Localization of mRNA for low density lipoprotein receptor and a cholesterol synthetic enzyme in rabbit nervous system by in situ hybridization

(3-hydroxy-3-methylglutaryl-coenzyme A synthase/apoprotein E/myelination/Watanabe heritable-hyperlipidemic rabbit)

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ABSTRACT The low density lipoprotein receptor and one of its ligands, apoprotein E, are known to be synthesized in the central nervous system. In the current study, we used in situ hybridization to localize the receptor mRNA in selected neurons and glia throughout the nervous system of 9-day-old rabbits. Particularly high levels were found in sensory ganglia, sensory nuclei, and motor-related nuclei. The same regions contained high levels of mRNA for 3-hydroxy-3-methylglutaryl-coenzyme A synthase, a regulated enzyme in cholesterol biosynthesis. The distribution of low density lipoprotein receptor mRNA was similar in mature and immature rabbits. The data suggest that certain cells in the nervous system have high requirements for cholesterol, which they satisfy through cholesterol synthesis and through receptor-mediated uptake of cholesterol-carrying lipoproteins. The latter originate in astrocytes which synthesize and secrete apoprotein E. These data suggest that the nervous system of mammals contains an active system for continuous redistribution and recycling of cholesterol that is physically distinct from the lipoprotein transport system in plasma.

The low density lipoprotein (LDL) receptor binds cholesterol-carrying plasma lipoproteins that contain apoprotein (apo) B-100 or apoE. Binding leads to cell uptake by receptor-mediated endocytosis, thereby supplying cholesterol to cells (1). The production of LDL receptors is driven by a cell's cholesterol requirement. Cells that require large amounts of cholesterol, such as those in the liver and adrenal gland and those that grow in tissue culture, produce relatively large numbers of LDL receptors. Cells with low requirements, such as resting lymphocytes, produce few receptors. When the LDL receptor is genetically defective, as in patients with familial hypercholesterolemia or in Watanabe heritable-hyperlipidemic (WHHL) rabbits, LDL is not taken up by cells normally, and plasma LDL levels rise (2).

One of the ligands for the LDL receptor, apoB-100, is secreted almost exclusively by the liver (3). The other ligand, apoE, is secreted by hepatic and extrahepatic cells. One nonhepatic cell type shown to secrete apoE is the peritoneal macrophage (4). When loaded with excess cholesterol, macrophages secrete cholesterol-containing lipoproteins that contain apoE, which targets the lipoproteins to LDL receptors. ApoE-containing lipoproteins are also secreted by macrophages that enter damaged peripheral nerves and become engorged with cholesterol derived from degraded myelin (5, 6). When the nerve regenerates, the neurons and Schwann cells produce LDL receptors and this allows them to use the lipoprotein-bound cholesterol secreted from the macrophages. Thus, apoE and the LDL receptor play a role in the healing of peripheral nerves.

A role for apoE in the central nervous system (CNS) was demonstrated by Boyles et al. (5), who showed by immunocytochemistry that astrocytes produce apoE. This finding stimulated a search for LDL receptors in the brain. Hofmann et al. (7), using RNA blotting to measure LDL receptor mRNA, and Pitas et al. (8), using immunocytochemistry, identified LDL receptors in rabbit, bovine, rat, and monkey brain. The levels of mRNA and protein were higher in white matter than in cortex. The mRNA was present at particularly high levels in the medulla, pons, and spinal cord. The amount of mRNA did not decline when myelination of the CNS was completed, indicating that the brain required LDL receptors even in adult life (7). Pitas et al. (8) showed that LDL receptors were present on astrocytes abutting on the arachnoid space and in unidentified cells deeper in the brain. They also showed that cerebrospinal fluid contains relatively high levels of apoE. These studies suggest a hitherto unsuspected system for cholesterol transport in the adult CNS. ApoE-containing lipoproteins secreted by astrocytes would be taken up by other brain cells to supply them with cholesterol.

In addition to this system for cholesterol transport, the rabbit brain contains surprisingly high levels of mRNA for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, an enzyme in the cholesterol biosynthetic pathway (7). The level of mRNA declines only by 50–60% when myelination is complete, further suggesting that the brain has an ongoing requirement for cholesterol that is fulfilled by LDL receptors and by endogenous cholesterol synthesis.

In the current studies, we have used the technique of in situ mRNA hybridization to identify the cells that contain mRNA for the LDL receptor and HMG-CoA synthase. The data indicate that both of these mRNAs are distributed widely in neurons in the brain and in sensory ganglia.

METHODS

Animals. New Zealand white rabbits (9- or 10-day-old and adult males) were used for experiments as indicated. WHHL rabbits were raised in Dallas.

In Situ Hybridization. RNA probe synthesis was carried out as described by Melton et al. (9) by using either SP6 or T7 polymerase and DNA fragments cloned into pGEM-3 or pGEM-4 (Promega). Templates for LDL receptor RNA synthesis were derived from a 2.4-kilobase Xmn I–Xho I fragment corresponding to nucleotides 53–2475 of a cDNA

Abbreviations: apo, apoprotein; 32S-cRNA, 35S-labeled complementary RNA; CNS, central nervous system; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low density lipoprotein; WHHL rabbit, Watanabe heritable-hyperlipidemic rabbit.
for the rabbit LDL receptor (10) cloned into pGEM-3 and linearized with EcoRI (antisense probe) or cloned into pGEM-4 and linearized with HindIII (sense-strand control). This fragment contains nucleotide sequences corresponding to nearly the entire coding region of the receptor cDNA. Templates for HMG-CoA synthase RNA synthesis were derived from a HindIII fragment of plasmid p53-312 (11) corresponding to nucleotides 29–1581 of the hamster HMG-CoA synthase cDNA. This fragment was cloned into pGEM-3 in both orientations (for antisense probe and sense-strand control) and linearized with EcoRI.

Animals were anesthetized with phenobarbital sodium (50 mg/ml; 0.2 ml for 9-day-old animals, i.p.; 2 ml for adults, i.v.) and perfused transcardially with aldehydes by using a “pH-shift” method (12). Frozen sections (20 µm thick) were cut on a sliding microtome, mounted on poly(1-lysine)-coated slides, and air-dried under vacuum. Pretreatment, hybridization, and washing conditions were similar to those described (13, 14). Briefly, sections were treated with proteinase K (10 µg/ml, 37°C, 30 min) in adult tissues or Triton X-100 in neonatal tissues, acetylated, and dehydrated. After thorough drying, 70 µl of hybridization mixture containing 35S-labeled probe (106 cpm/ml, with 10 mM dithiothreitol) was spotted on each slide, sealed under a cover glass, and incubated at 55°C for 16 hr. Slides were then rinsed, digested with ribonuclease A (20 µg/ml, 37°C, 30 min), washed in 15 mM sodium chloride/1.5 mM sodium citrate at pH 7 for 30 min at 35°C, and dried. The sections were exposed to βmax Hyperfilm (Amersham) at 4°C for 4–6 days for preliminary analysis and to estimate liquid emulsion exposure time. The sections were then delipidized with xylene, rinsed with absolute ethanol, air-dried, and coated with Kodak NTB2 liquid emulsion diluted 1:1 with distilled water. After an exposure time of 2 weeks at 4°C, the slides were developed with Kodak D-19, fixed, and stained through the emulsion with thionin. Because of the ribonuclease A treatment, thionin stained only cell nuclei. Adjacent series of unhybridized sections were also stained with thionin to identify neuronal size and shape.

**RESULTS AND DISCUSSION**

Fig. 1 *Upper* shows autoradiograms of tissue sections from a 9-day-old rabbit that were incubated with a 35S-labeled antisense RNA complementary to the LDL receptor mRNA. As a positive control we first examined the adrenal gland, the richest known source of LDL receptors (1). Silver grains were present over the entire adrenal cortex, including the zona glomerulosa, zona fasciculata, and zona reticularis. There was no hybridization to the medulla. Fig. 1 *Upper* also shows abundant hybridization of the 35S-labeled complementary RNA (35S-cRNA) in two parts of the nervous system: the trigeminal ganglion (Middle) and the hippocampus (Right). Control incubations with a 35S-labeled sense-strand probe gave no signal in the adrenal or trigeminal ganglion (*Lower*). Some background hybridization was observed in the hippocampus, but it was much less intense than that obtained with the antisense probe.

Fig. 2 shows sections of the same three tissues incubated with the 35S-labeled antisense probe for HMG-CoA synthase mRNA. Again, the adrenal cortex showed abundant labeling, whereas the medulla was negative. However, in marked contrast to the results with the LDL receptor probe, the HMG-CoA synthase probe hybridized weakly to the zona glomerulosa in comparison with the other two zones. These studies suggest that the zona fasciculata and zona reticularis derive cholesterol both from endogenous synthesis and from LDL uptake, whereas the zona glomerulosa derives cholesterol primarily from LDL uptake. Glomerulosa cells require much less cholesterol than cells in the other two zones, since glomerulosa cells produce relatively small amounts of steroid (15). The trigeminal ganglion and the hippocampus showed extensive

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**Fig. 1.** Dark-field photomicrographs showing hybridization of antisense- and sense-strand 35S-cRNA probes for LDL receptor to tissue sections through the adrenal gland, trigeminal ganglion, and hippocampus of a 9-day-old rabbit. Note dense hybridization (silver grains appear white) to the entire thickness of the adrenal cortex (ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis), but not to the medulla (M). (*×60.*) In the trigeminal ganglion (and in dorsal root ganglia), many sensory ganglion cells are labeled, as are many small cells, presumably Schwann cells. (*×200.*) The pyramidal layer of fields CA1 and CA3 in the hippocampus are also heavily labeled, as are presumed glial cells in the corpus callosum (wide diagonal stripe in upper right corner). (*×15.*) No specific label was observed in sections hybridized with sense-strand probes.
hybridization with the HMG-CoA synthase cRNA probe, suggesting that cells in these regions synthesize cholesterol.

Table 1 lists those areas of the CNS that contained relatively high levels of mRNA for the LDL receptor and HMG-CoA synthase in neurons, as judged by clusters of silver grains over large pale nuclei (Fig. 3). Labeling for LDL receptor mRNA was very high in dorsal root ganglion sensory cells, the trigeminal ganglion, and the mesencephalic nucleus of the trigeminal nerve. LDL receptor mRNA was also present in various motor-related and other neurons in widespread parts of the brain (Fig. 4) and in glia in many regions of the nervous system. The distribution of mRNA for HMG-CoA synthase paralleled that for the LDL receptor (Table 1). This finding suggests that cells in a single region synthesize cholesterol and take it up from lipoproteins. It is not yet clear whether the same cells carry out both processes.

In many areas of the CNS, hybridization signals for the receptor and synthase were no higher than the levels observed with sense-strand control probes. The most notable of these receptor and synthase-deficient areas were the cerebellum, striatum and lateral septum, isocortex, and lateral part of the reticular formation.

In Figs. 1-4 the sections were prepared from 9-day-old rabbits, which are actively engaged in myelination (16). We also studied the LDL receptor in sections from adult rabbit brain and obtained the same general results. We have not yet studied HMG-CoA synthase in sections from adult brain.

To confirm that the visualized mRNA was derived from the LDL receptor gene, we performed an S1-nuclease analysis on mRNA obtained from normal rabbits and from WHHL rabbits, which have a deletion of 12 base pairs in the coding region of the gene (10). A uniformly 32P-labeled cDNA probe was allowed to hybridize to the various RNA samples, and the mixture was then digested with the single-strand specific S1 nuclease (Fig. 5). The products were subjected to electrophoresis and autoradiography. Adrenal mRNA from normal animals protected the entire coding region of the probe from S1-nuclease digestion. Only the vector sequences at the 3' end were digested. Similar full-length protection was obtained with RNA from the superior colliculus, hippocampus, and trigeminal ganglion. The amounts of mRNA were similar in the trigeminal ganglion in an adult animal and a 10-day-old animal, as judged from the intensities of the protected bands.

Table 1. Neuron populations that contain mRNA for LDL receptor and HMG-CoA synthase in 9-day-old rabbits

<table>
<thead>
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<th>Neuron populations</th>
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<tr>
<td>Sensory ganglion cells: dorsal root, trigeminal, and mesencephalic n. of trigeminal</td>
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<tr>
<td>Somatic motor neurons: ventral horn (spinal cord), hypoglossal n., n. ambiguus, facial n., and trigeminal motor n.</td>
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<tr>
<td>Preganglionic parasympathetic neurons: dorsal motor n. of vagus and Edinger-Westphal n.</td>
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<tr>
<td>Motor-related cell groups: globus pallidus, subthalamic n., compact part of the substantia nigra, ventral tegmental area, red n., and lateral reticular n.</td>
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<tr>
<td>Sensory relay nuclei: vestibular nuclei, external cuneate n., and superior colliculus</td>
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<tr>
<td>Thalamus and hypothalamus (all parts)</td>
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<tr>
<td>Basal forebrain cholinergic regions: medial septal n., diagonal band n., substantia innominata, and magnocellular preoptic n.</td>
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<tr>
<td>Amygdala (all parts) and bed n. of stria terminalis</td>
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<tr>
<td>Cerebral cortex: piriform cortex and olfactory tubercle</td>
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<tr>
<td>Reticular formation and related cell groups: large and medium-sized neurons throughout length of medial half of reticular formation, all Raphé nuclei, and locus coeruleus</td>
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n., nucleus.
When the mRNA was obtained from WHHL rabbits, the probe was cleaved by the S1 nuclease to yield fragments of 292 and 249 nucleotides (Fig. 5). Inasmuch as the site of this cleavage corresponds to the known site of the deletion in the WHHL gene, this result demonstrates that the mRNA in the CNS is produced by the authentic LDL receptor gene (10).

The current results, when coupled with previous data (5–8), imply that the brain has an active system for transporting and synthesizing cholesterol. This system appears to be physically distinct from the lipoprotein transport system in plasma. Cholesterol transport in the CNS originates in astrocytes, which synthesize and secrete apoE-containing lipoproteins. The cholesterol in these lipoproteins is most likely derived from the turnover of myelin and plasma membranes of neurons and glia. The apoE-containing lipoproteins secreted by the astrocytes are targeted to cells that express LDL receptors. As shown in the current studies and in the studies of Pitas et al. (8), LDL receptor-bearing cells, including neurons and glia, are distributed widely in the CNS. This transport mechanism would allow continuous recycling of cholesterol from one cell to another in the brain. It would also allow cholesterol to be exported out of the brain through the movement of apoE-containing lipoproteins from cerebrospinal fluid to plasma (8). Cells in sensory ganglia may also be able to use their LDL receptors to take up circulating LDL. The capillary endothelia in these ganglia contain fenestrae (18) that may permit the passage of plasma lipoproteins.

Cells in the brain also have the ability to produce their own cholesterol. In rabbits, the overall rate of cholesterol synthesis, when averaged throughout the brain, is lower than the rate in the adrenal gland, but higher than it is in kidney and skeletal muscle (19). The current studies raise the possibility...
that cholesterol synthesis may be much higher in certain regions of the brain. In general, the regions that show high levels of mRNA for HMG-CoA synthase also show high levels of LDL receptor mRNA. These regions must have a particularly high turnover of cholesterol that they satisfy through endogenous synthesis and uptake of apoE-containing lipoproteins.

The question arises as to whether the high requirement for cholesterol in certain regions of the brain is attributable solely to the cholesterol required for structural purposes, or whether cholesterol might be converted into other products, such as steroid hormones, in these regions. LeGoascogne et al. (20) have shown that the cholesterol side-chain cleavage enzyme is present in oligodendrocytes and other cells in the brain, particularly in the white matter and in the olfactory bulb. This enzyme produces pregnenolone. It is possible that some of the LDL receptor activity and cholesterol biosynthetic activity in the nervous system is used to provide a substrate for this steroid synthesis.

If the LDL receptor plays a physiologic role in neural tissues, backup systems must exist. Genetic defects in the LDL receptor in humans or in WHHL rabbits do not cause obvious malfunctions of the CNS (2). Brain cells that ordinarily use LDL receptors may thus develop increased cholesterol synthesis when LDL receptors are deficient. Evidence for such a backup was obtained in the adrenal gland of the WHHL rabbit, which has a 5-fold elevated rate of cholesterol synthesis (21). It will be important to determine whether cholesterol synthesis and levels of mRNA for cholesterol biosynthetic enzymes are elevated in certain cell types of the CNS of WHHL rabbits.

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