Schilder's Disease: Abnormal Cholesterol Retention and Accumulation in Cultivated Fibroblasts

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Extract

Schilder's disease (SD) with adrenal insufficiency is a fatal X-linked recessive disorder for which the basic defect is as yet unknown. To investigate the hypothesis that Schilder's disease might be associated with a generalized defect in cholesterol metabolism, the uptake and disappearance of labeled cholesterol and cholesterol esters were studied in cultivated fibroblasts from patients with Schilder's disease, patients with familial hypercholesterolemia, and normal control subjects. Over a 10-12-day period of exposure to the labeled compound, SD fibroblasts accumulated significantly more cholesterol than did normal fibroblasts ($P < 0.001$). In addition, after a 24-hr exposure to the labeled cholesterol, the SD fibroblasts retained significantly more of the label than did control cells over a 5-day period of study ($P < 0.001$). The retained label was still located in the cholesterol fraction on thin layer chromatography in both SD and normal cells. The total cholesterol content of SD cells when measured directly after 12 days in tissue culture was significantly greater than that of normal cells.

Speculation

The results of these studies support the concept that Schilder's disease may indeed be related to a generalized defect in cholesterol metabolism or transport. An alternative explanation for these findings is that Schilder's disease may be associated with a defect in membrane structure or turnover that is not directly related to cholesterol metabolism but can be followed *in vitro* by cholesterol accumulation and retention.

Introduction

Schilder's disease, also called diffuse cerebral sclerosis or sudanophilic leukodystrophy, is a heterogeneous group of fatal degenerative neurologic disorders associated with morphologic findings similar to those observed in multiple sclerosis [2, 14, 17]. One entity within this group is inherited as an X-linked recessive disorder and may be associated with adrenocortical atrophy with adrenal insufficiency of varying degrees ranging from classic Addison's disease to isolated un-

responsiveness to exogenous ACTH administration [5, 7, 8, 16, 18, 19].

Recently, Schaumburg *et al.* [16] have examined the adrenal glands of male patients with Schilder's disease with and without clinically demonstrable adrenal insufficiency. In all cases studied, distinctive changes were noted in the adrenal cortex including the presence of nests of ballooned, nonsudanophilic cortical cells containing cytoplasmic aggregates of birefringent crystals. Similar findings had previously been reported in Wol-

man's disease [12] and in aniline intoxication [10], both of which are characterized by abnormalities in cholesterol metabolism. Biochemical studies of the brain in a number of patients with Schilder's disease have revealed an absolute and relative increase in esterified cholesterol [5]. Eto and Suzuki [4] have demonstrated the presence of cholesterol esters with an abnormal fatty acid composition in the brains of patients with Schilder's disease and other disorders associated with sudanophilic demyelination. Menkes and Eviatar [13] reported the presence of an abnormal sterol with the same number of carbon atoms as cholesterol in the white matter of the brain in one patient with Schilder's disease with adrenal insufficiency.

On the basis of this evidence, we hypothesized that Schilder's disease might be related to a generalized defect in cholesterol metabolism and that such a defect might be demonstrable in tissue culture. We present here the results of our investigations of the handling of labeled cholesterol and cholesterol esters by cultivated fibroblasts from normal individuals and patients with Schilder's disease and our measurements of the total cholesterol content of these cells. The data obtained support the concept that Schilder's disease may indeed be related to a generalized defect in cholesterol metabolism.

Materials and Methods

Tissue Culture

Fibroblasts were cultivated from three male patients with SD, all of whom had a family history of males with a degenerative neurologic disease compatible with the diagnosis of Schilder's disease. One of these patients had overt manifestations of adrenal insufficiency; a second demonstrated normal base-line function but unresponsiveness to exogenous ACTH; the third had normal base-line function with an equivocal response to ACTH administration. Cells were also cultivated from two patients with familial hypercholesterolemia (*type II* hyperlipoproteinemia), and from normal age-matched control subjects. Cells were grown in minimum essential medium [20] supplemented with 15% fetal calf serum and were incubated at 37° in an atmosphere containing 5% CO₂. At the time of our studies, all cells had undergone 5–15 passages in tissue culture.

Preparation of Labeled Medium

(4-¹⁴C)Cholesterol and cholesteryl (1-¹⁴C)palmitate [21] were added to heat-inactivated fetal calf serum.

The labeled serum was allowed to equilibrate for several hours at 37°. Five percent fetal calf serum which contained labeled cholesterol or cholesterol ester was then added to minimum essential medium [20] for use in the disappearance studies while 15% fetal calf serum was added for the uptake studies. All media was counted before use. Initial activity ranged from 0.6–2.0 × 10⁴ cpm/ml.

Disappearance Studies

This series of experiments was performed using both confluent cells and cells that had been subcultured 1 day before initiation of the experiment. To each 250-mm flask of fibroblasts were added 10 cu cm medium which contained labeled cholesterol or cholesterol ester. After 24 hr of exposure to the labeled medium, the cells were washed once with Puck's saline A solution and 10 cu cm nonlabeled medium which contained 15% fetal calf serum were added to each flask. Each subsequent day, the medium was removed, the cells were washed once with Puck's saline, and 10 cu cm medium were again added.

Duplicate flasks of cells from each cell line were harvested on *day 1*, after 24 hr of exposure to the labeled medium, and on each subsequent day for a total of 5 days. Cells to be harvested were washed three times with normal saline and then removed from the flasks with 0.1% trypsin. The suspended cells were removed from the flasks, centrifuged, and washed twice with normal saline. The cell pellet was dissolved by heating in 0.5 cu cm 2.5 N NaOH. Aliquots were removed and counted in Bray's solution in a liquid scintillation counter [22]. The remainder of each sample was saved for determination of protein content [11].

Uptake Studies

Medium was removed from cells that had been subcultured 1 day previously and replaced by 20 cu cm medium containing 15% fetal calf serum and the labeled compound. Duplicate flasks of cells were harvested and counted as in the disappearance studies, at 1- or 2-day intervals for a total of 10–12 days. The medium was removed every 4 or 5 days during the course of the study and was replaced with fresh medium also containing the labeled compound.

Thin Layer Chromatography

An additional flask of each cell line was harvested on *day 3* of the disappearance study for lipid analysis. The cell pellets were extracted with chloroform-meth-

anol and thin layer chromatography was performed in accordance with the method of Gloster and Fletcher [6]. Each lipid fraction was scraped from the plate and washed several times with chloroform. The eluted samples were evaporated and counted in Bray's solution in a liquid scintillation counter [22].

Determination of Total Cellular Cholesterol

Fibroblasts were grown in medium containing 15% fetal calf serum for periods of 4, 10, and 12 days after subculture. The medium was changed at 3–4-day intervals throughout the period of study. Ten flasks of each cell line were harvested, as in the disappearance studies, and pooled into a single cell pellet. The cells were washed two times with normal saline. Each cell pellet was resuspended in distilled water to a total of 2 ml. Two-tenths milliliter was removed for protein determination [11].

To the remaining 1.8 ml, 5 ml chloroform-methanol (2/1) were added. The sample was then allowed to stand overnight in the refrigerator. The following day, the top aqueous layer was removed and discarded. Each sample was filtered through glass filter paper and was washed in chloroform-methanol. The solvent was evaporated and the samples were redissolved in 2 ml chloroform. One milliliter was removed and saved for possible future studies. The remaining 1 ml was evaporated. The samples were hydrolyzed with alcoholic KOH and extracted with ligroin. Total cholesterol was determined by the process of Abell *et al.* [1].

Results

Disappearance of (4-¹⁴C)Cholesterol

The results of the disappearance studies using (4-¹⁴C)-cholesterol are illustrated in Figure 1. The data are expressed as counts per minute per milligram protein. Duplicate flasks of each cell line were harvested each day and the values displayed represent an average of the two values obtained. The uptake of cholesterol by the end of the 24-hr period of exposure, designated *day 1*, was similar in all cell lines studied. Over the following 4 days, however, cells from all three patients with Schilder's disease retained significantly greater amounts of label than did cells from control subjects. The data illustrated in Figure 1 reveal that the Schilder's cells contain an average of 80% more labeled cholesterol than do normal cells on *days 2 through 5* ($P < 0.001$). These differences are actually higher than those in a typical experiment in which only one Schilder's cell

Fig. 1. Disappearance of (4-¹⁴C)cholesterol over a 5-day period in fibroblasts from three patients with Schilder's disease (---) and three control subjects (—). The values are expressed in counts per minute per milligram protein.

line was studied with one or more controls. In each of the individual experiments, the Schilder's cells retained at least 25–40% more label than did the normal cells. The data presented in Figure 1 were selected for illustration because they represent the simultaneous study of the three mutant cell lines.

Cells from patients with familial hypercholesterolemia, not illustrated here, behaved as controls. Similar results were obtained when confluent or nonconfluent cells were used and when various concentrations of labeled cholesterol were added to the medium.

Disappearance of Cholesteryl (1-¹⁴C)Palmitate

No significant differences in cholesterol ester retention could be demonstrated between SD fibroblasts and controls during the period of the disappearance study. Valid data was difficult to obtain, however, due to technical difficulties. The labeled cholesterol ester could not be evenly dispersed throughout the original medium, so that it was impossible to be sure that each flask of cells was exposed to the same concentration of the labeled compound. In many instances, poor duplicate values were obtained. In addition, significant quantities of label were retained by all cell lines over the 5-day course of study. It is possible that differences not obvious here might become evident if the observations were extended over longer periods of time.

Thin Layer Chromatography

Thin layer chromatography was performed on extracts of SD and control fibroblasts on *day 3* of the disappearance studies to determine whether the retained label in each cell line represented the compound administered (cholesterol or cholesterol ester) or another major lipid. The results of this study are presented in Tables I and II. The data are expressed as the percentage of the total activity of the cell preparations (counts per minute per milligram protein) present in each lipid fraction.

Essentially all of the label administered to the cell as (4-¹⁴C)cholesterol was still detectable in the cholesterol fraction in both SD and control fibroblasts. Only 0.5–1.0% had been converted to cholesterol esters. Of the label administered as cholesteryl (1-¹⁴C)palmitate, 88% and 90% was still located in the cholesterol ester fraction in SD and control fibroblasts, respectively. Small quantities of label were located in each of the other lipid fractions. No significant differences in the distribution patterns were noted between SD and control fibroblasts.

Uptake of (4-¹⁴C)Cholesterol

The uptake of (4-¹⁴C)cholesterol by cultivated fibroblasts is illustrated in Figure 2. The data were originally expressed as counts per minute per milligram protein in each cell sample. It is presented here as percentage of maximum control activity *versus* time.

Table I. Percentage of total counts which remain in each fraction on *day 3* of cholesterol disappearance curve

	Schilder's disease	Control
Phospholipid	0.5	1.5
Cholesterol	98.0	98.0
Free fatty acids	0.5	0.0
Triglycerides	0.0	0.0
Cholesterol esters	1.0	0.5
Total	100.0	100.0

Table II. Percentage of total counts which remain in each fraction on *day 3* of cholesterol ester disappearance curve

	Schilder's disease	Control
Phospholipid	2.0	1.0
Cholesterol	4.0	3.0
Free fatty acids	2.0	4.0
Triglycerides	4.0	2.0
Cholesterol esters	88.0	90.0
Total	100.0	100.0

Fig. 2. Uptake of (4-¹⁴C)cholesterol in fibroblasts from two patients with Schilder's disease (---) and three control subjects and two patients with hypercholesterolemia (—). —, composite of values for the control subjects and hypercholesterolemia patients. The data is expressed as percentage of maximum control activity *versus* time. Horizontal bars represent ranges of activity in control subjects and hypercholesterolemic patients.

This method of expression of the data was selected so that uptake studies performed on different occasions with various concentrations of labeled cholesterol could be consolidated and compared on a single graph. The data presented in Figure 2 represent a composite of two different studies using fibroblasts from two patients with SD, two patients with familial hypercholesterolemia, and three control subjects. Similar results were obtained using various concentrations of labeled cholesterol in the original medium.

The uptake of (4-¹⁴C)cholesterol was similar in SD and control fibroblasts over the first 4–5 days of the study period. After this time, however, the control fibroblasts reached a plateau and uptake ceased to increase appreciably. The SD fibroblasts exhibited a small but steady increase in uptake over the remaining 6–7 days of the study. On *day 11*, SD fibroblasts contained, on the average, 25–40% more label than did control cells ($P < 0.001$). Fibroblasts from patients with familial hypercholesterolemia behaved as controls.

Uptake of Cholesteryl (1-¹⁴C)Palmitate

Fibroblasts from patients with Schilder's disease and from normal control subjects all continued to accumulate the cholesterol ester steadily over the 10-day period of study. This study was difficult to repeat and the data difficult to interpret because of the technical difficulties encountered in dealing with the labeled ester.

From our limited data, however, no difference could be observed in the uptake of the ester by control and SD fibroblasts.

Total Cellular Cholesterol Content

Results of the measurements of total cholesterol in fibroblasts from one patient with SD, two patients with familial hypercholesterolemia, and four control subjects are summarized in Table III. All values listed represent an average of two separate determinations. Four days after subculture, the cholesterol content of all cell lines was similar. After 10 days, the cholesterol content of the Schilder's cells was somewhat higher than that observed in control cells, but the difference was not statistically significant. After 12 days, however, the total cholesterol content of the SD cells was significantly higher than that of normal and hypercholesterolemic cell lines with a $P < 0.01$.

Discussion

These data suggest evidence that Schilder's disease may be related to a generalized defect in cholesterol metabolism. The defect would not appear to be that of either increased cholesterol uptake from the tissue culture medium or increased endogenous synthesis of cholesterol or cholesterol esters. A likely possibility is that the defect observed in the handling of cholesterol in fibroblasts from patients with Schilder's disease is one of either intracellular metabolism or transport of cholesterol or a related compound.

Little is known of the metabolism of cholesterol, or of steroids in general, in cultivated human fibroblasts. It has previously been demonstrated that these cells have the ability to incorporate acetate into cholesterol [3] and to convert the cholesterol precursor desmosterol to cholesterol [9]. Other cell types in tissue culture are known to have the ability to esterify cholesterol, to

hydrolyze cholesterol esters to free cholesterol and fatty acids, to cleave the first carbon atom from the fatty acid of cholesterol esters to yield CO_2 , and to take up both cholesterol and cholesterol esters from the tissue culture medium but to release only free cholesterol [15].

At the present time, no data is available with regard to the ability of cultivated human fibroblasts to synthesize other sterols from cholesterol. It is possible that Schilder's disease might be related to a metabolic block involving an intermediate in sterol metabolism rather than cholesterol itself. In this case, the label derived from cholesterol might accumulate within the cells either as the sterol or as cholesterol. An alternative hypothesis is that the retention and accumulation of labeled cholesterol observed in Schilder's fibroblasts is related to a nonspecific defect in membrane turnover or transport. Cholesterol is an important constituent of cell membranes and a defect in membrane structure or degradation might be reflected by a relatively normal rate of incorporation of the labeled compound into membrane structures but delayed release. Likewise, a defect in membrane transport mechanisms might be reflected by difficulty in the extrusion of cholesterol and of other compounds from the cell.

It is clear that further studies of cholesterol transport and metabolism in normal fibroblasts, fibroblasts of patients with Schilder's disease, and fibroblasts from patients with other degenerative neurologic diseases are essential to the further interpretation of the data presented here. Such studies may help to elucidate the basic biochemical defect in this disorder.

Summary

The uptake and disappearance of labeled cholesterol and cholesterol esters has been studied in cultivated fibroblasts from patients with Schilder's disease, patients with familial hypercholesterolemia, and normal control subjects. The initial uptake of cholesterol was similar in control and Schilder's fibroblasts but, on continuous prolonged exposure to the labeled compound, Schilder's fibroblasts accumulated significantly greater amounts of label than did control cells. When, after a 24-hr exposure to labeled cholesterol, the labeled compound was removed from the tissue culture medium, fibroblasts from patients with Schilder's disease retained significantly greater amounts of label than did control cells. The retained label remained in the cholesterol fraction on thin layer chromatography. Measurements of total cholesterol in fibroblasts after pro-

Table III. Total cellular cholesterol

Cell line	4 Days	10 Days	12 Days
	mg/mg protein		
Schilder's disease	0.030	0.052	0.069
Control			
1	0.040	0.047	0.048
2	0.036	0.045	0.038
3	0.038	0.042	0.042
4		0.046	
Hypercholesterolemia			
1	0.035		0.051
2	0.038		0.053
Mean of Controls	0.038	0.045	0.043

longed periods in tissue culture revealed an absolute increase in the total cholesterol content of Schilder's cells as compared with normal cells. No significant differences were observed between Schilder's and normal cells in the uptake and disappearance of cholesterol esters. Fibroblasts from patients with familial hypercholesterolemia behaved as controls in all studies.

These data indicate clearly that Schilder's disease may be related to a generalized defect in cholesterol metabolism or transport.

References and Notes

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