Lipid metabolism of isolated oligodendrocytes maintained in long-term culture mimics events associated with myelogenesis

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ABSTRACT Oligodendrocytes isolated from ovine white matter according to a published procedure (Szuchet, S., Stefansson, K., Wollmann, R. L., Dawson, G. & Arnason, B. G. W. (1980) Brain Res. 200, 151–164) were cultured for up to 35 days and their capacity to incorporate precursors into lipids was investigated. At various times, cultures were double labeled with [3H]glycerol/[^14C]acetate or [3H]galactose/[^35S]OAc. The cells were harvested 72 hr later and lipids were fractionated using standard procedures. The time course of incorporation for each precursor was distinct. In the days after attachment to substratum, oligodendrocytes preferentially incorporated [^3H]glycerol into phospholipids and [^14C]acetate into cholesterol while uptake of [^35SO4]− and [3H]galactose into glycolipids was modest. A switch in phospholipid metabolism from preferential incorporation into phosphatidylincholine to incorporation into phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol occurred at about the 10th day in vitro. After 20 days, uptake of [^3H]glycerol into phospholipids and [^14C]acetate into cholesterol had stabilized but incorporation of [^35S]OAc into glycolipids had increased. [^35S]OAc incorporation into glycolipids was even greater at 35 than at 20 days. Uptake of [^3H]galactose did not change over time. An attempt was made to correlate changes in lipid metabolism with morphologic developments. High incorporation into phospholipids and cholesterol coincided with the extensive membrane synthesis required for cell attachment and process extension. Differentiation of these newly formed membranes, as assessed by the incorporation of myelin-characteristic glycolipids, galactocerebrosides, and sulfatides, occurred at a time when an intricate network of processes had already been established. The sequence of metabolic events observed in vitro parallels that observed at the onset of myelogenesis in vivo. We postulate that mature oligodendrocytes can reenact those early events associated with myelogenesis.

Oligodendrocytes (OLG) synthesize membranes that enwrap axons; by fusion these membranes form myelin (1). Many issues pertaining to this function of OLG are poorly understood. In particular, little attention has been paid to the fact that OLG are not a homogeneous population of cells.

During studies on the morphology of OLG and their relation to axons, del Río-Hortega (2) discovered pleomorphism among these cells in terms of size, the disposition of processes, and in interaction with axons. OLG range from small cells connected with numerous axons to large cells attached to single axons. It has been calculated that an OLG wrapping a large axon may synthesize an order of magnitude more membrane than a small cell enshathing 39 axons (3). This poses a question as to the metabolism of these two cell types: Are there quantitative differences between their metabolisms or do qualitative variations distinguish them? This question can be rephrased to address yet another puzzle: Can OLG polymorphism also account for the compositional heterogeneity of adult myelin (4, 5)?

Another type of OLG heterogeneity, based on ultrastructural features, has come to light: three prototypes have been described (6, 7). It is believed that these types represent stages in the process of maturation of OLG. Although these observations have been made only on murine species and their general validity remains unproven, the fact that myelin changes in composition after its initial deposition, a process referred to as maturation (4), has led to the hypothesis of specialization among the OLG subgroups. It has been suggested that young OLG assemble myelin while mature ones maintain it (8, 9). This formulation presupposes that OLG change from a metabolism suitable for membrane synthesis to one geared to myelin maintenance. Whether these changes are reversible is a question of significance, because it bears on the issue of the capacity of OLG to remyelinate.

There is evidence that the onset of myelination is heralded by a cascade of events, morphological, ultrastructural, and biochemical, in OLG. These events reach their maxi-}

Abbreviations: OLG, oligodendrocytes; PL, NL, and GL, phospho-
neutral, and glycolipids, respectively; PtdCho, phosphatidy-
choline; PtdEtn, phosphatidylethanolamine; PtdSer, phospha-
dylserine; PtdIns, phosphatidylinositol; GalCer, galacto-
cerebrosides.
tention, ultrastructural (18) and immunocytochemical (17) data are reported separately.

MATERIAL AND METHODS

Isolation of OLG. OLG were isolated from ovine white matter according to a published procedure (9, 11) with the following modification: the crude cell suspension (P-1) was applied to a linear gradient of 1.0 M sucrose to 1.15 M sucrose/4% (wt/vol) dextran 70, all dissolved in Hanks' balanced salt solution (Hanks' solution) at half strength, and centrifuged at 1,020 × g for 10 min. Three bands separate on this gradient (11); only cells from band 3 were used for the experiments described here. Cells were removed from the gradient, diluted by slow addition of 1 vol of Hanks' solution, centrifuged at 700 × g for 5 min, washed once with Hanks' solution, and centrifuged again.

Cell Culture. Detailed descriptions have been given elsewhere (12, 17). Briefly, a pellet of freshly isolated cells was suspended in Dulbecco's modified Eagle's medium/20% horse serum/2 mM glutamine supplemented with antibiotics (Amphotericin B at 0.3 μg/ml and garamycin at 12.4 μg/ml) and plated at 2 × 10^5 cells/ml in Petri dishes. Cultures were maintained in an incubator at 37°C in 95% CO₂/5% air at 90% humidity. Approximately 40% of the cells attach to the Petri dish under these conditions; the remainder form small floating clusters that we refer to as B-3,f OLG. These cells were left to float for 96 hr, then the supernatant containing them was removed and centrifuged, and the cell pellet was collected. Pelleted cells were resuspended gently in culture medium and replated onto polylysine-coated plastic Petri dishes. The cells attached within 24 hr and extended processes. Cultures were fed twice weekly.

Isotope Labeling. At selected times, OLG cultures were double labeled with carrier-free H₂¹⁵SO₄ at 15 μCi/ml (78.2 mCi/mmol; 1 Ci = 37 GBq) and [³H]galactose at 1 μCi/ml (11.5 Ci/mmol) or with [²³H]glycerol at 1.5 μCi/ml (500 mCi/mmol) and [¹⁴C]OAc at 2 μCi/ml (56.7 mCi/mmol). All radiochemicals were from Amersham. Cultures were harvested after 72 hr of labeling and washed thrice with 10 ml of Hanks' solution, centrifuging after each wash. The final pellet was processed immediately or frozen at −25°C for subsequent analysis.

Analysis of Lipids. Pellets were suspended in a small volume of H₂O and sonicated, and 30-μl aliquots were removed for protein determination by the BioRad microassay. The remaining cell homogenates were adjusted to 0.5 ml with H₂O. Lipids were extracted by addition of 6 ml of CHCl₃/CH₃OH (2:1) (19), spun on a Vortex three times, and centrifuged for 10 min at 2,000 × g. The organic phase was removed with a pipette, three drops of 0.1% 2,6-di-t-butyl-p-cresol in CHCl₃ was added to each tube to prevent autoxidation of lipids, and the samples were taken to dryness under N₂ on a 40°C water bath. Each dried pellet was suspended in a drop of CHCl₃ and applied on a 20 × 6 mm silicic acid column. Neutral lipids (NL), glycolipids (GL), and phospholipids (PL) were separated by sequential elution from this column with 10 ml of CHCl₃ (NL), 12 ml of acetone/methanol, 9:1 (vol/vol) (GL), and 10 ml of CH₃OH (PL) (20). All samples were dried on a water bath under N₂. Final separation was achieved by TLC on 250-mm silica gel G plates (Analtech) as follows: NL, 20 × 20 cm plates, developed in n-hexane/diethylther/acetetic acid (90:10:1); GL, 20 × 20 cm plates, developed in CHCl₃/CH₃OH/H₂O (100:42:6); PL, 10 × 10 cm plates, developed in n-propanol/methylethyl ketone/H₂O/HCOOH (48:36:18:6) for the first dimension, dried 30 min in a hood and developed in CHCl₃/CH₃OH/H₂O (100:42:6) for the second dimension. All plates were activated at 110°C for 1 hr prior to use. Phosphatidylethanolamine (PtdSer) and phosphatidylethanolamine (PtdEtn) were visualized with a ninyhydrin spray (0.2% ninyhydrin in acetic acid/HOAc/pyridine [99:0.5:0.5]); all other lipids were visualized by I₂ vapor staining. NL and PL were identified by chromatography with lipid standards (Sigma); GL were identified by chromatography with GL isolated from sheep brain (21). The lipid spots were scraped from the plates and placed in 7-ml minivials, and 400 μl of H₂O and 5 ml of ACS fluid were added (Amersham 1962590). Radioactivity was determined in a Beckman LS-7000 scintillation counter. All data were normalized by dividing cpm by mg of protein.

RESULTS

The incorporation of ³⁵SO₄²⁻, [³H]galactose, [¹⁴C]OAc, and [³H]glycerol into GL, NL, and PL by B-3,f OLG was followed as a function of time in culture for up to 35 days. ³⁵SO₄²⁻ and [³H]galactose were metabolized into GL, [¹⁴C]OAc was preferentially into NL and [³H]glycerol into PL. The uptake of [³H]galactose was essentially constant for the first 20 days, with a moderate upsurge thereafter (Fig. 1). In contrast, uptake of the other precursors registered increases from the start (Fig. 1).

Examining first the metabolism of [³H]glycerol into PL, we find a 4-fold increase in incorporation from day 4 to day 7 (Fig. 1). The increase in the uptake of [¹⁴C]OAc chiefly into cholesterol (see below), over this same time span, is modest but, by 10 days, the intake of [¹⁴C]OAc is equivalent to that of [³H]glycerol. From then on, the two precursors run a parallel course that peaks at day 20. The incorporation of ³⁵SO₄²⁻ into sulfatide is still increasing at day 35.

It is important to correlate these observations with morphological events: the measurements taken at day 4 correspond to B-3,f OLG in the floating state (Fig. 2a); at day 7, the cells have attached and begun to extend processes. Thus, the burst in synthesis of PL coincides with drastic morphological changes that require substantial formation of membranes. The short lag in the synthesis of cholesterol at this time is, per se, intriguing.

![Fig. 1. Incorporation of labeled precursors into PL, NL, and GL by OLG over time in culture. At the indicated times, cultures were labeled with either [³H]glycerol/[¹⁴C]OAc or H₂¹⁵SO₄/[³H]galactose for 72 hr. Lipids were isolated following standard procedures and the label found in each fraction was normalized per mg protein. ○, [³H]glycerol into PL; □, [¹⁴C]OAc into NL; ◻, ³⁵SO₄²⁻ into sulfatide; □, [³H]galactose into total GL. (Bar = SEM; n = 6–9.)](image-url)
FIG. 2. Morphological changes accompanying attachment of OLG to substratum and their further development with time in culture. (a) OLG after 4 days in vitro plated under conditions in which they do not attach. Note the absence of processes. (Stain, orcein; bar = 5 μm.) (b–d) Phase-contrast micrographs of OLG after 18–25 days (b, 18; c, 20; d, 25) in culture. Note the profuse network of processes and alignment (→) of cells in rows. (Bar (see a) = 20 μm.)

The morphological development of the cells peaks at 20–25 days as judged by the profuse network of processes seen at this time (Fig. 2 b–d) and by the formation of tight junctions between adjoining cells (18). Events at the biochemical level are consistent with the idea that this represents a turning point towards membrane differentiation.
The distribution of \(^{3}H\)glycerol among the various PL is also of significance (Fig. 3A): for the first 10 days in vitro, B-3, f OLG direct the bulk of this label into phosphatidylcholine (PtdCho), only small amounts being incorporated into other PL. Beyond day 10, dramatic changes take place: incorporation of \(^{3}H\)glycerol into PtdCho decreases while incorporation into PtdEtn, PtdSer, and phosphatidylinositol (PtdIns) increases. After 20 days, the intake of label for most PL examined remains relatively constant except for PtdIns, which decreases, and PtdEtn plus PtdSer, which increase, though more slowly than before (Fig. 3A). Little \(^{3}H\)glycerol is incorporated into either sphingomyelin or lysolecithin.

A different perspective is obtained when incorporation of \(^{14}C\)OAc is followed (Fig. 3B). Here, sphingomyelin appears as an important acceptor of label, second only to PtdCho. The time course of the latter is basically the same for both labels, though the magnitudes differ. The same holds for PtdIns. Little \(^{14}C\)OAc is incorporated into PtdEtn and PtdSer (Fig. 3B).

Among the NL, cholesterol is the leading compound for both precursors—i.e., \(^{14}C\)OAc and \(^{3}H\)glycerol—the patterns of incorporation with time are similar but the magnitudes are distinct (Fig. 4). Although in terms of cpm/mg of protein, there is a significant intake of radiolabel into other NL, they are overshadowed by cholesterol (data not shown).

Turning now to myelin-characteristic GL, we note the following. (i) The uptake of \(^{35}S\)OAc into sulfatides is much higher than that of \(^{3}H\)galactoside into either sulfatides or galactocerebrosides (GalCer) (Fig. 5). (ii) The time course of incorporation of these precursors differs. Thus, whereas the incorporation of \(^{35}S\)OAc registers a significant increase, very little change is seen in the incorporation of \(^{3}H\)galactoside over the same time span. (iii) Within experimental error, the ratio of radiolabel taken up into GalCer and \(\alpha\)-OH-GalCer is maintained at 1 (at least during the period examined; Fig. 5B Inset). (iv) The constancy of this ratio suggests that both pools—i.e., \(\alpha\)-OH-GalCer and GalCer—contribute comparably to the synthesis of sulfatides. Bands corresponding to hydroxysulfatide and nonhydroxysulfatide were visualized in the chromatogram; no attempt to separate them was made.

Even if only relatively small amounts of the other precursors are taken up by these GL, the manner in which this is done is interesting. \(^{3}H\)OAc intake exceeded that of \(^{3}H\)glycerol (data not shown), presumably because the former comes in via fatty acid chain elongation, an important premyelination step.

It has been suggested that the GL/PtdCho ratio is a measure of myelin maturation (4). In this context, it is pertinent to compare the evolution of sulfatide taken as a representative for GL (the contribution of GalCer would merely add a constant and therefore not affect the shape of the curve) and PtdCho as OLG mature in culture (Figs. 1 and 3A). While the plots represent incorporation and not necessarily de novo synthesis and the numbers are cpm and, hence, not directly comparable, each curve is self-consistent. Hence, the fact that the uptake of label into sulfatide increases with time in culture, whereas that of PtdCho decreases, must be significant.

**DISCUSSION**

We have investigated the incorporation of precursors into PL, NL, and GL over time by cultured OLG. Lipid metabolism parallels morphological development. Thus, at the time of cell attachment and process extension, a period of extensive membrane synthesis, OLG synthesize PL and cholesterol preferentially (Fig. 1). Further differentiation of these newly formed membranes, as assessed by incorporation of myelin-characteristic GL, GalCer, and sulfatides, occurs at a slower rate. These
findings fit well with what has been observed in vivo (4, 22, 23).

Insight into the lipid metabolism of cultured OLG and its relatedness to that expressed by OLG in situ is obtained from examination of the variation in PL composition with time in culture (Fig. 3A). PL, NL, and GL account for almost all lipids present in OLG and myelin (4, 24, 25). However, their relative proportions differ: myelin is enriched relative to OLG in cholesterol and GL at the expense of PL. Within the latter, there is an unequal preponderance of classes; PtdCho is the major PL of OLG, PtdEtN is that of myelin (4, 24–26). Lipid composition changes slowly during development and transforms what was an extension of OLG plasma membrane into myelin. Maturation of myelin can be measured by two indices: the molar GL/PtdCho and PtdEtN/PtdCho ratios. Both increase with maturation (4). Although these conclusions stem from studies on murine species, the fact that myelin composition varies little across species (4, 22) justifies the extrapolation made here.

Figs. 1 and 3A indicate that cultured OLG recapitulate, at least qualitatively and in the proper sequence, events associated with myelogenesis. This conclusion is strengthened by the findings that the ultrastructural characteristics of these cells also match those found in situ (1, 27, 28). Thus, the cultured cells have numerous microtubules and a highly developed Golgi, align themselves in rows as interfacicular OL, and form tight junctions between adjoining cells and at points where processes make contact (18). The cells express myelin basic protein (15) and myelin-associated glycoprotein (17). We have measured the specific activity of 2',3' cyclic nucleotide 3'-phosphodiesterase as a function of time in culture; specific activity stays high throughout, typically, 5–6 (μmol/min)/mg of protein (17). Drastic changes in the levels of incorporation of [3H]-leucine into myelin-characteristic proteins follow cell attachment to substrate and process development (unpublished results).

It is important to stress that these myelinogenetic activities of cultured B-3,f OLG are expressed in the absence of neurons or astrocytes. Thus, if a signal is required to alert OLG that their metabolism has to be changed, it must be programmed within the OLG. This is not to say that OLG might not function better in the presence of other cell types. Bhat et al. (16) have reported that OLG need a non-OLG signal for enhanced expression of their activities. No myelin is formed in these cultures. However, when B-3,f OLG are cocultured with rat dorsal root ganglion neurons, myelin is formed (29). Whether this is myelination or remyelination remains to be elucidated.

The metabolism of myelin-typical GL is interesting, if puzzling. Note that incorporation of 35SO4 into sulfatide is much higher than that of [3H]galactose into either GalCer or sulfatide. This may indicate a complex mechanism of sulfation. Results obtained by Benjamins and co-workers (30, 31) using rat support this idea. Those authors found little labeling of sulfatides with [3H]galactose and concluded that there must be two GalCer pools. Our data are also consistent with a more rapid turnover of sulfatides than of GalCer. Rapid turnover of sulfatides has been postulated (32, 33).

B-3,f OLG incorporate similar levels of [3H]galactose into α-OH-GalCer and GalCer, thus maintaining their ratio at essentially 1. A value of 1.4 was reported for this ratio in sheep white matter (34), but the composition of mature white matter is doubtless different from the membranes synthesized initially by the OLG.

We have shown that OLG isolated from white matter of young brains and maintained in vitro exhibit a lipid metabolism that qualitatively and temporally mimics that seen at the onset of myelination. From these results, we postulate that mature OLG can revert to a lipid metabolism compatible with myelogenensis. Preliminary observations (unpublished) suggest that this holds also for protein metabolism. These findings are relevant to remyelination and, hence, to those diseases such as multiple sclerosis. In addition, our results show that OLG function well in the microenvironment provided and that the absence of other cell types or specific humoral factors does not lead to metabolic aberrations. We believe that this culture system offers a unique model to address questions concerning OLG and the molecular and cellular interactions that lead to myelination.

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