Current Status of Prenatal Diagnosis and Heterozygote Detection of Cystic Fibrosis

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ABSTRACT

The hallmark for the laboratory diagnosis of cystic fibrosis is an increased sodium and chloride concentration in the exocrine secretory fluid, sweat. This abnormality is an early and invariable consequence of the cystic fibrosis gene in homozygous, affected individuals but is not a suitable diagnostic marker for detection of the heterozygote. In the past five years, laboratory investigations of ion transport in cultured skin fibroblasts and evaluations of the effects of the Na⁺,K⁺-ATPase inhibitor, ouabain, suggested that cellular expression of the electrolyte transport defect in cystic fibrosis might be detectable in vitro. Enhanced resistance of fibroblasts from cystic fibrosis patients to the cytotoxic effects of ouabain and dexamethasone and abnormalities in fibroblast sodium transport have been reported as has a difference in calcium transport in these cells. Confirmation is lacking both with regard to the diagnostic utility of these findings and to the relationship of the phenomena to the expression of the cystic fibrosis gene.

Since 1967 the accumulation of biologically active “factors” has been suggested to occur in body fluids from cystic fibrosis patients. Although the specificity, chemical identity, and relationship of such “factors” to the pathophysiological changes in cystic fibrosis remain to be established, the possibility of their occurrence has led to the postulated existence of a lectin-like component in serum which might have utility in heterozygote detection. Confirmatory results have not been reported. Investigations from Nadler’s laboratory have suggested a deficiency in an isozyme of a protease with arginine esterase activity is associated with cystic fibrosis and could result in the accumulation of biologically active peptides. This quantitative abnormality in arginine esterase holds promise for prenatal diagnosis of cystic fibrosis but has not been validated by other laboratories. Not all individuals with cystic fibrosis may demonstrate this deficiency. This enzymatic approach is investigative and does not now have general clinical applicability. At present, there still is no generally accepted technique which can be used to establish definitively the prenatal diagnosis of cystic fibrosis or the carrier state in this disease.
Introduction

With an incidence of 1 in 1600 to 2000 newborns, cystic fibrosis is the most frequent of the severe inherited single gene defects in Caucasians. This autosomal recessive disease has a calculated carrier frequency of about one in 20 individuals in Caucasian populations. Cystic fibrosis is typified by early childhood onset. Its presentation includes failure to thrive, malabsorptive disease, recurrent bacterial respiratory infections, and chronic progressive obstructive pulmonary disease. Death of these patients results from pulmonary failure or its complications and in the United States now occurs at an average patient age of 18 years.

This disease can be viewed as a generalized exocrinopathy. Functional and, in some glands, histological abnormalities are regularly found in the exocrine pancreas, goblet cells of the trachea and intestine, most but not all of the salivary glands, secretory glands of the male genital tract, and the eccrine sweat glands. The expression of the secretory dysfunction in these glands varies from one gland type to another. For example, exocrine pancreatic hypossecretion is found in about 85% of cystic fibrosis patients, yet tracheal goblet cells appear to hypersecrete mucus, and the sweat glands produce sweat with an abnormal electrolyte concentration but at flow rates comparable to those found in normal individuals. The physiological mechanisms which lead to these diverse functional changes are unknown at present.

The primary biochemical defect underlying these several changes in the cystic fibrosis patient has remained elusive despite intensive investigation. This lack of knowledge has precluded development of therapeutic approaches aimed at ameliorating the basic biochemical defect and has frustrated attempts to develop assays both for prenatal diagnosis of the disease and for heterozygote detection. One approach to the latter two problems has been to attempt to discover biochemical markers which are specific enough to identify cystic fibrosis cells or body fluids. Although such a marker might be relatively remote from the basic biochemical lesion, it could have significant diagnostic value. Several distinct quantitative biochemical differences between specimens from cystic fibrosis patients and other individuals recently have been proposed as diagnostic markers experimentally applicable to prenatal diagnosis and heterozygote detection of cystic fibrosis (table I). Here the biochemical bases of these assays are reviewed as are their possible relationship to the abnormalities which clinically characterize cystic fibrosis and the validity of the diagnostic tests as shown by clinical and experimental evaluations. Literature cited in this review was selected from that published through September 1, 1981.

Heterozygote Detection and Prenatal Diagnostic Tests Based Upon Abnormalities in Sodium Transport

The increased quantities of sodium and chloride found in the eccrine sweat of cystic fibrosis patients are independent of age or clinical status of the patient. The

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NADH = Reduced nicotinamide adenine dinucleotide
constancy of this finding and the magnitude of the increase have made the quantitation of sweat electrolytes an essential clinical laboratory test for the diagnosis of cystic fibrosis. Early and constant expression of abnormal sweat electrolyte levels suggests that the electrolyte abnormality is a primary manifestation of the basic molecular defect underlying cystic fibrosis. The changes in sweat electrolytes reflect a disturbance in cation transport which occurs in several different exocrine glands and is restricted to them. Increased electrolyte levels occur in mixed salivary gland, submandibular and parotid saliva, and quantitative abnormalities in ion composition of other exocrine secretions also occur. However, plasma sodium, potassium, and chloride levels in individual cystic patients are in the normal range, and there is no alteration of renal sodium handling in patients with cystic fibrosis. The physiological mechanism causing these electrolyte abnormalities may be specific to exocrine glands and may involve an inhibitor of sodium transport specifically. An alternate explanation for the observed organ specificity of the electrolyte defect is that compensatory mechanisms may mask the expression of this defect in other tissues.

Carriers of the cystic fibrosis gene are reported on average to have sweat electrolyte levels which are statistically elevated compared to age and sex matched normal individuals. However, the overlap in absolute sweat electrolyte values between homozygous normal individuals and obligate heterozygotes is so great that this test cannot be used reliably in individuals to establish the carrier state (despite implications to the contrary in a widely used clinical chemistry text). The inability of the sweat test to distinguish heterozygotes consistently is implicit in the statement of the need for development of a suitable means for carrier detection by the U.S. National Academy of Sciences/National Research Council Report of the Committee for a Study for Evaluation of Testing for Cystic Fibrosis.

A marked difference in sodium accumulation in fibroblasts recently reported by Breslow, McPherson, and Epstein seemed to promise a link between the well-established in vivo sodium transport abnormality in exocrine glands and another cell type which could be obtained readily for heterozygote detection and for prenatal diagnosis. These investigators reported that confluent fibroblast cultures exposed to Na for six hours accumulated significantly more of the isotope if the cell lines were derived from cystic fibrosis patients than if they were derived from normal individuals. Further, upon exposure to the Na+,K+-ATPase inhibitor, ouabain, normal cells accumulated much higher levels of Na than did cystic fibrosis fibroblasts. The latter observation suggested that cystic fibrosis cells are relatively resistant to the effects of ouabain when compared to normal cells. This result was in keeping with Epstein and Breslow's earlier findings that ouabain-treated cystic fibrosis fibroblasts had increased colony-forming capacity compared to treated normal cells. After equilibration with Na, the ratio of cell-associated Na in ouabain-treated cells to that in untreated cells was 2.3 ± 0.1 in 13 fibroblast lines from normal subjects and 1.3 ± 0.1 in eight lines from cystic fibrosis patients. Fibroblasts from eight obligate heterozygotes had a ratio of 1.4 ± 0.1. While there was considerable overlap between cell lines from cystic fibrosis homozygotes and heterozygotes, no overlap in the ratios was seen when any of these lines were compared to normal lines. This technique appeared to provide a definitive diagnostic test for both heterozygous and homozygous states of the cystic fibrosis gene.

Disappointingly, the difference in ouabain-sensitive Na accumulation appears
to result from an unfortunate sampling of fibroblast cell lines, not from the action of the cystic fibrosis mutation. Upon blind analysis of fibroblast cultures obtained from additional cystic fibrosis patients and normal individuals, these investigators were unsuccessful in their attempt to correlate the $^{22}$Na accumulation ratio with the clinical phenotype of the individual from whom the fibroblast cultures had been derived. The diagnostic utility of this $^{22}$Na accumulation assay has been retracted.

Another possible approach to differentiating cystic fibrosis fibroblasts from normal ones arose from Epstein, Breslow, and co-workers explorations of the ouabain sensitivity of Na$^+$/K$^+$-ATPase. When the ouabain susceptibility of a limited number of fibroblast lines from cystic fibrosis patients' and normal individuals were compared on potassium-deficient medium, patient's cells were relatively resistant. These cystic fibrosis fibroblast strains also were found to be more resistant to the cytotoxicity of dexamethasone and sex steroids. The selective effect of dexamethasone has been proposed as the basis of a diagnostic test for this disease both in fetuses and for the detection of individuals heterozygous for the trait. As yet, these findings have not been confirmed by the evaluation in a blind fashion of a larger number of independently obtained strains from both cystic fibrosis and normal individuals. More destructive to this approach as a useful technique for prenatal diagnosis and heterozygote detection is the report by Kurz, Perkins, and Buchwald that assays based on the counting of total cells and colony-forming cells failed to demonstrate that dexamethasone kills human fibroblasts under a variety of conditions. Their results contradict the previously described studies in which dexamethasone killed a higher percentage of fibroblasts from normal individuals than from patients with cystic fibrosis. The basis for the differences obtained by the different groups of investigators remains unresolved.

**Arginine Esterase Deficiency as a Prenatal Diagnostic Marker of Cystic Fibrosis**

Several biologically active "factors" have been reported to be present in body fluids of cystic fibrosis patients. Based upon very preliminary evidence it was suggested that these factors might be small basic polypeptides. The inhibition of sodium reabsorption in the rat parotid gland by sweat or saliva from cystic fibrosis patients can be mimicked by poly-lysine and other cationic polypeptides. This prompted a search for a protease deficiency which could be expected to result in the accumulation of biologically active peptides. Rao and Nadler first described the marked decrease of trypsin-like esterase activity in mixed saliva from cystic fibrosis patients. This relative deficiency of arginine esterase followed a pattern in families which was consistent with a simple genetic basis. Plasma treated with ellagic acid to activate protease zymogens also revealed a deficiency of arginine esterase-like activity in specimens from cystic fibrosis patients.

On the basis of physical properties and response to inhibitors, there are several species of arginine esterase in plasma. In cystic fibrosis patients, the fraction of arginine esterase activity inhibited by soybean trypsin inhibitor was reduced to about 30 percent of the activity in the corresponding plasma fraction of controls. More recently, Nadler and co-workers have utilized methylumbelliferylguanidinobenzoate, a substrate for serine proteases, to demonstrate significant differences in protease activity among fibroblast lines from cystic fibrosis, obligately heterozygous and normal individuals.

The deficiency of arginine esterase activity detected with methylumbelliferylguanidinobenzoate also extends to am-
niotic fluid from pregnancies in which the fetus is affected with cystic fibrosis. Normal amniotic fluid contains arginine esterase with specific activity, pH optimum, isoelectric points and substrate and inhibitor specificities similar to that found in plasma. However, in contrast to plasma, there are two species separable by molecular weight (100,000 and 200,000), neither of which is present in zymogen form. A survey of about 1900 control midtrimester amniotic fluid samples indicated an average specific activity of 2.35 ± 0.46 (range 1.5 to 3.8). When the specific activity of this enzyme was determined retrospectively in amniotic fluid samples from seven obligate heterozygotes whose child was found to have cystic fibrosis after birth, it was much lower (1.2 ± 0.2, range 1.06 to 2.34). In 13 pregnancies at risk for cystic fibrosis in which prospective studies were carried out, three affected fetuses were detected. The outcome of all 13 pregnancies was predicted correctly. These exciting results have prompted Nadler and co-workers to offer to monitor pregnancies at risk for cystic fibrosis on a research basis.

Unfortunately, more recent studies correlating a deficiency of methylumbelliferylguanidinobenzoate-reactive proteases in amniotic fluid with cystic fibrosis are not so unequivocal. Nadler has reported* the occurrence of several false negative results, i.e., normal protease levels in amniotic fluid, of affected newborns. Two different mechanisms may explain this result. One possibility is that the clinical entity cystic fibrosis can be produced by either of two different mutant genes, one which reduces the catalytic activity of this protease and one which does not. A second possibility is that the arginine esterase abnormality is associated with a structural gene which is only genetically linked to the cystic fibrosis mutation but is not causally related to the disease. In this situation, the allele specifying deficient arginine esterase activity would reflect a genetic polymorphism in the human population with genetic coupling to the mutant allele at the cystic fibrosis locus. An example of such a genetic polymorphism is the restriction endonuclease-sensitive cleavage site frequently associated with the sickle cell variant of the human β-globin gene. Until larger numbers of at risk pregnancies can be screened, it will not be possible to establish how seriously this latest finding jeopardizes the utility of the arginine esterase assay as a prenatal marker for cystic fibrosis. At present the arginase esterase assay must be considered experimental and at least somewhat equivocal.

β-glucuronidase, were significantly elevated in lymphocytes cultured for 48 hours in the presence of patient or heterozygote sera but without mitogen.28 (The number of individual obligate heterozygotes and patients providing specimens and the number of lymphocyte cultures analyzed were not specified in this study.) These increases in the presence of sera from cystic fibrosis or heterozygous individuals resulted from reduced cellular loss of protein and acid hydrolase activities into the medium. A similar effect upon intracellular retention of total protein and enzyme activities occurred with phytohemagglutinin. Both a component of cystic fibrosis serum and the mitogen appeared to stabilize cellular enzyme content rather than to induce accumulation. With little rigorously specified data, the reduced lymphocyte responsiveness to phytohemagglutinin was proposed as “useful for the detection of cystic fibrosis heterozygosity.”

This in vitro effect on lymphocyte enzyme activity, an apparent blunting of their response to mitogens in the presence of patient or heterozygote sera, is distinct from the mitogen-induced proliferative response. Stimulation of 3H-thymidine incorporation into deoxyribonucleic acid (DNA) by phytohemagglutinin or concanavalin A is neither intrinsically impaired in cystic fibrosis lymphocytes nor significantly altered by exposure of lymphocytes to plasma from cystic fibrosis patients.44 (It should be pointed out, however, that the conditions for this investigation differ from those used by Lieberman et al.) However, lymphocytes from cystic fibrosis patients may show in vitro hyporesponsiveness to Pseudomonas aeruginosa and other gram-negative bacteria as judged by reduced 3H-thymidine incorporation after exposure to these antigens.43,44 This lymphocyte unresponsiveness is associated with advanced disease, specifically with Pseudomonas aeruginosa infection, and it does not result directly from the genetic lesion in cystic fibrosis.43,44,45

Lectins are divalent or multivalent carbohydrate binding proteins which can agglutinate cells containing more than one specific saccharide of appropriate complementarity.4 The superficial similarity of action between sera from patients and heterozygotes and the lectin mitogens (e.g., phytohemagglutinin) and the known mucosecretory action of lectins,18 led Lieberman et al to conjecture that these sera contained a lectin-like component.29,30 They have reported the ability of sera from cystic fibrosis patients and heterozygotes to agglutinate mouse erythrocytes.26,29 This agglutinating activity has been proposed as a simple, rapid screening test for detecting heterozygosity for the cystic fibrosis gene.29 A test is scored as positive when hemagglutination occurs at titers of 1:160 or greater and can be blocked by fructose. A lectin-like activity was detected in 25/25 patients with cystic fibrosis, 70/70 obligate heterozygotes and four percent of 675 normal individuals and contrast patients.29 However, these investigators were unable to score accurately the samples received in a blind fashion from other laboratories. Technical difficulties in specimen handling and maintenance may contribute to this unsuccessful attempt to validate the method. The data supporting this hemagglutinin assay for cystic fibrosis must be considered preliminary until such blind analyses have been carried out successfully and until the method has been verified independently in other laboratories.

Altered Mitochondrial NADH Dehydrogenase in Fibroblasts from Patients and Carriers with Cystic Fibrosis

The exocrine secretory anomalies in cystic fibrosis and the importance of calcium to stimulus-secretion coupling suggested to Feigal and Shapiro17 the possi-
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...ble existence of a defect in cellular calcium homeostasis in cystic fibrosis. Cystic fibrosis cell lines (n = 5) and heterozygote cell lines (n = 3) exhibit increased $^{45}$Ca accumulation when compared with their respective controls. The magnitude of the difference is probably too small to allow this approach to heterozygote screening. A small but statistically significant difference in $^{45}$Ca efflux also was observed. Cystic fibrosis cells retain a higher percentage of their initial $^{45}$Ca content than do control cells.

Cell fractionation studies have been used to establish the site of the difference in calcium handling. Mitochondria isolated from cystic fibrosis fibroblasts (the number of lines tested is unclear) accumulated 78 percent more $^{45}$Ca per mg of protein than do normal fibroblasts. There was no difference in $^{45}$Ca binding to the microsomal fraction obtained from these fibroblasts. These observations and the specific effects of an inhibitor of mitochondrial calcium uptake suggested that the difference in fibroblast calcium uptake was specifically associated with an alteration in mitochondrial calcium transport.

Although the accumulation of calcium in mitochondria is regulated in a complex manner, one biochemical step known to be implicated is the proton gradient generated during electron transport. Increased sequestration of calcium in cystic fibrosis fibroblasts might reflect, therefore, an intrinsic increase in mitochondrial terminal oxidation activity. Oxygen consumption by patient fibroblasts is increased about two-fold compared to normal cells and, in these mutant cells, $O_2$ consumption is inhibited to a greater extent by rotenone, a specific inhibitor of reduced nicotinamide-adenine dinucleotide (NADH) dehydrogenase. This effect of rotenone led to an examination of the properties of the NADH dehydrogenase in the fibroblast lines. Total NADH dehydrogenase activity was quantitatively similar but major differences in pH optima for catalytic activity were seen when cystic fibrosis, heterozygote, and normal cell lines were compared. These differences in the pH optima for NADH dehydrogenase activity occurred both in crude extracts and in partially purified enzyme preparations. Although these investigators have been able successfully to identify both heterozygote and cystic fibrosis cell lines in blind analyses, this approach to carrier detection and prenatal diagnosis awaits confirmation by the documented demonstration of correct identification of a large number of cell lines in a blind fashion and the independent confirmation of these results by other laboratories.

Summary

Although no highly efficient assay for the prenatal diagnosis of cystic fibrosis and for heterozygote determination has been established at the time of this writing, the arginine esterase deficiency is the most thoroughly characterized biochemical marker which may have significant value for prenatal diagnostic testing. Much effort will be required to document the actual utility of the other biochemical abnormalities suggested as markers for this disease.

References


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