Developmental Lag in Superoxide Dismutases Relative to Other Antioxidant Enzymes in Premyelinated Human Telencephalic White Matter

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Abstract. Periventricular leucomalacia (PVL) involves free radical injury to developing oligodendrocytes (OLs), resulting from ischemia/reperfusion, particularly between 24 and 32 gestational weeks. Using immunocytochemistry and Western blots, we tested the hypothesis that this vulnerability to free radical toxicity results, in part, from developmental lack of superoxide dismutases (SOD)-1 and -2, catalase, and glutathione peroxidase (GPx) in the telencephalic white matter of the human fetus. During the period of greatest PVL risk and through term (≥37 weeks), expression of both SODs (for conversion of O$_2^-$ to H$_2$O$_2$) significantly lagged behind that of catalase and GPx (for breakdown of H$_2$O$_2$), which, in contrast, superseded adult levels by 30 gestational weeks. Our data indicate that a developmental “mismatch” in the sequential antioxidant enzyme cascade likely contributes to the vulnerability to free radical toxicity of the immature cerebral white matter, which is “unprepared” for the transition from a hypoxic intrauterine to an oxygen-rich postnatal environment. All enzymes, localized to astrocytes and OLs, had higher-than-adult expression at 2 to 5 postnatal months (peak of myelin sheath synthesis), suggesting an adaptive mechanism to protect against lipid peroxidation during myelin sheath (lipid) synthesis. The previously unrecognized dissociation between the expression of the SODs and that of catalase and GPx in the fetal period has potential implications for future antioxidant therapy in PVL.

Key Words: Antioxidant; Human brain; Myelination; Oligodendrocyte; Periventricular leucomalacia; Prematurity.

INTRODUCTION

About 55,000 premature infants (<37 gestational weeks) are born in the United States each year (1). With modern neonatal intensive care, nearly 90% of these infants survive, although 10% go on to develop cerebral palsy. Between 25% and 50% of these premature infants will also develop cognitive and behavioral deficits. Thus, 5,000 new cases of cerebral palsy and 10,000 to 20,000 children with serious learning disabilities result yearly. Despite the magnitude of the problem, there are no clinical therapies for the brain injury leading to cerebral palsy, other than the prevention of prematurity itself. The substrate for cerebral palsy is periventricular leucomalacia (PVL), which refers to focal necrosis in deep white matter (WM) in the cerebral hemispheres associated with WM gliosis, occurring in the setting of lung immaturity, blood pressure lability, and impaired cerebrovascular autoregulation, which leads in turn to cerebral ischemia/reperfusion (2). Intrauterine infection and the fetal inflammatory response may be contributory in certain cases (3–6).

We recently demonstrated that nitrative and oxidative injury to developing oligodendrocytes (OLs) plays a major role in PVL (7). The age at which premature infants are at greatest risk for PVL (24 to 32 gestational weeks) coincides with the time interval when premyelinating (O$_4^+$ and O$_1^+$) OLs, especially O$_4^+$/O$_1^+$ cells, preferentially populate the telencephalic WM (8). Thus, we postulate that these cells in particular are susceptible to free radical injury and death (2, 9, 10). This susceptibility has been demonstrated in rat OL cultures, in which premyelinating but not mature myelin basic protein (MBP)-positive OLs are particularly vulnerable to glutathione deprivation (10). The transition of the developing human brain from a relatively hypoxic environment in utero to one rich in oxygen in postnatal life requires upregulation of antioxidant enzymes (AOEs) to handle the increased reactive oxygen species (ROS) (11–13). By inference, AOE expression is likely to be increased during synthesis of lipid-rich myelin sheaths to protect against accelerated lipid peroxidation due to superoxide-generating mitochondrial activity (14–16). The main AOE are copper-zinc-containing and manganese-containing superoxide dismutases (CuZnSOD or SOD-1, and MnSOD or SOD-2, respectively; E.C. 1.15.1.11), catalase (E.C. 1.11.1.6), and glutathione peroxidase (GPx; E.C. 1.11.1.9). The SODs are required for the initial dismutation of superoxide anion (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$), which, in turn, breaks down into oxygen and water by the action of catalase and GPx. Thus, all 4 enzymes are considered essential for the sequential catalysis of ROS (11, 17).

In the following study, we hypothesized that a developmental lack of antioxidant capacity exists before birth,
and underlies, at least in part, the OL maturation-specific vulnerability to free radical injury of the cerebral WM of the human premature infant, particularly during the period of highest PVL risk. We further hypothesized that enzyme expression progressively increases over the last part of gestation in preparation for birth and the transition of the fetus to an aerobic environment. We postulated that the temporal increases of the 4 key AOE s occur in parallel because of the need for the complete, sequential degradation of ROS. Finally, we hypothesized that AOE expression continues to increase postnatally, during the most active and dramatic period of myelin sheath (lipid) synthesis in the telencephalic WM.

MATERIALS AND METHODS

Study Population

For Western blots and immunocytochemistry (ICC), 42 brain specimens were collected prospectively from the fetal and pediatric autopsy populations of the Brigham and Women’s Hospital, Children’s Hospital, Boston, and University of Maryland Brain Tissue Bank for Developmental Disorders. Adult WM from 3 non-neurologic patients, aged 55 to 75 years, autopsied at the Brigham and Women’s Hospital, was pooled for Western blot standards. The approval of the hospitals’ Institutional Review Boards was obtained for all studies undertaken. Gestational ages were determined on the basis of foot length. At autopsy, the parieto-occipital poles (the site at which the sequence of OL maturation has been defined (8), and for which PVL has a predilection) were removed from 29 cases without PVL, blocks of parietal lobe WM and overlying cortex at the level of the atrium were immersed in freshly prepared 4% paraformaldehyde (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS; Ambion, Austin TX) for ICC, and were also paraffin-embedded for histopathologic assessment; 5 of these blocks were taken from cases that also had frozen samples. Standard neuropathologic evaluations (i.e. gross and microscopic examination of cerebral cortex, WM, diencephalon, hippocampus, brainstem, and cerebellum) were performed in each case.

Western Blot Analysis of AOE and Mitochondrial Protein Expression

Fresh-frozen WM samples (n = 29) were prepared as previously described (18, 19). Immunostaining of blots was performed with antibodies to the 4 AOE s and to the mitochondrial proteins cytochrome c oxidase (subunit I) and mitochondrial protein AB2, which recognizes a 60-kDa non-glycosylated protein component of human mitochondria (Table). Staining with the latter 2 antibodies was performed to determine a relationship (if any) between tissue levels of MnSOD and of proteins specific for mitochondria, in which the enzyme is localized along the inner membrane. For primary antibodies, the buffer was PBS containing 5% normal goat serum (Vector Laboratories, Burlingame, CA) /3% BSA/0.05% Triton X-100, while for the secondary antibodies, PBS containing 0.05% Triton X-100 was used. Immunostaining controls were achieved with omission of primary or secondary antibodies, and, for CuZnSOD, GPx, and catalase, preabsorption with the intact proteins (all from Sigma, St. Louis, MO). Although we were unable to perform preabsorption with the anti-MnSOD used in our study (the immunogenic protein being unavailable due to proprietary reasons), our Western blots demonstrated a single band of 24 kD, which corresponds to the known molecular weight of the enzyme; there was virtually no nonspecific background staining (see Results). Preabsorption with GPx, catalase, and CuZnSOD proteins blocked all immunostaining with the respective antibodies (data not shown). Detection of labeled bands for MnSOD, GPx, catalase, cytochrome c oxidase, and mitochondrial

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<table>
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<th>Antibody</th>
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Abbreviations: Poly = polyclonal; mono = monoclonal; dilutions are those used for double-label ICC, unless otherwise indicated; SOD = superoxide dismutase; GFAP = glial fibrillary acidic protein.
protein AB2 was attained by chemiluminescent methods (Western Lightning Chemiluminescence Reagent Plus, Perkin Elmer, Wellesley, MA), using digital imagery by Kodak Image Station 440CF. Kodak ID3.5 (Eastman Kodak, Rochester, NY), with quantitation performed according to established protocols (20), using a Bio-Rad QuantityOne apparatus. For CuZnSOD, detection was performed by densitometry of bands revealed after autoradiography, using standard techniques in our laboratory (21). Values obtained from densitometry of bands on all gels (analyzed for each AOE) were normalized to the adult standard (expressed as the percent of the adult standard level), and plotted as a function of postconceptional age (gestational age plus postnatal age).

**Immunostaining**

The technique for ICC follows that previously published by us (7). Briefly, blocks (1 to 1.2 cm diameter) of periventricular WM and overlying cortex were cut on a vibratome (Leica VTS-1000, Leica Microsystems AG, Wetzlar, Germany) at 40 to 50 μm as free-floating sections, incubated in the primary antibody (all antibodies listed in the Table), and subject to detection using established avidin-biotin complex methods (kit from Vector Laboratories). The EnVision detection system (Dako, Carpin-teria, CA) was also employed by placing free-floating or mounted, air-dried sections in the species-specific secondary polymer solution for 30 to 60 min at room temperature with agitation. Sections were developed using 3,3′-diaminobenzidine (DAB; Sigma), mounted on charged slides and coverslipped with permanent mounting medium. Negative control slides were achieved as outlined above for Western blots. Preabsorption with GPx, catalase, and CuZnSOD proteins blocked all immunostaining with the respective antibodies on ICC (data not shown). Between 8 and 12 cases aged 20 to 49 postconceptional weeks were single labeled for each AOE.

For double-label ICC of sections with selected OL markers and MnSOD, CuZnSOD, or catalase, a 2-step procedure was required (modified from the procedure of Back et al [8], as previously described [7]). After blocking, tissues were incubated in primary antibody to O4 or O1 (Table), washed, and incubated with appropriate fluorescent secondary antibody (Molecular Probes, Eugene, OR). Sections labeled with O4 or O1 were then incubated with the desired AOE antibody (Table), using techniques previously described (7). For double labeling of enzymes with antibodies other than O1 or O4, co-incubation was acceptable (Table). After labeling with appropriate secondary fluorochrome probes (Molecular Probes), sections were mounted on charged slides and coverslipped using Fluormount-G (Southern Biotechnology, Birmingham, AL). Because of cross-reactivity between commercially available primary and secondary fluorescent antibody isotypes, double labeling of GPx with OL markers was not successful. Slides were viewed using epifluorescence microscopy on a Nikon Eclipse 800 microscope (Nikon, Melville, NY) with image capture using Spot Software (Diagnostic Instruments Inc., Sterling Heights, MI). Between 4 and 11 cases aged 20 to 49 postconceptional weeks were double stained for each AOE.

**Quantitation of Single-Labeled Cells in Tissue Sections**

Immunopositive cells for CuZnSOD and glutathione peroxidase in the parietal WM were identified as cytoplasmic staining, whereas for MnSOD, positivity was noted as perikaryal clusters of punctuate cytoplasmic staining, presumably representing mitochondrial localization. For catalase, a less distinct, very fine punctate pattern corresponded to localization in peroxisomes. Three high-power (×400; 0.17 mm² area) microscopic fields in 1 to 4 serial sections of each case were counted, similar to a protocol previously published by us (7). For all immunostains, positive cells were counted only when distinct from blood vessels. The immunoreactive WM cells on single-labeled sections were presumed to be glia (astrocytes and possibly OLs) on the basis of morphologic criteria: small to intermediate cell size and short cytoplasmic processes, some of which extended to surround penetrating vessels. While a quantitative determination of the proportion of definitively identified cells (whether astrocytic or oligodendroglial) as determined by double labeling was of interest, this step could not be reliably performed because cells deep in the 40- to 50-μm tissue section demonstrated fainter immunostaining for those antigens than those at the surface. This observation suggested a significant variability in the penetration and binding of the antibodies in these thick sections, rendering cell counting of double-labeled slides subject to unacceptable inaccuracies.

**Statistical Analysis**

To determine if there was an effect of postmortem interval (PMI) upon the levels of AOE expression in either Western blots or cell counting of single-labeled sections, regression analyses of the PMI were performed. Analyses of the relationships between AOE levels on Western blots and postconceptional age were performed by considering 2 age ranges: 18 to 64 postconceptional weeks and 110 to 214 postconceptional weeks. These age groups were separated because different trends in enzyme levels were observed. There were no cases with ages between 65 and 109 weeks. Thus, regressions (linear and, if appropriate, quadratic) were fit to the enzyme levels separately for the 2 age groups, with a dotted line connecting them to indicate that data are lacking in the intermediate age range. Analyses of the relationships between numbers of AOE-positive cells (by single labeling) per 3 high-power microscopic fields (cell density) and postconceptional age in weeks were performed by linear regression. Since values in the age range 20 to 26 weeks are clearly 0, or low, an average value was assigned. For the older age group, where the cell densities are clearly increasing, a linear regression was fit. In all analyses, a p < 0.05 was considered significant.

**RESULTS**

**Clinicopathologic Correlations**

The 42 subjects from which data from Western blot, ICC, or both were obtained ranged from 18 to 214 postconceptional weeks, with a median of 34 weeks. Not including stillbirths (n = 10) and terminations (n = 3), the postnatal survivals ranged from a few hours to 3.5 years, median less than 1 day of age. Causes of death of live-born infants included extreme prematurity (n = 8), congenital heart disease (n = 8), acute chorioamnionitis/sepsis (n = 2), acute pulmonary hemorrhage (n = 2), bronchopulmonary dysplasia, acute bronchopneumonia,
dehydration from gastroenteritis, hematologic malignancy, trauma, drowning, necrotizing enterocolitis, pulmonary hypoplasia due to renal agenesis, or congenital muscular dystrophy (n = 1 each). The majority of cases (n = 27) were histologically normal. Due to the extreme difficulty in obtaining controls in the premature age range, cases (n = 15) with minimal gliosis of the telencephalic WM, a finding which in isolation is of uncertain significance, were also used. Cases with WM necrosis (the focal component of PVL) were specifically excluded, to be the subject of further separate study. Cases for ICC ranged in age from 20 to 49 postconceptional weeks, with a median of 33 weeks. Frozen tissue used for immunoblotting ranged in age from 18 to 214 postconceptional weeks, with a median of 34 weeks. Postmortem intervals ranged from 0 to 72 hours, with a median of 12 hours. There was no effect of PMI upon the level of protein expression for the 4 AOEs sampled (by Western blot), or on the densities of immunopositive cells using regression analyses (data not shown). There was no effect of PMI on the intensity of immunostaining in tissue sections by visual inspection (data not shown).

Western Blot Analysis of Human Parietal WM

To examine the developmental expression of each AOE from fetal ages through infancy, with comparison to adult cases as indices of maturity, we used Western blots probed with the specific antibodies. An MnSOD-immunostained Western blot of parietal WM demonstrates the pattern of expression for this protein across development (Fig. 1), and is representative of the immunoblot technique used for all proteins evaluated in this study. Densitometric quantitation of bands for each AOE across the time period of interest resulted in the regression curves shown (Fig. 2A, B). These curves demonstrate that the levels of expression of MnSOD and

![Fig. 1](image)

**Fig. 1.** Representative Western blot of human parietal lobe WM immunostained for human MnSOD. Adult standard (std) bands, undiluted and diluted to 1/4 concentration, with molecular weight standards at 20.7 and 29.9 kDa are indicated; MnSOD molecular weight = 24 kDa.

**Fig. 2.** Regression curves of normalized WM values of AOE expression, and of expression of MnSOD and mitochondrial markers, obtained by Western blot analysis. There are statistically significant differences in the expression levels of MnSOD, CuZnSOD, and catalase (Cat) (p < 0.001), but not for Gpx, with age, across all ages studied (A). At term, the levels of the SODs are below adult levels, in contrast to those of catalase and Gpx, as shown across 20 to 60 postconceptional weeks (B, A, B): Vertical dotted line denotes term birth at 38 weeks, and horizontal dotted line the adult level of 100%. Levels of expression of mitochondrial markers cytochrome c oxidase (Cyt Ox) and protein AB2 parallel those of MnSOD, which increase at the time of onset of maximal myelin sheath synthesis postnatally (C).
CuZnSOD between 18 weeks and term are lower than the adult standard (reaching 76% for MnSOD and 65% for CuZnSOD at 37 weeks), in contrast to the levels for GPx and catalase, which supersede adult levels by 37 weeks (149% for GPx and 118% for catalase). During the fetal period (between 20 weeks and term), catalase levels, while exceeding adult levels beginning at 30 weeks, are significantly below those for GPx (Fig. 2A, B), suggesting a dissociation between the developmental regulation of these H$_2$O$_2$-degrading enzymes. Interestingly, all enzymes have higher-than-adult expression in the postnatal period of myelin sheath synthesis (2 to 5 postnatal months). The linear regression $p$ values for the change in expression between 18 and 64 postconceptional weeks are $p < 0.001$ for MnSOD, catalase, cytochrome c oxidase, and mitochondrial protein AB2; the $p$ values for CuZnSOD are $p = 0.007$ and $p < 0.001$ using linear and quadratic terms, respectively (Fig. 2A-C). There is no statistically significant change in the expression of GPx during this time interval. After 64 weeks, the general trend for all enzymes is to decrease by 110 postconceptional weeks. Due to the lack of cases in this age range, however, no conclusions can be drawn as to the precise profile of this decrease (denoted as dotted portions of the regression lines). After 110 weeks, enzyme expression seems to remain fairly constant at around adult levels and does not change significantly for any of the enzymes or mitochondrial markers (Fig. 2A-C). Because of the limited availability of cases between 110 and 214 weeks, however, conclusions about the trend in change of expression with age are tentative across this interval. It is possible that CuZnSOD, which has markedly higher expression during the postnatal months as compared to the other AOE, decreases to adult levels more slowly than the other enzymes.

For MnSOD, the changing levels of expression parallel the changing levels of the mitochondrial markers cytochrome c oxidase and protein AB2 with age (Fig. 2C). As with all of AOE, the mitochondrial markers reach high levels by 64 postconceptional weeks, though, as above, firm conclusions about the age of peak expression cannot be drawn.

**Immunocytochemistry of Human Parietal WM**

Having found that AOE expression in whole WM homogenates is developmentally regulated, we next aimed to identify the timing of expression and the cellular localization of each enzyme. Single-label studies demonstrated only very rare, faintly staining MnSOD- and CuZnSOD-positive cells in WM parenchyma and blood vessels between 21 and 27 weeks, in contrast to the definite immunopositivity for catalase and GPx at these ages (Fig. 3A, B, D, E). At 29 weeks, strongly SOD-positive cells were more numerous in the WM, similar to the intensity of staining for catalase and GPx at this same age. For all AOE, intense staining was detected after 33 weeks in cells with the morphology of glia and in blood vessel walls (Fig. 3C, F). Statistically significant increases in small to medium-sized cells bearing short processes (presumed to be glia) immunoreactive for the SODs and catalase were noted between 21 and 49 weeks ($p = 0.001$). The change with age in the number of GPx-positive glia, however, was not statistically significant, after excluding the single outlier at 49 weeks (Fig. 4). This outlier case was excluded because it seemed unreasonable to conclude that there was a significant increase with age.
based solely on 1 case. Additional data would be needed for a more definitive conclusion, however. Densities of positive cells for MnSOD and CuZnSOD began to increase between 27 and 29 weeks, and between 26 and 29 weeks, respectively. Catalase and GPx already had higher cell densities at slightly earlier ages, between 24 and 28 weeks, and between 24 and 26 weeks, respectively.

By double labeling, the MnSOD-positive cells in the WM were predominantly O4+ beginning at 29 weeks (Fig. 5A), although rare O1+ cells also expressed MnSOD (Fig. 5B). At later ages (>33 weeks), more MnSOD-positive OLs are O1+ cells (data not shown). By double labeling, catalase and CuZnSOD were localized to rare developing OLs beginning at 29 gestational weeks, and qualitatively increased thereafter (Fig. 5C, D). Many MnSOD-expressing WM cells are astrocytes, based on co-labeling with GFAP (Fig. 5E), especially in cases at 33 weeks and older. This observation held true for CuZnSOD and catalase as well (Fig. 5F). Astrocyte foot-process staining (by double labeling with GFAP and MnSOD, CuZnSOD, or catalase) along blood vessels was also observed (data not shown). Co-labeling of scattered microglia with CD68 and MnSOD was present at all ages examined (data not shown). By CD34/MnSOD double labeling, the blood vessel positivity detected with single-label ICC was confirmed at all gestational ages analyzed (Fig. 5G). For technical reasons as mentioned above, double labeling for GPx and OL markers was not successful.

**DISCUSSION**

This study demonstrates that fetal AOE expression in human cerebral WM differs significantly between the fetal period and infancy, while in turn the expression in infancy varies significantly from that in the adult. These results have important implications with respect to the pathogenesis and eventual treatment of PVL, a devastating disease affecting the brains of premature infants and leading to cerebral palsy. We hypothesized that fetal WM would have lower AOE content than the adult, reflecting the lower antioxidant capacity of immature tissues before the transition to an oxygen-rich extrauterine milieu (12). We also expected that the expression of the 4 AOE2s would parallel each other across early development, given their sequential and interdependent actions in detoxifying ROS in both physiologic and pathologic situations (11, 17). Indeed, animal studies indicate that the balance of all the AOE2s in the brain must be optimized for proper protection from oxygen-free radicals in hypoxic-ischemic injury, although this balance may differ between mature and immature brain (22–25). We found, surprisingly, that the peroxide-degrading enzymes catalase and GPx were expressed at higher than adult levels by 30 gestational weeks, while the peroxide-generating SODs paradoxically lagged in expression until postnatal ages. This developmental delay in the expression of the SODs relative to catalase and GPx represents, in our opinion, a crucial, “rate-limiting” factor in the protection of premyelinating OLs from superoxides.

**Implication of Delayed SOD Expression in Pathogenesis of PVL**

The lag in SOD expression in human fetal, as compared to postnatal, WM likely underlies, at least in part, the known vulnerability of human premyelinating OLs to ROS damage during the risk period for PVL (7). Several studies indicate major importance for the SODs and their regulation in protection of the brain from free radical injury. In animal models of stroke, CuZnSOD is neuroprotective when overexpressed (22), or given exogenously (23). Neurons expressing nitric oxide synthase (NOS), the synthetic enzyme for nitric oxide (NO), are protected from peroxynitrite-related damage by their co-expression of MnSOD (26). Overexpression of MnSOD confers protection against both NO and N-methyl-D-aspartate toxicity in neuronal cell culture systems (27) and from ischemia-induced neuronal apoptosis and brain injury in transgenic animals (28), as mediated through mitochondrial release of cytochrome c (29). We previously demonstrated that both protein nitration and apoptosis occur in OLs in the immature human brain in PVL (7). Thus, our observation of increasing MnSOD expression in OLs with gestational age suggests a maturational mechanism for protection from nitrative as well as oxidative injury and, possibly, cell death.

In experimental systems investigating cerebral ischemia/reperfusion, excitotoxicity, and/or cytokine toxicity, all major cell types of the brain, including neurons (30–33), OLs (34), and/or astrocytes (32, 35, 36) respond by upregulation of MnSOD, CuZnSOD, and/or catalase.
Fig. 5. Double-label ICC for AOE expression and WM cell markers. MnSOD (green) colocalizes with O4 (red) in human parietal lobe WM at 29 postconceptional weeks (A, colocalization seen as yellow signal in all merged images, arrows). MnSOD (green) is also present in rare O1+ cells (red) as early as 26 weeks (B), becoming more numerous after 31 weeks (data not shown). Catalase (Cat) and CuZnSOD (both green) are expressed in O4+ cells (red), here shown at 35 weeks (C and D, respectively). Astrocytes immunostained for GFAP (red) contain MnSOD (green) at 29 weeks (E) and catalase (green) at 35 weeks (F) (scale bar = 45 μm). Blood vessels express both CD34 (red) and MnSOD (green) at all ages studied, here shown at 31 weeks (G) (scale bar = 20 μm, unless otherwise noted).

Thus, many of the very conditions thought to contribute to the pathogenesis of PVL result in induction of protective AOE expression in the brain. Our demonstration that MnSOD, CuZnSOD, and catalase are present in human OLs near term gestation may reflect a need for similar protection, which is highest during the time of transition to extraterine life.

Timing of AOE Expression Relative to Markers of OL Maturation

We found SOD expression to be low in the premyelinating phase of the fetal period (20 to 29 weeks, during which OLs are O4+, O1+, MBP+), to begin to increase at the same time as the acquisition of the O1 marker (29 to 33 weeks), and to achieve higher-than-adult levels in the
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Fig. 6. Schematic relating postconceptional age (top line; period of greatest PVL risk noted), stages of OL maturation as defined by sequential expression of markers O4, O1, and MBP (ref 7), expression of each AOE, and phases of myelination (bottom line) (ref 41). Please see Discussion for complete explanation.

Timing of AOE Expression Relative to Birth and Myelination

At birth, the transition from a relatively hypoxic intrauterine environment to one rich in oxygen results in compensatory upregulation of AOE expression in the lung (40, 41) and in the liver (12, 13). The presumption that a similar upregulation occurs in brain is based on several lines of evidence, including developmental changes in AOE expression in the developing rodent brain (11, 17), increasing oxygen consumption in studies of minced human fetal brain from 20 gestational weeks through term (42), and increased postnatal expression of ketone-body-utilizing enzymes (allowing the incorporation of ketone-body carbon into cerebral lipids and proteins) in human brain (43). Thus, our findings of rising levels of AOE expression around the time of birth are consistent with a response to the increasing oxidative capacity of the brain as it prepares for and/or responds to extrauterine life. The reasons for the differences in the exact perinatal timing of attainment of adult levels of expression among the 4 AOE s, however, remain obscure.

In rodents, the brain is vulnerable to lipid peroxidation with the onset of increased metabolism of precursor-free fatty acids into the myelin sheath (16, 44). Our finding of a marked upsurge in mitochondrial cytochrome oxidase expression at the same time as the highest AOE levels likely reflects this increased metabolic activity. Respiring mitochondria generate ROS (15) and accumulate superoxide in the setting of glutathione depletion (10), which results in depolarization of the mitochondrial membrane potential, leading to cell death in developing OLs (38). MnSOD, in particular, localized to the inner mitochondrial membrane, is likely to play a critical part in protection of the OL at the onset and peak of myelination when mitochondrial respiration and levels of iron, a major generator of free radicals, are at their highest (45–47). While evidence supports MnSOD as the primary scavenger of superoxide generated by mitochondrial respiration, we report a striking increase in CuZnSOD with development, particularly during this highly metabolic interval of myelin sheath synthesis. We speculate that the assembly of myelin phospholipids along the cytosolic face of the endoplasmic reticulum (48) requires superoxide scavenging capability in the cytosol, provided by CuZnSOD. Catalase-containing peroxisomes synthesize plasmalogens, comprising a third of myelin phospholipids (49), and are localized to OL cell bodies and processes during active myelin sheath formation in the postnatal rat (50). Thus, all three of the AOE s showing developmental regulation in the human are importantly related, both temporally and functionally, to the process of myelogenesis.

Our double-labeling studies demonstrate AOE expression not only in O4+ and O1+ cells, but also significantly in astrocytes, which are more numerous after 33 weeks of gestation.
even in “control” brains (7). We speculate that astrocytes, known to regulate antioxidant levels in the extracellular space (51, 52), serve as “sinks” for ROS generated by OLs as they initiate the metabolism required for the onset of myelination.

Expression of AOE s in Humans as Compared to Experimental Models

Human studies of AOE s in the developing brain are limited to single-label immunohistochemical surveys for catalase (53) and MnSOD (54). While the former described “glial” staining in the deep WM beginning at 31 to 32 postconceptional weeks, double labeling to determine the cell type was not performed (53). For MnSOD, only gray matter sites were described (54). Thus, our investigation is the first to systematically analyze all 4 major AOE s in human WM, using the combined techniques of Western blotting, single- and double-label ICC, and statistical analysis across pre- and postnatal development.

As in the human, AOE s are also developmentally regulated in the brains of experimental animals. In the study of postnatal rat brain by Mavelli et al (17), a gradual increase in Mn- and CuZnSOD and catalase activities occurs with increasing postnatal age, versus a constant activity of GPx from birth. This result is similar to our findings, although our findings are based on protein expression by Western blot analysis of WM rather than on enzyme activity in whole brain. Khan and Black (11) correlated protein levels with enzyme activity for all 4 AOE s in whole rat brain at E18 and from P1 to P21. Interestingly, they found disparities between the age at which activity of a given enzyme was maximal, and that at which its protein level was highest: for MnSOD, they ascribed the disparity to developmental differences in mitochondrial uncoupling protein levels (55), and for CuZnSOD and GPx, post-translational modification and bioavailabilities of their copper and selenium cofactors, respectively (11). Neither of these studies documented the expression of any of the AOE s specifically in OLs or WM. We did not determine the activities of the AOE s in our human cerebral WM samples, due to concerns about postmortem autolysis which would likely lead to spurious changes in them. In work by colleagues in our laboratory, however, developmental trends in activity and protein level are concordant for all 4 AOE s in cultured rat OLs (38, 56). Both protein expression and activity of CuZnSOD, a cytosolic enzyme, are similar across OL lineage development. In contrast, mitochondrially localized MnSOD protein and activity levels increase with development, stabilizing the mitochondrial membrane potential in mature (MBP+) cells, rendering them more resistant than MBP− cells to glutathione depletion-induced toxicity (38). For catalase, both protein expression and activity are stable across OL maturation, while GPx shows a 2- to 3-fold increase in expression and activity. A key role for GPx in the resistance of mature OLs to peroxide-induced oxidative injury is thought to be due to its regulation of catalase activity at high H2O2 levels (56).

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