Prenatal Diagnosis of Neurolipidoses

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ABSTRACT

The prenatal diagnosis of Tay-Sachs disease is presented to illustrate the procedures, problems, and some methodological pitfalls associated with prenatal diagnosis of neurolipidoses.

Introduction

There exists a group of metabolic diseases characterized by the accumulation of complex lipids in lysosomes. They are variously categorized either according to the accumulated compounds (sphingolipidoses, gangliosidoses), the site of accumulation (lysosomal storage diseases), or the tissue affected, (e.g., neurolipidoses).

In table I are listed those diseases in which there is neurological impairment caused by the accumulation of complex lipids. They are all genetic diseases inherited as autosomal recessive traits, and in each case the lipid accumulation is the result of the enzyme deficiency shown.

Each of the conditions exhibits a fairly wide spectrum of clinical severity, ranging from severe infantile forms in which symptoms appear early and death usually occurs before age five, to juvenile or adult forms where symptoms appear later and life expectancy is longer.

Prenatal diagnosis of each of the diseases is accomplished by biochemical analysis of cultured amniotic fluid cells and where appropriate, cell-free amniotic fluid. Cells are removed from the amniotic fluid, grown in tissue culture, and when sufficient cells are available (2.5 to four weeks) they are harvested and assayed for the enzyme in question. The assay procedures have been described in several recent publications.1,7,14

Each of the enzymes can be assayed by several different procedures, some of which, however, may be unsuitable for prenatal diagnosis, and some difficult to interpret. An important distinction exists between the ability to perform an enzyme assay and the ability to make a correct diagnosis. The purpose of this presentation is to emphasize this distinction by describing precautions and pitfalls to help convey the need to understand the limitations of the methods, the heterogeneity of the enzyme under study, and the clinical heterogeneity of the disease. Tay-Sachs disease will be used as an example to explain the requirements for successful diagnosis of the neurolipidoses because it is clinically representative of the lipid storage diseases, its prenatal diagnosis il-
lustrates some typical pitfalls, and it is the disease with which the most experience has been gained.

Tay-Sachs disease is a lysosomal storage disease caused by a deficiency of the glycolytic enzyme hexosaminidase A (hex A). Because of the deficiency of hex A, its natural substrate (GM₃ ganglioside) accumulates in lysosomes of nerve tissue bringing about the clinical manifestations of the disease characterized by progressive neurological impairment resulting in blindness, deafness, and loss of motor function. The disease is invariably fatal with death usually resulting from respiratory illness before age five. Children affected with Tay-Sachs disease show an absence of hex A in all tissues. This absence of enzyme activity is also demonstrable in cultured amnion cells and in cell-free amniotic fluid of affected fetuses so both tissues are useful for prenatal diagnosis of Tay-Sachs disease.

Obtaining and Preparing Samples

The success of a diagnosis is heavily dependent on the quality of the sample. Because many people are usually involved (patient, obstetrician, tissue culturist, biochemist, genetic counselor), good communication is necessary to assure that an adequate sample is obtained and then handled and processed properly. Sufficient fluid should be obtained (20 ml) to maximize the chance for good cell growth. The fluid should be free of blood for the reason noted later.

The usual precautions must be observed in cell culture to avoid contamination and to produce physiologically sound cells. Enzyme activity is influenced by the culture conditions and the stage of growth at the time of harvest. To control this as well as possible, amnion cells derived from the fetus at-risk should be grown under identical conditions with fibroblasts known to harbor the enzyme defect in question and with normal control cells.*

Biochemical Analysis

Hex A can be readily assayed with the fluorogenic substrate 4-methylumbelliferonyl-N-acetylglucosaminide. However, its analysis in prenatal diagnosis for Tay-Sachs disease is complicated by the presence of other hexosaminidase isozymes which also react with this substrate. The known human isozymes are B, I, I₂, A, C, and S, listed in order of decreasing pI. Because these isozymes all differ in charge, they can be separated by ion-exchange chromatography, electrophoresis, and isoelectric focusing. Some commonly used methods are diethylaminoethyl (DEAE) cellulose chromatography, and electrophoresis by starch gel, Cellogel, and polyacrylamide gel. Still another way to distinguish hex A is by the heat inactivation method which relies on the relative heat lability of hex A. Hex B and A are the major forms and are found in all tissues including cultured amnion cells and amniotic fluid. Some of the minor forms are also detectable in cultured amnion cells and amniotic fluid and must be taken into consideration in prenatal diagnosis. Therefore, the key to successful prenatal diagnosis of Tay-Sachs disease is

TABLE I

<table>
<thead>
<tr>
<th>Disease</th>
<th>Enzyme Defect</th>
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<tbody>
<tr>
<td>Gaucher (infantile)</td>
<td>Glucocerebrosidase</td>
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<tr>
<td>Niemann-Pick (types A-D)</td>
<td>Sphingomyelinase</td>
</tr>
<tr>
<td>Krabbe</td>
<td>Galactocerebrosidase</td>
</tr>
<tr>
<td>Metachromatic leukodystrophy</td>
<td>6-galactosidase</td>
</tr>
<tr>
<td>Generalized gangliosidosis</td>
<td>Arylsulfatase A</td>
</tr>
<tr>
<td>Tay-Sachs disease</td>
<td>6-Galactosidase A &amp; B</td>
</tr>
<tr>
<td>Sandhoff disease</td>
<td>Hexosaminidase A</td>
</tr>
<tr>
<td></td>
<td>Hexosaminidase A &amp; B</td>
</tr>
</tbody>
</table>

* Affected cell lines can be obtained from the Institute for Medical Research, Camden, NJ.
a command of the techniques which separate or distinguish hex A from other isozymes and a thorough knowledge of the properties and relationships of the isozymes to avoid an error in diagnosis by confusing them.

Optimally, a diagnosis will be made on both cell-free fluid and cultured cell extracts using several different tests. The procedure followed in our laboratory is to remove by centrifugation cells from fresh fluid for planting in tissue culture. Part of the cell-free fluid is dialyzed and analyzed directly by DEAE chromatography, and another portion is concentrated and analyzed by Cellogel, starch gel, and polyacrylamide gel electrophoresis. When cultured cells have grown they are analyzed with the same three electrophoretic procedures, and by heat inactivation.

Use of Cultured Cell Extracts

The two major isozymes of cultured cell extracts are hex A and B which are clearly distinguished from one another by all methods. In figure 1 is shown the typical pattern obtained upon polyacrylamide gel electrophoresis of normal cultured cell extracts. In affected fetuses, the hex A peak is absent. Because hex A is such a major component in cultured cell extracts, its absence is very easily detected. Cultured cell extracts also contain two other minor isozymes which may or may not be detectable depending on the sample, the method used and the conditions of assay.

One of these minor isozymes is hex C which, like hex A, is heat labile and anodic. Electrophoretically it migrates ahead of hex A but is frequently not detectable either because there is too little present or because it is obscured by the large amount of hex A. For this reason it is most easily detected in extracts of Tay-Sachs cells which contain no hex A to mask it. It then appears as a faint but distinct band in the hex A region of the gel and can be confused with hex A without proper controls and an awareness of its existence.

The other minor isozyme occasionally encountered is seen most distinctly in polyacrylamide gels and is indicated by the arrow in figure 1. This activity peak is usually minor and may or may not be detectable in any given sample. In an occasional sample, it is present in larger amounts and becomes significant in interpreting the results. An example of this is shown in figure 2a. Most of the enzyme activity (indicated by the arrow) fell squarely between the hex A and B peaks on repeated electrophoresis with different controls. Hex B appeared as only a minor component in the region of slices 4 to 6, and there was no peak of hex A activity. (Starch gel electrophoresis and heat inactivation of this extract also showed the absence of hex A, as did prior studies on
the cell-free amniotic fluid, indicating an affected fetus). An extract prepared from a later passage of that same cell line showed the pattern in figure 2b, which still contained the intermediate peak but also a more pronounced B peak. A second amniocentesis was performed prior to termination of the pregnancy, and cultured cell extracts from that fluid showed the pattern in figure 2c, which approached the typical pattern seen in an affected fetus: the B peak was prominent, the intermediate peak was only a broad shoulder, and hex A was still absent.

From a review of all our polyacrylamide gel data of other cell extracts, it seems that this intermediate peak of activity is a normal component, usually present in small amounts and, because of this, frequently obscured by hex A and B. Occasionally however, as described previously, it can be a dominant component that could be confused with hex A. This rather striking difference in isozyme patterns of three different cell batches of the same individual illustrates the earlier comment that the isozyme pattern is dependent on the physiological state of the culture at harvest.

A third potential pitfall leading to misclassification of the isozymes may be encountered in the heat inactivation assay in which hex A can be distinguished from the others by its heat lability. When tissue extracts are heated at 52°C for two hours, all or nearly all hex A activity is destroyed while the other isozymes remain active. Hence, the difference in activity between heated and unheated samples is a measure of the amount of hex A originally present. This method is valuable because it is based on a different physical property than all the other methods which rely on the charge of the isozymes to distinguish between them. It has, however, an inherent danger. If the conditions of heat inactivation are not optimal, it is possible to get a significant amount of hex B or I inactivation which, by definition, is interpreted as hex A. For example, heated and unheated samples from an affected fetus should be identical because no hex A is present to be inactivated. In practice, however, even under optimal conditions, a slight difference is usually found (three to ten percent). Therefore, considerable experience and caution are needed when interpreting these data, and the use of a posi-
tive control (preferably several) is absolutely imperative.

Another possible source of error involving the heat inactivation method has been suggested by Penton et al. Because hex C is heat labile, it would be destroyed by heat inactivation and possibly mistaken for residual hex A. It is important to emphasize these potential pitfalls in the heat inactivation test because the method is probably the best known (because of its use in heterozygote screening) and the simplest (requiring the least specialized equipment), resulting in its widespread use.

Use of Amniotic Fluid in Prenatal Diagnosis

After amniotic fluid has been centrifuged to obtain cells for tissue culture, the cell-free supernatant fluid itself can be used for diagnosis. Normal amniotic fluid appears to contain three isozymes. On DEAE column chromatography, starch gel, Cellogel, and polyacrylamide gel electrophoresis, the isozyme pattern corresponds to hex B, I, and A. In figure 3 is shown the pattern obtained by polyacrylamide gel electrophoresis of normal amniotic fluid and fluid from an affected fetus. Hex B and A separate well in this system, but hex I is seen only as a shoulder on the hex B peak. Note the relative difference in the amount of hex A and B compared to cell extracts and the characteristic absence of hex A in fluid from an affected fetus.

On isoelectric focusing of amniotic fluid, Christomanou et al have also found three isozymes which they identified as hex B, A, and a new isozyme, hex X. This hex X, with a pI of 4.4 compared to 5.2 for hex A, separated well from B and A on isoelectric focusing but comigrated with hex B on polyacrylamide gel electrophoresis, which suggests it may be the same as the isozyme referred to as hex I. Hex X has the same heat stability, pH dependence, and substrate specificity as hex B and I.

Although the true isozyme composition of fluid may not yet be known, all the separation methods discussed previously will reliably detect the presence of hex A provided proper controls are run to distinguish it from others. Different tests, or slightly different conditions of performing the same test can result in altered isozyme patterns, making it necessary for the investigator to know what to expect under his conditions and not rely solely on published observations of others.

Because hex A is a minor component in normal amniotic fluid, a few special precautions are necessary to avoid misleading results. (The amount of hex A seen in samples from unaffected pregnancies at-risk will often be even lower because 66 percent will be heterozygotes.) Care must be taken to obtain blood-free fluid, for the
presence of maternal serum in the fluid will introduce hex A into the sample with the possibility of a false negative result. Conversely, false positive results can be obtained if fluid is allowed to stand at ambient temperature for prolonged periods during which time hex A may be inactivated. Finally, it is necessary to concentrate the fluid because the hex A concentration in undiluted fluid may be too low to detect.

The heat inactivation method is not used by us for amniotic fluid analysis where, because of the characteristically low level of hex A, it would sometimes be difficult to distinguish real from spurious activity even with adequate controls.

Comparison of Cultured Cell Extracts and Amniotic Fluid

Both cultured cell extracts and amniotic fluid should routinely be used. Each offers certain advantages and they serve as a valuable cross check. The advantage of cultured cell extracts is the larger proportion of hex A which makes its presence or absence easier to detect. However, cells suffer the potential disadvantage of poor growth yield and contamination by bacteria, mycoplasma, or maternal cells, all of which can introduce non-fetal hex A. The advantages of amniotic fluid are the speed with which a diagnosis can be made, and the ease of control over contamination. Its disadvantage is the low level of hex A requiring more stringent methodology.

Cell extracts are frequently described as being inherently more reliable than fluid for prenatal detection of hex A. There is no real justification for this preference which probably stems from the fact that the lower levels of hex A in fluid are more difficult to detect. It would indeed be unwise to recommend the use of fluid without stressing the importance of technique, but there is no documented evidence of its unreliability. Our results between amniotic fluid and cultured cell extracts have always been in agreement.

Choice of Substrate

Hexosaminidase is usually measured with the synthetic substrate 4-methylumbelliferyl-N-acetylglucosaminide. This substrate is readily available, simple to use, and generally satisfactory. There is a small risk associated with its use in prenatal diagnosis in that it will fail to detect that rare variant in which a mutation has altered the enzyme's activity toward the natural substrate but not toward the synthetic substrate, in which case a false negative would result, or the reverse situation which would produce a false positive result. This problem can be avoided by using natural radioactive substrate. However, the natural substrate is difficult to assay, and not commercially available.

Summary

While there is no single best procedure for performing prenatal diagnosis, there is a rationale or strategy which will produce correct, reliable results. The investigator should be experienced with all the tests and know what to expect from each. At least two of these tests should be used (more if there is ambiguity) on amniotic fluid and cultured cell extracts. Which tests, and how many, are not as important as the skill and experience of the investigator performing them. Proper controls should be used. Probably the biggest single factor in successful prenatal diagnosis is the use of both negative and positive controls run simultaneously. No method, no matter how good or how well performed, can be counted upon to give sufficiently reproducible results to interpret without these controls. Finally, it is necessary for the investigator to be thoroughly familiar with the enzyme and its isozymes and the clinical heterogeneity of the disease.
Although the foregoing details pertain specifically to Tay-Sachs disease, similar or related problems exist in the prenatal diagnosis of any of the neurolipidoses. The need for care of the samples, appreciation of biochemical and clinical heterogeneity, the need for adequate techniques, and the importance of proper controls are requirements for diagnosing any of the neurolipidoses.

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References