of glial cells – oligodendrocytes and astrocytes– as well as the axons of retinal ganglion cells. The oligodendrocytes myelinate the axons, while the astrocytes provide structure and help control the environment in the nerve. The astrocytes develop from the neuroepithelial cells that form the optic stalk, the primordium of the nerve. Their numbers are mainly determined by controls on cell division, which depend on uncharacterized signals from the axons (Burne and Raff, 1997).

The oligodendrocytes are postmitotic cells, which develop from proliferating precursors that migrate into the developing nerve from the brain (Small *et al.*, 1987). Thus the final number of oligodendrocytes in the optic nerve will depend on the number of precursors that migrate in, the number of times each precursor divides before it terminally differentiates, and the number of precursor cells and oligodendrocytes that normally undergo PCD. We do not know how many precursor cells migrate into the developing nerve, but we do know something about how many times they divide, how many oligodendrocytes normally die, and how cell division and survival in this cell lineage are controlled. We shall first review the controls on oligodendrocyte survival and then discuss the controls on oligodendrocyte precursor cell division.

Controls on oligodendrocyte survival

Both developing oligodendrocytes and their precursors undergo PCD if they are cultured on their own in the absence of other cells or signaling molecules (Barres *et al.*, 1992). A number of cytokines promote the survival of these cells in culture, including platelet-derived growth factor (PDGF), insulin-like growth factor-1, ciliary neurotrophic factor, and neurotrophin-3; whereas any one of these supports short-term survival in culture, at least three are required for longer-term survival (Barres *et al.*, 1993b). Although it is uncertain whether all of these factors normally promote oligodendrocyte survival *in vivo*, in principle, there is an advantage in having the survial of a cell depend on multiple signals rather than just one: by using signals in combinations an animal can control the survival of its cells in specific ways using a relatively small number of signals.

About 50% of the oligodendrocytes produced in the developing optic nerve normally undergo PCD soon after they differentiate (Barres *et al.*, 1992). Experimentally increasing the levels of any of the four cytokines that promote survival in culture greatly decreases this normal cell death, indicating that all of these cytokines are normally present in limiting amounts in the developing nerve (Barres and Raff, 1994). Thus the normal death of oligodendrocytes may reflect a competition for these and/or other survival signals.

Although the four cytokines are made by astrocytes in the developing nerve, it is clear that the survival of newly formed oligodendrocytes also depends on axons: if the developing nerve is cut behind the eye, the axons in the nerve rapidly degenerate, and, as a result, the oligodendrocytes undergo PCD (Barres *et al.*, 1993a). Moreover, purified retinal ganglion cells, the source of axons in the optic nerve, promote the survival of purified oligodendrocytes in culture (Barres *et al.*, 1993a). To explain both the large-scale death of oligodendrocytes during normal development and the dependence of oligodendrocyte survival on axons, Barres *et al.* (1992) proposed that when oligodendrocyte precursors stop dividing and differentiate their survival requirements change so that they now require survival signals from axons: about half the cells normally find an axon and survive, while the other half fail to do so and die. This scheme would provide a simple mechanism for matching the number of oligodendrocytes to the number and length of axons requiring myelination. Several predictions follow from this hypothesis. One is that most of the oligodendrocytes should undergo PCD when the axons in a developing nerve

degenerate following nerve transection, and, as just mentioned, this is the case (Barres *et al.*, 1993a). Another is that, if the number of axons in the nerve is experimentally increased, fewer oligodendrocytes should die and, as a result, oligodendrocyte numbers should automatically adjust upward to match the increase in axon numbers, and this too is the case (Burne *et al.*, 1996). Yet another prediction is that if the number of oligodendrocytes is experimentally increased by providing excess PDGF, the main mitogen for oligodendrocyte precursor cells, extra oligodendrocyte death should bring the number back to normal, and Richardson and his colleagues have shown that this is the case in the embryonic mouse spinal cord Calver *et al.*, 1998). Trapp *et al.* (1997) have recently shown that many oligodendrocytes also undergo PCD in the developing rat brain, and the ones that die seem to be those that fail to contact axons, providing further support for the hypothesis. The nature of the putative axon-derived survival signals are unknown.

Control of oligodendrocyte precursor cell division

When oligodendrocyte precursors are cultured on their own with survival signals but without mitogens, they prematurely stop dividing and differentiate (Temple and Raff, 1985; Barres *et al.*, 1994). They can be stimulated to divide by a number of mitogens, but PDGF is the most important, both *in vitro* (Noble *et al.*, 1988, Raff *et al.*, 1988, Richardson *et al.*, 1988) and *in vivo* (Calver *et al.*, 1998). In mice in which the *PDGF A* gene has been inactivated, for example, oligodendrocyte precursors do not proliferate and very few oligodendrocytes develop (Calver *et al.*, 1997). Both astrocytes (Noble *et al.*, 1988; Richardson *et al.*, 1988) and retinal ganglion cells (Mudhar *et al.*, 1993) make PDGF-AA, and it is not known which is the main source of the mitogen in the developing optic nerve. Just as newly formed postmitotic oligodendrocytes seem to compete for limiting amounts of survival factors in the developing optic nerve, so Richardson and his colleagues have shown that dividing oligodendrocyte precursor cells compete for limiting amounts of PDGF mitogen in the embryonic mouse spinal cord, before oligodendrocyte differentiation has begun (Calver *et al.*, 1997).

The timer that controls oligodendrocyte differentiation

The first oligodendrocyte precursor cells in the developing rat optic nerve stop dividing and differentiate around the time of birth (Miller *et al.*, 1985), and new oligodendrocytes continue to differentiate from dividing precursor cells for another six weeks (Barres *et al.*, 1992). This timing of oligodendrocyte differentiation can be reconstituted in cultures of dissociated embryonic optic nerve cells, as long as there is sufficient PDGF to drive the proliferation of the precursor cells (Raff *et al.*, 1985, 1988). The reason that precursor cells stop dividing and differentiate in these cultures is not because PDGF becomes limiting or that PDGF receptors disappear or can no longer be activated: adding excessive PDGF does not alter the timing of oligodendrocyte differentiation (Raff *et al.*, 1988), and newly formed oligodendrocytes still express large numbers of PDGF receptors (Hart *et al.*, 1989b), which can induce both an increase in cytosolic Ca^{2+} (Hart *et al.*, 1989a) and immediate-early gene expression when stimulated with PDGF (Hart *et al.*, 1992).

Clonal analyses in culture suggest that the oligodendrocyte precursors in the postnatal day 7 optic nerve are heterogeneous in the number of times they divide in response to PDGF stimulation, dividing from 0 to 8 times, but the progeny of an individual precursor tend to stop dividing and differentiate at approximately the same time (Temple and Raff, 1986; Barres *et al.*, 1994). The similar behavior of sister cells transferred to different microwells suggests that an intrinsic mechanism operates in the precursor cells to limit their proliferation to a certain period of time or a certain number of cell divisions (Temple and Raff, 1986). The finding that precursors cultured at 33°C divide more slowly but stop dividing and differentiate earlier, after fewer divisions, than when they are cultured at 37°C, suggests that the intrinsec mechanism does not operate by counting cell divisions but instead measures time in some other way (Gao *et al.*, 1997).

The influence of thyroid hormone

The intrinsic timer seems to consist of at least two components –a counting component that measures time and an effector component that stops the cell cycle and initiates differentiation when time is up (Barres *et al.*, 1994; Bögler and Noble, 1994). This can be shown, for example, by studying the influence of thyroid hormone (TH) on the timer. When purified oligodendrocyte precursor cells isolated from the optic nerve of postnatal day 8 rats are grown at clonal density in the presence of mitogens and TH, the cells divide a limited number of times before they stop and differentiate; in the absence of TH, by contrast, many of the cells divide more than 8 times and very few differentiate into oligodendrocytes (Barres *et al.*, 1994). If, however, purified postnatal day 8 precursor cells are cultured at clonal density in mitogens in the absence of TH for 8 days, and then TH is added, most of the cells stop dividing and differentiate within 4 days, suggesting that the cells can count time in the absence of TH (Barres *et al.*, 1994). It seems therefore that TH helps the effector mechanism stop the cell cycle and initiate differentiation when the counting mechanism indicates that it is time. Although retinoic acid can substitute for TH in culture (Barres *et al.*, 1994), TH is required for normal oligodendrocyte development in the optic nerve: hypothyroid rats (Ibarrola *et al.*, 1996) and mice (Ahlgren *et al.*, 1997) have many fewer oligodendrocytes in their optic nerves at postnatal day 7. As TH influences the differentiation of many types of vertebrate precursor cells, it may help to coordinate the timing of cell differentiation in tissues throughout the body, just as it coordinates the events of amphibian metamorphosis (Tata, 1993).

The role of p27/Kip1

How does the timer count time, and how does it stop the cell cycle and initiate differentiation when time is up? In principle, such a timer could depend on (1) the decay of stimulatory proteins such as cyclins or Cdks that normally drive the cell cycle, (2) the accumulation of inhibitory proteins such as the Cdk inhibitors that normally suppress cell-cycle progression, or (3) both mechanisms. It seems likely that both a decrease in stimulators and an increase in inhibitors will turn out to be involved, as this would make the stopping mechanism robust and precise: if one component were to fail, for example, such a mechanism could still stop the cell cycle, although not at precisely the right time.

Several lines of evidence suggest that the accumulation of the Cdk inhibitor p27/Kip1 is part of the timer in oligodendrocyte precur-

sor cells (Durand *et al.*, 1997, 1998). First, p27 is high in all oligodendrocytes, whereas it is variable in their precursors. Second, when precursor cells are deprived of PDGF in culture, the rate at which p27 rises is indistinguishable from the rate at which the cells commit to cell-cycle withdrawal and differentiation, raising the possibility that the increase in p27 is part of the effector mechanism that stops the cell cycle. Third, p27 progressively increases as precursor cells proliferate in culture in the presence of PDGF and the absence of TH, even though most of the cells do not stop dividing and differentiate in these conditions. It reaches a plateau value at around the time when most of the cells would have stopped dividing and differentiated were TH present, suggesting that the progressive rise in p27 is part of the timing mechanism. Since the cells continue to divide with this high level of p27, however, it is clear that the rise in p27 is not enough on its own to stop the cell cycle. Fourth, as mentioned earlier, the timer runs faster at 33° than at 37°; as would be expected if p27 accumulation is part of the timing mechanism, p27 levels rise faster at 33° than at 37°, perhaps because p27 degradation is decreased more than p27 synthesis is decreased at the lower temperature (Gao *et al.*, 1997). Fifth, precursor cells isolated from the optic nerves of p27-deficient mice go through one or two more divisions in clonal culture in the presence of PDGF and TH before they differentiate than do precursors from wild-type mice (Durand *et al.*, 1998).

p27-deficient mice are larger than normal and have more cells in all organs that have been examined, suggesting that p27 is required in many cell lineages for normal exit from the cell cycle (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). On the other hand, oligodendrocyte precursors, as well as other cells affected in p27-deficient mice, still stop dividing and differentiate, suggesting that p27 is only one part of a multi-component mechanism that is responsible for stopping the cell cycle and initiating differentiation at the appropriate time in development. Interestingly, the p27-like protein Dacapo helps cells exit the cell cycle in developing flies, suggesting that Cdk inhibitors may be involved in stopping precursor cell division in all animals (de Nooij *et al.*, 1996; Lane *et al.*, 1996).

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