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Forum Review

Redox Regulation of Precursor Cell Function: Insights and Paradoxes

MARK NOBLE, MARGOT MAYER-PRÖSCHEL, and CHRIS PRÖSCHEL

ABSTRACT

Studies on oligodendrocytes, the myelin-forming cells of the central nervous system, and on the progenitor cells from which they are derived, have provided several novel insights into the role of intracellular redox state in cell function. This review discusses our findings indicating that intracellular redox state is utilized by the organism as a means of regulating the balance between progenitor cell division and differentiation. This regulation is achieved in part through cell-intrinsic differences that modify the response of cells to extracellular signaling molecules, such that cells that are slightly more reduced are more responsive to inducers of cell survival and division and less responsive to inducers of differentiation or cell death. Cells that are slightly more oxidized, in contrast, show a greater response to inducers of differentiation or cell death, but less response to inducers of proliferation or survival. Regulation is also achieved by the ability of exogenous signaling molecules to modify intracellular redox state in a highly predictable manner, such that signaling molecules that promote self-renewal make progenitor cells more reduced and those that promote differentiation make cells more oxidized. In both cases, the redox changes induced by exposure to exogenous signaling molecules are a necessary component of their mode of action. Paradoxically, the results obtained through studies on the oligodendrocyte lineage are precisely the opposite of what might be predicted from a large number of studies demonstrating the ability of reactive oxidative species to enhance the effects of signaling through receptor tyrosine kinase receptors and to promote cell proliferation. Taken in sum, available data demonstrate clearly the existence of two distinct programs of cellular responses to changes in oxidative status. In one of these, becoming even slightly more oxidized is sufficient to inhibit proliferation and induce differentiation. In the second program, similar changes enhance proliferation. It is not yet clear how cells can interpret putatively identical signals in such opposite manners, but it does already seem clear that resolving this paradox will provide insights of considerable relevance to the understanding of normal development, tissue repair, and tumorigenesis. *Antioxid. Redox Signal.* 7: 1456–1467.

MODULATING THE BALANCE BETWEEN SELF-RENEWING DIVISIONS AND DIFFERENTIATION IN OLIGODENDROCYTE GENERATION

THE PROBABILITY WITH WHICH A PRECURSOR CELL (a term used to generically include both stem cells and lineage-restricted progenitor cells) undergoes either self-renewing division or differentiation into a terminally differentiated cell type has a profound impact on the development of an organism. If differentiation happens too early, then the number of precursor cells available for generating an organism may be reduced below the levels needed for normal development to occur. If the probability of continued division is too high, in contrast, then precursor cell numbers may continue to increase inappropriately, as occurs in cancer. A great deal has been learned about how final cell number is regulated by cell

Department of Biomedical Genetics, University of Rochester Medical Center, Rochester, NY.

death, including identification of many of the relevant cytokines and signaling pathways. In contrast, we still know relatively little about how the alterations in cell number that can be achieved simply by altering parameters of self-renewal and differentation are modulated in normal and abnormal development.

The general importance of regulating the balance between self-renewal and differentiation suggests that mechanisms of broad general application are involved in this regulation, for this is a problem that would have to have been solved early in evolutionary history. At a minimum, this was a problem that had to have been solved with the appearance of multicellular organisms in which specialized cell types occur. Thus, there is a hope that if the mechanisms involved in controlling these probabilities could be identified in a single precursor cell lineage, such insights might have broad general applicability.

We have been studying the balance between self-renewal and differentiation in the cellular lineage that gives rise to the myelin-forming oligodendrocytes of the central nervous system (CNS), a cellular system particularly suitable for such analysis. The precursor cell that gives rise to oligodendrocytes has been exceptionally well studied. This cell is a lineage-restricted progenitor cell that can also generate a second cell type *in vitro,* the type-2 astrocyte, leading to this cell being originally named an oligodendrocyte–type-2 astrocyte (O-2A) progenitor cell (64). [As uncertainty about the *in vivo* generation and role of type-2 astrocytes (18, 73) caused some laboratories to rename this same cell an oligodendrocyte precursor cell (OPC), we refer to these cells as O-2A/OPCs.] O-2A/OPCs can be purified from all regions of the CNS [except possibly the olfactory bulb (6)], but in particular can be isolated from such tissues as the optic nerve at a time when they are physiologically poised to be highly responsive to environmental cues (a topic that will be considered later in this review). Multiple developmentally relevant signals have been identified that modify the balance of self-renewal and proliferation in dividing O-2A/OPCs, allowing analysis of this balance *in vitro.* A further very useful feature of this lineage for cellular biological analysis is that visual examination allows accurate determination of whether a particular cell is an oligodendrocyte or a progenitor cell (*e.g.,* 35, 74, 79). Thus, clonal analysis of individual cellular families allows a detailed dissection of the balance between self-renewal and differentiation.

The first experiments suggesting that the study of dividing O-2A/OPCs might provide novel insights into the control of the balance between self-renewal and differentiation demonstrated a remarkable conservation of the timing of oligodendrocyte generation *in vivo* and *in vitro* (65). For example, the first oligodendrocytes are generated *in vivo* in the rat optic nerve at the time of birth, which corresponds to the 21st day of embryogenesis (E21). If cells from the E18 optic nerve are grown in culture in medium supplemented with platelet-derived growth factor (PDGF), then the first oligodendrocytes are generated 3 days later. In contrast, if cells from the E19 optic nerve are grown in identical conditions, they first generate oligodendrocytes 2 days later. Similarly, cultures of optic nerve cells generated from E20 rats produce their first oligodendrocytes within 24 h. The remarkable accuracy with which this timing is maintained led to the suggestion that dividing O-2A/OPCs contain a cell-intrinsic clock that regulates the first generation of oligodendrocytes. A number of features of this clock have been identified (see, *e.g.,* 11, 12, 26, 35, 81, 82, 85, 87), but we still do not have a satisfactory understanding of how such remarkable biological time-keeping occurs.

Distinct from the cell-intrinsic clock that regulates the initiation of oligodendrocyte generation is a second and distinct mechanism that controls the balance between self-renewal and differentiation in dividing precursor cells once oligodendrocyte generation has begun (35). Thus, this second mechanism controls the extent to which oligodendrocytes are generated at any given cell division after the first cell-intrinsic timer induces or enables a clonal family of cells to begin generating oligodendrocytes.

Multiple cell-extrinsic signaling molecules have been identified that can modify the balance between self-renewal and differentiation in dividing O-2A/OPCs, pushing this balance mildly or sharply in either direction. The degree to which exposure to signaling molecules can alter the balance between self-renewal and differentiation in dividing O-2A/OPCs is very extensive. Growth of O-2A/OPCs in the presence of PDGF, which appears to be the basal division conditions for these cells, is associated with the spontaneous generation of oligodendrocytes in the absence of inductive signals (59, 69). In cells derived from the postnatal day 7 (P7) rat optic nerve, growth in such conditions is associated with a self-renewal probability of ~0.5 for at least the first several days of *in vitro* growth (86), a probability that allows continued generation of new precursor cells and differentiation to occur at about equal yields. The extent of asymmetric division and differentiation is modified by combining PDGF with other cell-extrinsic regulatory molecules (35). It is possible to induce nearly synchronous differentiation of all clonally related cells into oligodendrocytes when cells are exposed to thyroid hormone (TH) and type-1 astrocytes (66, 80). In contrast, almost complete suppression of differentiation with continuous promotion of self-renewal occurs when cells are grown in the presence of both PDGF and fibroblast growth factor-2 (FGF-2) (12), or, to a somewhat lesser extent, in the presence of PDGF + neurotrophin-3 (NT-3) (35, 74). At least a subset of these signaling molecules are also known to be of importance *in vivo*. For example, hypothyroid animals show reduced oligodendrocyte generation (2, 35), and animals in which NT-3 levels are artificially increased show increases in O-2A/OPC proliferation (8). Both of these results are consistent with the outcomes of *in vitro* studies on the effects of TH and NT-3 on O-2A/OPC division and differentiation (7, 8, 35).

INTRACELLULAR REDOX STATE CONTROLS THE RESPONSE TO SIGNALING MOLECULES, AND ACTS IN OPPOSITE MANNERS ON DIVISION OR SURVIVAL PATHWAYS VERSUS DIFFERENTIATION OR DEATH PATHWAYS

The key insights connecting our studies on self-renewal to intracellular redox state emerged from earlier studies

demonstrating that the effects of exposing cells to a variety of signaling molecules were highly dependent on the redox state of the target cell.

It was first noted by Fidelus and co-workers that reducing the glutathione content of lymphocytes lessened their responsiveness to mitogens and other activators (19, 20). The potential importance of these observations with respect to regulation of immune function began to be appreciated as it became clear that there appeared to be a relationship between the decline in T-cell glutathione levels in AIDS patients and the abnormal function of these T-cells. As the major reduced thiol present in cells, a reduction in glutathione content—without compensation by other regulatory pathways—would be associated with a more oxidized status. The extent to which reduced glutathione levels contribute directly to the pathology of AIDS has been the subject of extensive study (see, *e.g.,* 17, 32, 57, 76).

Complementary to the demonstration that making cells more oxidized could decrease their response to some cell-signaling molecules, we found that making cells more reduced greatly enhanced their responsiveness to other signals. Our first studies showed that mild reduction of oligodendrocytes or neurons greatly enhanced responsiveness to promoters of cell survival (52). In these experiments, we found that exposing cells to a combination of suboptimal amounts of such survival factors as ciliary neurotrophic factor (CNTF), insulin-like growth factor-I, or nerve growth factor in the presence of antioxidants was associated with a markedly synergistic enhancement in cell survival. A particularly potent antioxidant used in these studies was *N*-acetyl-L-cysteine (NAC). This cysteine pro-drug is readily taken up by cells and converted to cysteine, the increased availability of which enhances production of glutathione, which is the most prevalent reduced thiol within cells and is crucial for maintaining a reduced intracellular environment (54, 55). We also obtained similar results with other antioxidants, such as Trolox (a water-soluble analogue of vitamin E) and vitamin C (52), indicating that multiple modulators of redox status were able to alter cellular function in this manner.

Relatively small changes in redox state can have profound effects on cell function. For example, we have seen that 10–15% increases in the glutathione content of oligodendrocytes are associated with dramatic increases in responsiveness to signals that modulate cell survival (52). Stall *et al.* reported that as little as a 10% decrease in average glutathione levels significantly decreases calcium influx in peripheral blood lymphocytes stimulated with anti-CD3 antibody (77). As discussed later in this review, similarly small changes in redox state can sharply alter the balance between self-renewal and differentiation in dividing O-2A/OPCs (74). This ability of relatively small changes in key components of the redox regulatory network to modulate cell function would enable this fundamental aspect of cellular physiology to function as a highly sensitive central rheostat that integrates cell-intrinsic states with cell-extrinsic signals.

Perhaps most importantly for analyzing the effects of redox state on cell function, the results of our studies on O-2A/OPCs, oligodendrocytes, and neurons demonstrated that altering the redox balance of a cell has opposing effects on different kinds of signaling pathways. Specifically, our collective studies indicate that making cells mildly more *reduced* is associated with a markedly *enhanced* response to inducers of *cell survival and cell division* and with *suppression* of the response to inducers of *differentiation or apoptosis*. In contrast, making cells mildly more *oxidized suppressed* the response to inducers of *cell survival and division*, and *enhanced* the response to inducers of *differentiation or apoptosis*. In the context of cell survival, exposure of oligodendrocytes to NAC or other antioxidants confers protection against the cytotoxic effects of tumor necrosis factor-α and glutamate.

EVIDENCE THAT INTRACELLULAR REDOX STATE IS A PHYSIOLOGICALLY RELEVANT REGULATOR OF PRECURSOR CELL FUNCTION

Continued analysis of the effects of oxidant status on signaling pathways revealed that responsiveness of a variety of cell types to mitogens was enhanced by coexposure to antioxidants. When these studies were conducted on O-2A/OPCs, we further found not only that cell division was enhanced at suboptimal concentrations of PDGF, but also that differentiation appeared to be inhibited. Based on these observations, we initiated a methodical dissection of the contribution of intracellular redox state to precursor cell function (74).

The first experiments indicating that intracellular redox state might be a highly relevant physiological modulator of precursor cell function demonstrated that O-2A/OPC self-renewal *in vitro* could be predicted based on the redox state of these cells at the time they were isolated from the organism. In these experiments, cells were labeled with dihydrotetramethyl-rosamine (DHTM-Rosamine), a dye that becomes fluorescent when it is oxidized in the cytoplasm. At low dye concentrations, the oxidized dye translocates to the mitochondria, but at higher concentrations this oxidized dye appears to be more diffusely distributed in the cell (84). Other dyes can be similarly used, as described (74), but DHTM-Rosamine was initially the only dye we found that had no apparent effects on precursor cell function simply as a consequence of labeling (74). For those interested in conducting similar experiments, our current studies indicate that dihydrocalcein-AM gives results equivalent to those obtained with DHTM-Rosamine, and has the added advantage of providing stable results for at least twice the time period of analysis as is possible with DHTM-Rosamine.

In considering the different dyes that are available for analyzing intracellular redox state, it is important to note that there is no single dye that is completely satisfactory. There are probes that trap or otherwise react with singlet oxygen, hydroxyl radicals, or superoxide, but many of the dyes are quite specific in the species with which they react. Thus, different dyes may be more or less suitable for analysis of different kinds of changes. Dyes can also have other disadvantages. For example, calcein has the disadvantage that it can be quenched by Fe^{3+}, Co^{2+}, Ni^{2+}, and Cu^{2+} at pH 7. Considera-

tions of which dye to use also are driven by whether the labeled cells are going to continue to be studied after the labeling, as in our studies in which cells are purified according to their intracellular redox state (74), in which case it is essential to screen for dyes that do not themselves perturb the function of interest. As new dyes are being introduced at a rapid pace, it is strongly advised that *The Handbook—A Guide to Fluorescent Probes and Labeling Technologies* (from Molecular Probes) and other relevant resources are checked regularly to identify those dyes that may be of the greatest use for particular research efforts, and then to compare several dyes in the assays of interest.

Results obtained following redox-based purification of O-2A/OPCs were striking, and demonstrated a clear correlation between the redox state of a cell at the time of its isolation and its self-renewal properties (74). DHTM-Rosamine^{low} cells (*i.e.,* cells thought to be more reduced *in vivo*) grown in the presence of 10 ng/ml PDGF generated clones in which self-renewing divisions were prevalent. Five days after plating, ~70% of these consisted only of dividing progenitor cells, and the average number of cells per clone was 11. In contrast, DHTM-Rosamine^{high} cells, which would have had a higher intracellular level of oxidizing equivalents *in vivo*, underwent very little division in these same conditions. With rare exceptions, the largest clones consisted of four cells, and only ~30% of clones contained any progenitors at all.

DHTM-Rosamine^{high} cells also exhibited a greatly increased tendency to generate oligodendrocytes. At day 5, approximately half of all the clones derived from DHTM-Rosamine^{high} cells consisted of one oligodendrocyte and no progenitor cells, and nearly 70% of all clones contained at least one oligodendrocyte. Thus, in agreement with results obtained for hematopoietic stem cells (HSCs) and hepatic precursor cells (*e.g.,* 10, 39, 56, 68), DHTM-Rosamine^{low} O-2A/OPCs appeared to be more able to undergo self-renewing divisions than DHTM-Rosamine^{high} cells.

It is important to stress that DHTM-Rosamine^{high} O-2A/OPCs isolated from the P7 rat optic nerve had not already irreversibly committed to differentiation *in vivo*. Exposure of these cells to NAC markedly enhanced the self-renewal of these cells when grown in basal division conditions *in vitro*, suggesting that alteration of intracellular redox state is a dynamic modulator of the balance between self-renewal and differentiation rather than merely being a secondary consequence of an irrevocable fate determination.

Further studies confirmed that the balance between self-renewal and differentiation in dividing O-2A/OPCs could be modulated by manipulation of intracellular redox state. Treatment with pharmacological agents that render cells more oxidized [either *tert*-butyl hydroperoxide, a potent prooxidant (61), or buthionine sulfoximine (BSO), an inhibitor of glutathione biosynthesis (51)] was associated with diminished progenitor cell division and increased oligodendrocyte generation (74). In contrast, exposure of cells to NAC or to polyhydroxyalkyl thiazolidine carboxylic acid pro-cysteine drugs (which also lead to increased intracellular glutathione levels) was associated with enhanced self-renewal and a marked reduction in oligodendrocyte generation in clonal cultures of O-2A/OPCs grown in the presence of PDGF.

REDOX STATE ALTERATION IS A NECESSARY COMPONENT OF THE MODE OF ACTION OF SIGNALING MOLECULES THAT ALTER SELF-RENEWAL PROBABILITIES

Although it is possible that variations in intracellular redox state *in vivo* might be due to cell-intrinsic properties or to direct exposure to pro- or antioxidants, it is also possible that variability is a result of modulation of redox state by exposure to cell-extrinsic signaling molecules. For example, exposure of cells to neurotrophins (25, 71), type 1 interferon (47), stem cell factor (44), transforming growth factor-β (37), inflammatory cytokines (*e.g.,* 30), and TH (62) can alter intracellular redox state. Some of these cytokines, such as the neurotrophins, cause cells to become more reduced. Others, such as transforming growth factor-β and TH, render cells more oxidized. Similar changes in the redox regulatory network can be achieved by direct activation of ras, a key component of several cytokine-induced signaling pathways (43).

Although prior studies had not revealed an obvious organizing principle distinguishing between cell-signaling molecules that made cells more oxidized and those that made cells more reduced, it became apparent in our studies that these effects could be distinguished based on whether the signaling molecules promoted division/survival or differentiation (74). Growth of O-2A/OPCs in the presence of PDGF supplemented with factors that promote self-renewal (*e.g.,* NT-3 or FGF-2) was associated with a more reduced intracellular redox state, as indicated by DHTM-Rosamine fluorescence, whereas supplementation with inducers of differentiation [*i.e.,* TH or bone morphogenetic protein-4 (BMP-4)] was associated with increased DHTM-Rosamine fluorescence, indicating an increase in oxidative levels. Such effects were seen within 18 h of exposure to the compounds of interest, well before it is possible to observe any effects on division or differentiation.

Consistent with our analyses and those of others demonstrating that small alterations in redox balance can be associated with marked alterations in cellular function, the effects of extracellular signaling molecules on redox state were not large (74). For example, after exposure of cells to NT-3 + PDGF for 12 h, aggregate DHTM-Rosamine fluorescence was ~15% lower than in the presence of PDGF alone, a change comparable to the effects of exposure to 1 m*M* NAC. By 18 h after exposure to NT-3, relative fluorescence was nearly 30% lower than for control cells. Similar results were associated with exposure to FGF-2. In contrast, exposure to TH, which induces oligodendrocyte generation, had the opposite effect to NT-3 and was associated with greater DHTM-Rosamine fluorescence, indicating an increase in oxidative levels. A similar increase in average oxidative activation of DHTM-Rosamine was also associated with exposure to BMP-4, even though this protein causes O-2A/OPCs to differentiate into type-2 astrocytes (49) and acts through receptor and signaling systems very different from those relevant to TH action. Further demonstration of the effects on redox state of NT-3 and TH, the two signaling molecules for

which the best evidence for an *in vivo* role has been provided (2, 8, 35), was obtained by analysis of cells with the JC-1 dye, which provides information on mitochondrial inner membrane potential ($\Delta\Psi$). Treatment with TH for 18 h resulted in an ~18% decrease in $\Delta\Psi$ (aggregate red:green fluorescence), indicative of a more oxidative state within the cells (74). In contrast, treatment with NT-3 for 18 h was associated with an ~16% increase in $\Delta\Psi$, indicative of a more reduced state within the cells. Despite the magnitude of all of the changes observed, they were all highly reproducible and statistically significant.

Despite the fact that the changes in redox levels (as determined by fluorescence analysis) appeared to be small, such changes seem to be necessary to the action of TH and NT-3. For example, if cells were grown in conditions where the oxidizing effect of TH exposure was antagonized with a reducing agent like NAC, cells exhibited a profile of self-renewal similar to that of cells grown in the presence of PDGF alone. Thus, NAC countered the effects of TH. This effect was also seen at the level of redox analysis; after 12 h in the presence of NAC + TH + PDGF, cells displayed mean DHTM-Rosamine fluorescence no different from control cells, compared with the ~23% increase observed for cells grown in just TH + PDGF. In complementary experiments, exposure of cells to PDGF + 1 μ*M* BSO inhibited the ability of NT-3 to enhance self-renewal and eliminated the ability of NT-3 to make cells more reduced.

The above results indicate strongly that the redox alterations caused by NT-3 and TH are essential to the mechanism by which cell-signaling molecules modulate the balance between self-renewal and differentiation in O-2A/OPCs (60, 74). The ability of NAC to protect against oxidative agents suggests that the increased intracellular oxidation associated with exposure to TH may be a necessary aspect of the mechanism by which TH enhances oligodendrocyte generation. Similarly, as BSO inhibits glutathione production, thus making cells more oxidized, our results suggest that the ability of NT-3 to render cells more reduced is necessary for this signaling molecule to promote self-renewal.

ANALYSIS OF THE BALANCE BETWEEN SELF-RENEWAL AND DIFFERENTIATION IN O-2A/OPCS FROM DIVERSE CNS REGIONS REVEALS REDOX-ASSOCIATED CELL-INTRINSIC DIFFERENCES THAT CORRELATE WITH THE TIMING OF MYELINATION IN THESE REGIONS

A further indication that regulation of intracellular redox state plays a key role in the regulation of precursor cell function is provided by studies on O-2A/OPCs isolated from different regions of the developing CNS. The qualifiers CX, OC, and ON are used throughout the following discussion to refer to O-2A/OPC populations isolated from cortex, optic chiasm, and optic nerve, respectively.

Our interest in comparing O-2A/OPCs from different regions stemmed from attempts to understand why different regions of the CNS develop according to different schedules, with great variations seen in the timing of both neurogenesis and gliogenesis. For example, neuron production in the rat spinal cord is largely complete by the time of birth, is still ongoing in the rat cerebellum for at least several days after birth, and continues in the olfactory system and in some regions of the hippocampus of multiple species throughout life. Similarly, myelination has long been known to progress in a rostral–caudal direction, beginning in the spinal cord significantly earlier than in the brain (*e.g.,* 23, 40, 50). The cortex itself shows the widest range of timing for myelination, both initiating later than many other CNS regions (*e.g.,* 23, 40, 50) and exhibiting an ongoing myelinogenesis that can extend over long periods of time. This latter characteristic is seen perhaps most dramatically in the human brain, for which it has been suggested that myelination may not be complete until after several decades of life (9, 86).

Our first clue that the differing temporal development of various CNS regions might be due to cell-intrinsic differences in the precursor cells present in these regions came with the demonstration of striking dissimilarities in the *in vitro* behavior of O-2A/OPCs isolated from optic nerve, optic chiasm, and cortex of P7 rats and grown in basal division conditions (*i.e.,* PDGF as the sole mitogen applied).

A subset of the results obtained in these studies (63) is discussed here to illustrate the extent of differences we observed and to enable discussion of the conclusions drawn from these studies.

We observed that the extent of oligodendrocyte generation in clones of ON cells grown in basal division conditions was much greater than that seen for OC progenitors and even more dramatically different from CX progenitors. For example, nearly 60% of clones of ON O-2A/OPCs contained at least one oligodendrocyte after 3 days of *in vitro* growth in these conditions, and this proportion increased to ~87% after 6 days of *in vitro* growth. In contrast, after 3 days of *in vitro* growth of OC cells, only ~26% of clones contained oligodendrocytes, less than half the value obtained for ON cells. Even after 6 days of *in vitro* growth, only ~40% of OC-derived clones contained oligodendrocytes. This relative absence of oligodendrocyte generation at the clonal level was associated with a greatly reduced contribution of oligodendrocytes to the total culture of OC as compared with ON progenitors.

Self-renewal was even more enhanced, and generation of oligodendrocytes even more reduced, in populations of CX cells than in cells derived from either the optic nerve or optic chiasm. In clones of CX cells grown in basal division conditions, only ~3% of clones contained at least one oligodendrocyte after 3 days of *in vitro* growth, and this proportion increased only to ~6% after 7 days of *in vitro* growth. Even after 10 days of *in vitro* growth, <20% of CX clones grown in PDGF contained one or more oligodendrocytes. Moreover, the overall percentage of oligodendrocytes in O-2A/OPC(CX) cultures remained <2% on days 3, 7, and 10 in these basal division conditions. Even in those clones that did contain oligodendrocytes, the proportion of these cells was still low, and these clones rarely contained more than one or two oligodendrocytes regardless of the number of O-2A/OPCs found within the clone. The proportionate representation of oligodendrocytes seen in these cultures was also lower than for OC

or ON cultures. For example, in cultures of O-2A/OPCs from CX, <2% of cells were oligodendrocytes even as late as 10 days in culture. In ON-derived cultures, in contrast, more than half of all cells were oligodendrocytes on day 6. These differences between CX, OC, and ON cells seem to be cell-intrinsic, as shown by experiments in which cells from one region were grown in medium conditioned by cells from another region. For example, conditioned medium from optic nerve cells did not cause premature differentiation of cortex-derived cells. Nor did conditioned medium from cortex-derived cells cause extended proliferation of O-2A/OPCs derived from the optic nerve.

In addition to differences in their basal self-renewal tendencies, O-2A/OPCs derived from different regions of the CNS also showed striking differences in their responses to inducers of oligodendrocyte generation. In the optic nerve, both TH and CNTF (7, 35, 52) are effective inducers of oligodendrocyte generation. For both OC and CX, exposure to either of these agents was a less effective inducer of this differentiation pathway (63). For example, after 7 days of growth in the presence of TH only, ~5% of CX cells were oligodendrocytes, as compared with >80% of cells in ON cultures. Similar results were obtained with CNTF, which was even less effective at inducing oligodendrocyte generation in cultures of cortex-derived O-2A/OPCs than was TH.

The biological differences between O-2A/OPCs from optic nerve, optic chiasm, and cortex bear a striking resemblance to the differences we found to be associated with the redox status of optic nerve-derived O-2A/OPCs. As discussed earlier, in our studies on optic nerve-derived cells, we found that freshly isolated O-2A/OPCs that possessed a relatively reduced intracellular environment were most likely to undergo continued self-renewing divisions when grown in the presence of PDGF. Moreover, we found that pharmacological manipulation to make cells more reduced antagonized the differentiation-inducing abilities of TH.

Experimental analysis confirmed the hypothesis that O-2A/OPCs isolated from optic nerve, optic chiasm, and cortex expressed different intracellular redox states that were consistent with biological properties expressed by these cells. We found that, at the time of their isolation from the animal, those cells that undergo the most self-renewal [*i.e.,* O-2A/OPCs(CX)] were the most reduced, those that undergo the least self-renewal [*i.e.,* O-2A/OPCs(ON)] were the most oxidized, and O-2A/OPCs(OC) were intermediate with respect to both self-renewal and intracellular redox state.

THE CASE FOR INTRACELLULAR REDOX STATE AS A CENTRAL INTEGRATOR OF O-2A/OPC FUNCTION

The results of our developmental studies on O-2A/OPCs point to a remarkable association between redox regulation and precursor cell function. In the work discussed thus far, we showed that intracellular redox state modulation appears to be a central biochemical/molecular regulator of the balance between self-renewal and differentiation. In particular, redox state modulation satisfies all of the following criteria required to support such a conclusion: (a) The proposed regulator should be altered in its level and/or function by cell-extrinsic signaling molecules that modulate the balance between self-renewal and differentiation, with signaling molecules that have opposite effects on self-renewal and differentiation exerting opposite effects on the proposed regulator. (b) Alterations like those caused by exposure to these signaling molecules should have the same effect as the signaling molecules themselves. (c) Substances that antagonize the alterations in the regulator caused by the cell-extrinsic signaling molecules should block their effects on this balance. (d) Progenitor cell populations isolated from developing animals on the basis of the state of the proposed regulator should exhibit predictably different self-renewal characteristics consistent with the outcome of the other analyses.

The potential importance of intracellular redox state in modulating the specifics of precursor cell function *in vivo* was highlighted by studies asking whether differing times of myelination in different regions of the CNS might be in any way associated with the properties of the resident precursor cell populations. The redox state of O-2A/OPCs from different CNS regions showed a striking correlation with the properties expressed by cells of the optic nerve in which redox state was experimentally manipulated. In particular, those cells with the greatest self-renewal potential (*i.e.,* cortical O-2A/OPCs), and the least response to inducers of oligodendrocyte generation, exhibited the most reduced redox state when examined as freshly isolated cells. The characteristics of these populations are such as to be consistent with the hypothesis that differences in the timing of myelinogenesis may be due, at least in part, to the local utilization of oligodendrocyte precursor cell populations with fundamentally different properties. Our results raise the intriguing possibility that at least one of the cell-intrinsic properties that regulate self-renewal and responsiveness to environmental factors is intracellular redox state (Fig. 1).

WHAT NEXT? THOUGHTS ON IMPLICATIONS OF OUR FINDINGS AND FURTHER CHALLENGES TO BE ADDRESSED

We are very much aware, from the vantage point of traditional cell biology, that our studies on the control of the cellular functions most central to the biological purposes of a precursor cell have entered a realm very different from that inhabited by most precursor cell biologists. In striking contrast with the many excellent studies in which specific transcriptional regulators have been identified as vital control elements in precursor cell function, our studies have demonstrated an equally important role of metabolic regulation. The multiple surprises emerging from our studies raise a number of questions of potential interest. We will close this review by focusing on several of these, including the intriguing paradox that arises when our results are compared with results obtained by studying other cell populations.

On a general level, our studies indicate the need to incorporate an understanding of precursor cell developmental

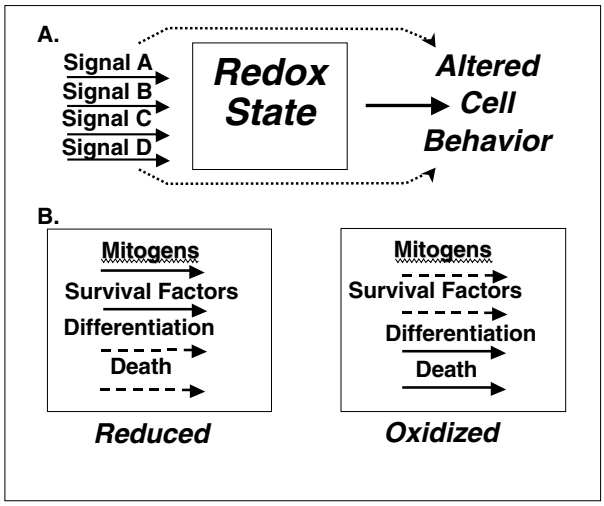

FIG. 1. Summary of the role of redox state in the function of O-2A/OPCs and oligodendrocytes. (**A**) Cell-extrinsic signaling molecules activate signaling systems, leading to alterations in intracellular redox state. Although activation of signaling systems obviously does more than simply alter redox state (indicated by dotted lines), our data indicate that the redox changes are necessary for the signaling systems to enhance self-renewal in dividing cells, to promote differentiation, or to induce cell death. Moreover, changing the redox state in dividing O-2A/OPCs is sufficient to alter cell behavior, to promote self-renewal (with reducing agents) or to promote oligodendrocyte generation (with oxidizing agents). (**B**) Signaling pathways are differentially modified in their efficacy by intracellular redox state. For O-2A/OPCs, cells that are slightly more reduced are more responsive to mitogens (or, for oligodendrocytes, to survival factors), but are less responsive to inducers of differentiation or death. Cells that are slightly more oxidized are less responsive to mitogens or survival factors, but more responsive to inducers of death or differentiation.

stages in designing and interpreting studies on the function of signaling molecules. For example, had we originally studied the effects of TH on cortical O-2A/OPCs rather than optic nerve O-2A/OPCs, we (and others) would not have observed the striking effects that have led to potentially important insights on the biology of development and developmental maladies. We believe a principal reason why studies in the optic nerve have been so successful in analyzing differentiation-related problems is that these cells are generally isolated at a time (P7) when they are physiologically poised to be maximally responsive to environmental signals. It is at 7 days after birth that myelination in the rat optic nerve is moving into its peak phase. Thus, these cells are isolated at a time when their normal function *in vivo* will be to generate oligodendrocytes. Within the highly defined architecture of the CNS, however, it is particularly important to generate the correct cell in the correct place at the correct time. In the context of myelination, there is no reason to generate an oligodendrocyte unless there is a length of unmyelinated axon for it to enwrap, creating the need for a high degree of sensitivity to the immediate microenvironment. Thus, isolating O-2A/OPCs from a tissue such as the optic nerve, at a time when myelination is about to commence, may simply provide cells that are physiologically poised to be particularly responsive to exogenous signals and thereby provide a particularly tractable population for experimental analysis. In contrast, in tissues (such as the cortex) in which myelination occurs over a much longer time period, and within a much larger volume of tissue, it seems very important to have progenitor cells that are less likely to cease proliferation early and differentiate into nondividing oligodendrocytes as such an outcome would be at odds with the goal of enabling the cortex to develop over a longer time frame.

The observation that the intracellular redox state of freshly isolated precursor cells is highly correlated with their self-renewal characteristics and responses to signaling molecules is somewhat surprising, but even more surprising are our observations that differences between different O-2A/OPC populations are maintained for extended periods even when cells are grown in ambient oxygen concentrations. Indeed, even after 6 weeks of growth of P7 cortex-derived O-2A/OPCs in standard *in vitro* growth conditions of atmospheric oxygen, these cells undergo much more self-renewal and less differentiation than do O-2A/OPCs freshly isolated from the P7 optic nerve. Thus, the redox properties of these cells are both robust and heritable.

We are far from understanding the molecular basis of the differences between O-2A/OPCs from different CNS regions, or the means by which the critical features of cortical O-2A/OPCs are maintained for extended periods of *in vitro* growth. A large number of mechanisms may prove relevant, including differences in mitochondrial function, expression and activity of redox-related enzymatic activities, iron uptake, and peroxisomal function. Our initial studies on this problem are revealing multiple differences between cortical and optic nerve populations, but the mechanistic importance of any of these differences is yet to be determined.

That the developmental stage of a tissue is associated with critical and heritable constraints on precursor cell function, and these constraints seem to be intimately associated with intracellular redox state, seems quite remarkable. How does the redox state of a cell provide the specificity to regulate such processes? There are multiple ways to think about this problem. For example, Nathan has recently suggested (58) that to understand the participation of reactive oxygen intermediates, and also reactive nitrogen intermediates, in specific signaling processes, one needs to recognize that intracellular signaling requires several types of specificity. Some of this specificity may actually lie in the ability of certain chemical species to regulate cellular pathways that are physically noncontiguous with each other. Moreover, any given mediator may participate in multiple reactions that represent different specificities. From this point of view, it is important to think of intracellular redox state in a very different manner than that applied to the analysis of linearly connected signaling arrays.

It is also important to point out that very little is known about the dynamics of redox interactions within a cell as this might apply to the modification of biological processes. Although there may be instances where it is the overall balance between reducing and oxidizing equivalents that is somehow sensed and that leads to changes in cellular function, it seems increasingly likely that there are other processes where changes in levels of reduced thiols, or thioredoxin status, or the presence of particular reactive oxidative species in particu-

lar subcellular locations are what is most important. An optimistic view is that the better we comprehend the biochemical pathways that are activated or suppressed by changes in intracellular redox state, and the better we are able to define changes in particular subcomponents of redox state, the more we will be able to recognize specific components that are crucial in particular situations. A pessimistic view (from the vantage point of the scientific desire to dissect complexity) would be that the redox regulatory system is so interwoven that a change in any component of the system leads to immediate reverberations throughout the system, with it being thus difficult to isolate different components as controllable variables.

THE CENTRAL PARADOX OF REDOX SIGNALING STUDIES: EVIDENCE FOR TWO PROGRAMS OF REDOX REGULATION OF SIGNALING SYSTEM FUNCTION THAT ACT IN OPPOSITE DIRECTIONS

Although the above ideas may be helpful in understanding the relationship between intracellular redox state and functioning of cellular signaling systems, they do not help to explain a most confusing paradox emerging from our results—this being that they are exactly the opposite of what would be predicted from multiple publications on the effects of oxidants on signaling systems. Multiple laboratories have found that making cells more oxidized increases the efficacy of mitogen signaling pathways, including receptor tyrosine kinase-based pathways, and have documented effects of prooxidants that are opposite in direction from what we have observed in our studies on glial precursor cells. For example, it has been suggested for lung cells that hydrogen peroxide (H_2O_2) induces phosphorylation of the epidermal growth factor (EGF) receptor, albeit only at a subset of the amino acids phosphorylated following EGF exposure (27). The idea that oxidative stress might be associated with increasing phosphorylation in signaling pathways critical in promoting cell division is consistent with a variety of observations that protein tyrosine phosphatases have a reactive cysteine in their active site, and oxidant stress can reversibly inactivate cellular tyrosine phosphatase activity (*e.g.,* 21, 46). A general inactivation of phosphatases would be expected to be associated with increased phosphorylation of their target proteins. Such results are also consistent with observations that increasing catalase levels [which would reduce levels of reactive oxygen species (ROS)] inhibits the ability of PDGF and EGF to stimulate extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in vascular smooth muscle cells and fibroblasts (5, 78). Similarly, 5-hydroxytryptamine exposure induces ROS generation in mesangial cells, fibroblasts, and smooth muscle cells that, in all cases, induces ERK activation (28, 45). The role of ROS in promoting cell-cycle progression has been well reviewed (14).

Consistent with observations of enhanced activation of proteins associated with proliferation, such as ERK1/2, multiple studies have also shown that ROS production can enhance cell division—a result that is the opposite of our own. For example, the ability of ROS production to enhance DNA synthesis and stimulate cell proliferation has been reported for vascular smooth muscle cells (22, 67) and for V79 cell Chinese hamster lung fibroblast cells (31). Within the CNS, Fike and colleagues found that neural precursor cells isolated from the hippocampus divided more in growth conditions where they were relatively more oxidized, and that treating cells with α-lipoic acid (which made them more reduced) was associated with a reduction of division (48). Similarly, in the hematopoietic system, Spangrude and colleagues (39) observed that cells with the antigenic phenotype of HSCs could be subdivided further by dyes that provide readouts of mitochondrial activation status. Those HSCs that were less brightly labeled, indicative of a lesser degree of mitochondrial activation, were more able than their brightly labeled counterparts to partake in reconstitution of the hematopoietic system following transplantation. That is to say, the less brightly labeled cells, which would be expected to be less oxidized, exhibited greater self-renewal activity. However, these less brightly labeled cells also appeared to be proliferatively quiescent. In contrast, the more brightly labeled cells appeared to be activated progenitor cells, engaging in a much more active proliferation than the dimly labeled quiescent stem cells. In sum, these multiple studies contrast with our own in indicating that being more reduced is associated with a lesser degree of cell division [although it should be noted that the results of the studies of Kim *et al.* (39) are in agreement with our conclusions (60, 63, 74) that more reduced cells have a greater self-renewal potential and that oxidation promotes differentiation (in the case of HSCs, from a quiescent stem cell to an activated transit amplifying cell)].

As our own results are very consistent in multiple experimental paradigms, and our ongoing signaling studies demonstrate that prooxidants reduce signaling intensity at every stage of signaling from the PDGF-α receptor in O-2A/OPCs (unpublished observations), it seems clear that are there two very different cellular growth control programs that need to be understood in the context of redox regulation. In one of these programs, making cells more oxidized reduces proliferation and induces terminal differentiation (including, as discussed later, the terminally differentiated phenotype of cellular senescence in fibroblasts). A similar program may also exist in T-cells, where it appears that ROS production negatively regulates ERK1/2 phosphorylation (41). In these cells, stimulation of the T-cell antigenic receptor (TcR) with anti-CD3 antibodies causes a rapid generation of ROS, which seems to feed back negatively to dampen antigen-mediated ERK activation. Interestingly, a rapid event in anti-CD3 stimulation of mature T-cells is growth arrest (4), suggesting that the oxidant-mediated inhibition of ERK signal may promote cell-cycle arrest in TcR-stimulated cells. In contrast, in the second program, making cells more oxidized is instead interpreted as a division-promoting signal.

Two likely contributors to the ability of an increased oxidative state in some instances to enhance signaling and promote cell division, and in other instances to reduce signaling and suppress cell division, are distinct signal-pathway and cell-type specificities. Both of these factors seem likely to be of importance. Activation of ERK1/2 by ROS does seem to be cell type-specific, with only some cell types exhibiting increased ERK1/2 phosporylation in response to becoming more oxidized (1, 3, 24, 29, 75, 83). But there are also path-

way-specific effects. For example, although H_2O_2 exposure enhances phosphorylation in human neuroblastoma SH-SY5Y cells stimulated by EGF, it impairs phosphorylation induced by carbachol stimulation of muscarinic receptors in these same cells (38). It also has been suggested that the length of the ROS signal determines whether the outcome is pro- or antiproliferative (14).

It also seems clear that a particular cell type can switch its program of response to becoming more oxidized, demonstrating that it is not simply the lineage of a cell that determines its response to redox changes. That such is the case has been demonstrated in studies on oncogene function.

It has been known for some time that expression of at least some immortalizing genes (*i.e.,* those oncogenes that confer on cells the ability to continue to divide indefinitely) can change the way in which a particular cell type responds to a given stimulus. When a constitutively active mutant ras allele is introduced into a cell that has been immortalized (for example, by expression of v-myc or SV40 large T antigen), the cell becomes fully transformed, able to grow in an anchorage-independent manner and without exogenously supplied mitogens (42). In contrast, when introduced into a normal cell, the identical ras protein causes the cell to undergo growth arrest (70). This observation was originally made in Schwann cells, the myelin-forming cells of the peripheral nervous system, and subsequent studies obtained comparable results with fibroblasts (33, 72).

Studies on normal and immortalized cells have shown that expression of an immortalizing gene can cause a reversal in the response to ROS like the reversal in the response to ras—and further indicate that the differential effects of ras may be related to this reversal in the response to oxidative stress. Normal fibroblasts respond to oxidative stress, or to expression of a constitutively active ras, by dropping permanently out of cell division and becoming senescent (15, 16, 34). The ability of ras to induce senescence in normal fibroblasts appears to be dependent on the production of oxidative stress, as growth of ras-expressing cells in the presence of NAC, or at oxygen concentrations of 1%, blocks the induction of premature senescence in these cells (43). In contrast, in NIH 3T3 fibroblasts (which are a spontaneously immortalized cell line), expression of a constitutively active H-ras allele promotes cell division, similar to what occurs in Schwann cells expressing an immortalizing gene. Moreover, in NIH 3T3 cells expressing ras, this expression still leads to increased levels of ROS production, but in this case the ROS production is essential to the ability of ras to promote cell division (36). In these cells, application of NAC significantly inhibits the growth-promoting activity of the mutant ras. Thus, normal fibroblasts interpret constitutively active ras and ROS production as a signal to become senescent, whereas immortalized cells respond to these same signals by having their proliferation enhanced.

How does a cell interpret the same data in different ways and why do these opposite programs exist? There is currently insufficient information to answer the former question, and only vague grounds for speculation on some aspects of the latter. One possibility that seems interesting enough not to dismiss outright is that one program might represent a developmental program and the other a tissue-injury program. It is clear that there are many cell types in the body for which part of their physiological function is to respond to injury by increasing their division, and injury is generally associated with oxidative stress. In this context, oxidative stress might be a signal for stem cell populations to generate the more rapidly dividing transit amplifying populations that are the intermediate step between a stem cell and the generation of differentiated cell types. Such a hypothesis is consistent with the increased division that occurs in the hippocampus dentate subgranular zone after a single treatment with radiation [which causes oxidative stress (48)]. It is also consistent with the observations of Kim *et al.* (39) that a relatively more oxidized status distinguishes quiescent HSCs from activated cells that are more engaged in extensive proliferation. Such a view may also have value in the context of understanding cancer cells, for a tumor represents a site of continuous tissue injury. There seems an obvious advantage to a cancer cell to activate a developmental program specifically associated with extensive cell division in the context of tissue injury. From the point of view of our studies on glial precursor cells, it is also of more than passing interest that, for a stem cell, the generation of a transit amplifying population represents a differentiation event, which would be consistent with our studies indicating that making a cell more oxidized tends to induce differentiation. We hasten to add, however, that these ideas represent only suggestions for how to begin thinking about this paradox from the point of view of developmental biology. The understanding that eventually will be achieved of this critical biological problem will certainly be more complex.

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ABBREVIATIONS

BMP-4, bone morphogenetic protein-4; BSO, buthionine sulfoximine; CNS, central nervous system; CNTF, ciliary neurotrophic factor; CX, cortex; DHTM-Rosamine, dihydrotetramethyl-rosamine; E, embryonic day; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; H_2O_2, hydrogen peroxide; HSC, hematopoietic stem cell; NAC, *N*-acetyl-L-cysteine; NT-3, neurotrophin-3; O-2A/OPC, oligodendrocyte–type-2 astrocyte/oligodendrocyte precursor cell; OC, optic chiasm; ON, optic nerve; P7, postnatal day 7; PDGF, platelet-derived growth factor; ROS, reactive oxidative species; TcR, T-cell antigenic receptor; TH, thyroid hormone; $\Delta\Psi$, mitochondrial inner membrane potential.

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Address reprint requests to:
Mark Noble, Ph.D.
Department of Biomedical Genetics
University of Rochester Medical Center
601 Elmwood Avenue, Box 633
Rochester, NY 14642

E-mail: mark_noble@urmc.rochester.edu

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