Investigations on Myelination in Vitro

REGULATION BY THYROID HORMONE IN CULTURES OF DISSOCIATED BRAIN CELLS FROM EMBRYONIC MICE

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Cultures of dissociated brain cells from embryonic mice were used to study the influence of thyroid hormone on myelination in vitro. Synthesis of myelin-associated lipids such as cerebrosides and sulfatides was used as an index for myelination. An experimental design, in which the cells were grown on medium containing serum from a thyroidectomized calf, was employed to demonstrate the direct effect of T₃, T₂, and T₄ on the biosynthesis of myelin lipids. The cells grown in the presence of hypothyroid calf serum which contained very low levels of thyroid hormones (T₁, thyroxine), 1.2 μg/ml; T₂, <25 ng/100 ml) compared to normal serum (T₁, 5.8 μg/ml; T₂, 110 ng/100 ml) showed a diminished synthesis of myelin-associated glycolipids. This reduced activity could be restored to normal by including T₃ (13 ng/ml) in the medium.

Neonatal thyroid status is known to affect the growth, development, and maturation of the mammalian central nervous system (1). These processes are dependent on the presence of thyroid hormones during the so-called "critical period" (2). This critical period of development is marked by the onset of active myelination. In the hypothyroid neonatal rat, the process of myelination is impaired (3, 4) and there is a decrease in the amount of myelin deposited (5, 6). In contrast, administration of thyroid hormones leads to precocious myelination both in intact rats (7) and in cultured cerebral explants obtained from newborn rats (8). These changes are reflected in the course of deposition of myelin-associated lipids like cerebrosides, sulfatides (6, 9, 10), and monogalactosyl diacylglycerol (11).

Most of the studies on the effect of hormonal imbalance on myelination have been carried out in vivo, thereby making it difficult to assess the primary role of the hormones. Manipulation with one hormone invariably affects the availability and concentration of many others. Animal cell culture offers a tool to resolve some of these problems and to delineate the actions of the hormones at the molecular level. We have initiated studies on the growth of primary cultures of dissociated embryonic mouse brain cells as a means of eventually studying the precise role and mechanism of interaction of thyroid hormones with the myelin-synthesizing components of the brain. The present report demonstrates the direct influence of T₃ on myelination as monitored by the synthesis of some of the lipids closely associated with myelin.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium, calf serum (heat-inactivated), and antibiotic mixture were obtained from GIBCO, Grand Island, N. Y. Serum from thyroidectomized calf was purchased from Rockland Farms, Gilbertsville, Pa. Sterile culture dishes and flasks were supplied by Fischer Scientific Co., Pittsburgh, Pa. Polylysine (M, 80,000) and 3,5,3'-triiodothyronine were from Sigma Chemical Co., St. Louis. H₂[¹⁴C]SO₄ (3.6 Ci/mmol) and [²H]galactose (2.1 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. Pregnant mice (ICR) were supplied by Charles River Laboratories, Boston, Mass.

Culture Conditions—Fifteen-day-old embryos numbering 11 to 14/litter were removed by Cesarean section (12) and placed in Dulbecco's modified Eagle's medium augmented with glucose (600 mg %), 0.23% sodium bicarbonate, 90 units/ml of penicillin, 90 μg/ml of streptomycin, and 0.225 μg/ml of fungizone, adjusted to pH 7.0. Cerebral hemispheres (cerebra) were dissected and temporarily placed in the medium. Cells were then dissociated mechanically (13) by passing through a nylon mesh (82 μ). The cells were collected in a small volume (~1 ml for three cerebra) of the above medium supplemented with 20% heat-inactivated calf serum. Aliquots (1 ml) of the cell suspension containing, on average, 10 to 15 × 10⁶ cells/ml were added to 250-ml polylamine-coated (14) plastic tissue culture flasks containing 9 ml of growth medium. Cultures were incubated at 37°C under an atmosphere of 90% air, 10% CO₂ and 90% relative humidity. The medium in the flasks containing nonattached cells was carefully removed on the 4th day and replaced by 10 ml of the fresh medium containing 20% calf serum. Thereafter, the medium was changed once a week.

Incorporation of Radioactive Precursors into Lipids—The cultures of surface-adhering cells were exposed for 16 h to either 400 μCi of H₂[¹⁴C]SO₄ (final specific activity, 50 μCi/μmol) or 10 μCi of [²H]galactose (the rate of incorporation was linear during this period). After 16 h, the radioactive medium was removed and the cultures were washed four times with 0.9% NaCl. The cells were removed from the surface with a rubber policeman and suspended in physiological saline. Lipids were extracted by Bligh and Dyer procedure (15) and analyzed for various lipids according to Neskovic et al. (16).

RESULTS AND DISCUSSION

The culture system used in this study, described here in detail elsewhere (17, 18), has proven suitable for studying the regulation, especially by hormones, of myelination in vitro. Initial studies (17, 18), however, showed no effect of T₃ on sulfolipid synthesis by dissociated brain cells grown on medium containing 20% calf serum. It should be noted that the endogenous concentrations of hormones and growth factors in the serum may be sufficiently high (19) to preclude observing an effect by exogenous hormones. This condition could not be differentiated from that in which the added hormone is completely nonassociated with the process being measured. The role of thyroid hormone may be tested best by using calf

1. L. L. Sarlieve, G. Subba Rao, and R. A. Pieringer, manuscript submitted for publication.
3. The abbreviations used are T₁, T₂, T₃, 3,5,3'-triiodothyronine; T₄, thyroxine.
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Table I

Values of thyroxine and triiodothyronine in normal and hypothyroid calf sera

<table>
<thead>
<tr>
<th>Hormone concentrations</th>
<th>T4</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf serum</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>Hypothyroid calf serum</td>
<td>&lt;25</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>

Table II

Effect of serum manipulation and T3 addition on sulfolipid synthesis by the dissociated brain cells treated on 4th day in culture

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total protein mg/flask</th>
<th>H4[35S]SO4 incorporated into lipids cpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf serum</td>
<td>2.09 ± 0.20</td>
<td>4154 ± 642</td>
</tr>
<tr>
<td>Calf serum + T3</td>
<td>2.10 ± 0.22</td>
<td>4112 ± 635</td>
</tr>
<tr>
<td>Hypothyroid calf serum</td>
<td>1.64 ± 0.31</td>
<td>2231 ± 300</td>
</tr>
<tr>
<td>Hypothyroid calf serum + T3</td>
<td>1.88 ± 0.25</td>
<td>4142 ± 416</td>
</tr>
</tbody>
</table>

Table III

Effect of serum manipulation and T3 addition on the incorporation of H4[35S]SO4 and [3H]galactose into glycolipids by dissociated brain cells treated on 11th day in culture

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total protein mg/flask</th>
<th>Incorporation of [3H]galactose and H4[35S]SO4 into lipids (cpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf serum</td>
<td>2.36</td>
<td>Cerbroside MGD(^a), Cer-SO(^a), MGD-SO(^a), Cer-SO(^b), MGD-SO(^b)</td>
</tr>
<tr>
<td>Calf serum + T3</td>
<td>2.36</td>
<td>4,670 902 22,265 3,644 14,207 2,928</td>
</tr>
<tr>
<td>Hypothyroid calf serum</td>
<td>2.86</td>
<td>4,187 1,143 25,428 4,504 15,120 3,146</td>
</tr>
<tr>
<td>Hypothyroid calf serum + T3</td>
<td>2.56</td>
<td>4,955 1,096 23,321 4,719 13,196 2,951</td>
</tr>
<tr>
<td>Hypothyroid calf serum + T3</td>
<td>2.50</td>
<td>4,171 616 24,735 3,042 22,142 3,876</td>
</tr>
</tbody>
</table>

\(^a\) MGD, monogalactosyl diacyl- and monoacylmonoalkylglycerol.

\(^b\) Cer-SO, cerebroside sulfate.

\(^c\) MGD-SO, monogalactosyl diacyl- and monoacylmonoalkylglycerol sulfates.

serum obtained from a thyroidectomized animal. Depressed activities observed in the hypothyroid state should be restored by exogenous hormones. This experimental design has been used successfully by Samuels et al. (20) to study the effect of thyroid hormone on the metabolism of pituitary tumor cell line in culture.

The hormone concentrations in the normal and hypothyroid calf sera as determined by radioimmunoassay are given in Table I. Both T4 and T3 values are far below normal in hypothyroid calf serum. In the experiments described, the growing brain cells in culture were challenged with hypothyroid calf serum and hormone supplementation. The accumulation of sulfatides (cerebroside sulfate and monogalactosyl diacylglycerol sulfate), galactocerebroside, and monogalactosyl diacylglycerol was studied by using H4[35S]SO4 and [3H]galactose as the labeled precursors. The synthesis of these lipids has been used as an index for following myelination (10, 11, 21–23).

Table II gives the results of an experiment in which the effect of serum manipulation on sulfolipid synthesis by dissociated brain cells was examined. The cells isolated from the embryonic mouse brain were grown on the medium containing calf serum (20%) for 3 days by which time most of the cells would have attached to the substratum. On the 4th day, the medium was replaced by fresh medium containing calf serum, calf serum + T3 (2 X 10^{-11} M), hypothyroid calf serum, hypothyroid calf serum + T3 (13 ng/ml). Cultures were grown for another week and then labeled with 400 nCi of H4[35S]SO4, for 16 h. The lipids isolated from the cultures were analyzed by thin layer chromatography and the radioactivity was determined. As is clear from the results, when T3 was added to the cultures grown on medium containing normal calf serum, hardly any effect was discernible. On the other hand, presence of hypothyroid calf serum caused a reduction in the synthesis of sulfolipids. This inhibition could be reversed by including T3 in the deficient medium.

In Table III is described the effect of hormone manipulations on myelin lipid synthesis at a later stage, namely, 11th day in culture. The cultures were exposed to the effectors for 3 days and then the synthesis of glycolipids was followed by labeling the cells with H4[35S]SO4 and [3H]galactose. The total lipid extract was analyzed for cerebrosides, monogalactosyl diacylglycerol, cerebroside sulfate, and monogalactosyl diacylglycerol sulfate. As expected, there was about a 3- to 4-fold increase in the rate of synthesis of sulfolipids on the 15th day as compared to 10 days in culture. The synthesis of all the four lipid classes studied appears to be affected by thyroid hormone level in the medium. The effective concentration of the hormone is rather low showing, thereby, the responsiveness of the culture system to near physiological levels of the hormone.

The results of the present study on the effect of thyroid hormone on myelin lipid synthesis correlate well with the in vivo changes induced under altered thyroid functions proving, thereby, the direct influence of T3 on brain maturation. Another in vitro system used to demonstrate such a direct effect of thyroid hormone on myelination was an explant culture of cerebella obtained from newborn rats. Using this system, Hamburgh (8) could show an acceleration of myelinogenesis by T3 (1.5 to 3 μg/ml) addition. The mechanism whereby T3
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influences myelination includes (a) differentiation of the neuroglial cell population responsible for myelin synthesis (3), (b) induction of such differentiated cells to synthesize myelin components, and (c) assembling of the various components to form the complex myelin membrane. Studies are underway to examine the possibilities.

Acknowledgment—We thank Mrs. C. C. Campbell of the serology department for determining T₄ and T₃ concentrations in the serum samples.

REFERENCES

Investigations on myelination in vitro. Regulation by thyroid hormone in cultures of dissociated brain cells from embryonic mice.
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