Cystic Fibrosis: Fractionation of Fibroblast Media Demonstrating Ciliary Inhibition

(cystic fibrosis/cilia/fibroblast/oyster)

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ABSTRACT A chromatographic fraction has been identified in media from fibroblast cultures derived from 15 of 18 cystic fibrosis patients and 8 of 10 heterozygotes that inhibits ciliary activity, as judged by the oyster gill assay. The analogous fraction of media from cultures of fibroblasts derived from 11 normal individuals did not inhibit ciliary activity under the experimental conditions used. The chromatographic fractions containing the ciliary inhibitory activity from both fibroblast media and sera of cystic fibrosis patients and heterozygotes eluted in the same position from diethylaminoethyl-cellulose at pH 8.5.

Although the serum ciliary inhibitor had consistently been detected in a fraction that contained predominantly immunoglobulin G, results of the fractionation of fibroblast media demonstrate that the ciliary inhibitor is present in a protein fraction containing three species of different molecular weight and in which IgG cannot be detected by the techniques used.

Cystic fibrosis, an autosomal recessive condition, is a severe chronic disease that occurs in approximately one out of every 2500 Caucasian births. A factor is present in serum of cystic fibrosis patients and heterozygotes that inhibits ciliary activity in oysters (1), rabbit trachea (2), and fresh water clams (3). Serum from homozygous normal individuals does not inhibit ciliary activity. The inhibitor has been fractionated from serum of cystic fibrosis patients and heterozygotes. It migrates with a cationic protein fraction, as judged by electrophoresis at pH 8.6 (4). The inhibitor is eluted during gel filtration with a fraction having a molecular weight of 125,000-200,000. On diethylaminoethyl (DEAE)-cellulose chromatography (4) and rivanol precipitation (5), it is fractionated with the immunoglobulin G (IgG) fraction. The analogous fraction from serum of normal individuals does not inhibit oyster ciliary activity.

Several abnormalities have been noted in cultured cells derived from cystic fibrosis patients and heterozygotes. On the basis of metachromatic staining, Danes and Bearn (6) divided the cultured fibroblasts from both cystic fibrosis homozygotes and heterozygotes into three classes. Studies of Matalon and Dorfman (7) have furnished evidence of a significant increase of acid mucopolysaccharides in fibroblasts from patients with cystic fibrosis. It has been recently demonstrated that media taken from some fibroblast cultures from cystic fibrosis patients and their parents inhibited ciliary activity in oysters (8). The media from cultures showing metachromasia showed inhibitory activity, whereas those from ametachromatic

cultures and from cultures of fibroblasts derived from normal individuals did not.

We have now identified the chromatographic fraction of the fibroblast media that inhibits oyster cilia. Our studies indicate that in fibroblasts derived from 15 of 18 cystic fibrosis patients and 8 of 10 heterozygotes a ciliary inhibitor is detectable that elutes from DEAE-cellulose in the same fraction as does the ciliary inhibitor from the serum of the patients and heterozygotes. The analogous chromatographic fraction from media of fibroblasts derived from 11 normal individuals did not inhibit ciliary activity. The ciliary inhibitor associated with fibroblasts derived from the patients and heterozygotes did not demonstrate apparent immunological reaction with antisera specific for IgG. These studies furnish evidence that the cystic fibrosis ciliary inhibitor is not an immunoglobulin.

MATERIALS AND METHODS

Identification of the Ciliary Inhibitor from Fibroblast Media.

Media from fibroblasts cultured in two laboratories (New York and Chicago) were studied. Skin fibroblasts were cultured in the Chicago laboratory according to the conditions described by Matalon and Dorfman (9) with the following modifications: fibroblasts were cultured in modified Eagle's medium (Gibco), supplemented with ascorbic acid (100 mg/liter), fetal-calf serum (10%), and calf serum (10%), unless otherwise stated. The cells were grown in 100-mm Falcon petri dishes at 37° and at 10% CO₂ tension. A pH of 7.2-7.4 was maintained. Cells used for these experiments had undergone two to eight transfers, and were grown 1-2 weeks to a cell density of 12 to 16 × 10⁶ cells per plate before use in experiments. Presence of mycoplasma contamination was monitored by routinely culturing cells and media in mycoplasma agar medium (Gibco). In the Chicago laboratory, fibroblasts were grown from skin biopsies from nine cystic fibrosis patients, two heterozygotes, and eight normal individuals.

In the New York laboratory the establishment of cell lines from skin biopsies by standard culture methods (6) required about 6 weeks, so that the cells studied had been grown 1-2 months as monolayer cultures (2-6 subcultures by trypsinization) before these studies. All cultures were grown in reinforced Eagle's medium containing 10% by volume of newborn-calf serum (10) with the pH maintained at 6.8-7.2. For these experiments the cells were trypsinized into suspen-

sion and about 10^4 cells were inoculated into 75-cm² Falcon flasks. After two culture weeks, the cells had formed a dense monolayer containing approximately 10^8 cells. 72 Hr after the medium was changed, an aliquot was taken to test for ciliary inhibitory activity. Fibroblast cultures were initially derived as skin biopsies from nine patients, eight heterozygotes, and three normal individuals.

Media received from both laboratories had been centrifuged, stored in dry ice, and shipped frozen. Media remained frozen until they were chromatographed. 2-5 Plates of medium from fibroblast cultures from the same individual were pooled. Volumes of fibroblast medium ranging from 30 to 150 ml were subjected to chromatography on DEAE-cellulose, as described for serum (4). After each batch of medium was chromatographed, the first protein peak eluted was concentrated under reduced pressure at 5° to concentrations ranging from 0.04 to 2.9 mg/ml, as determined by the method of Lowry *et al.* (11). The protein solution was then dialyzed against seawater for 16 hr at 5° before it was examined by the oyster cilia assay. Ciliary action was observed under phase microscopy as described by Bowman *et al.* (1) with gills from oysters, *Crassostrea virginica*. The time required for ciliary cessation was recorded for each sample. Controls consisting of normal and cystic fibrosis serum fractions were included in each experiment. The identity of each sample was unknown to the observer. Each fraction was examined 3-5 times on oyster cilia.

In the Chicago laboratory, fibroblast cultures derived from two patients, two heterozygotes, and two normal individuals

FIG. 1. (A) Elution from DEAE-cellulose at pH 8.5 of a serum protein fraction from cystic fibrosis patients that contains the inhibitor to oyster ciliary activity. Horizontal bar indicates fractions pooled for examination on oyster cilia. (B) Elution from DEAE-cellulose at pH 8.5 of a fraction prepared from media of cultured fibroblasts from patients with cystic fibrosis containing the ciliary inhibitor. Horizontal bar under the major protein peak indicates fractions pooled for examination of oyster cilia. The second minor peak appeared to be comprised of molecules of low molecular weight, and was lost during dialysis. The absorbance is designated by an unbroken line. In experiments where cystic fibrosis fibroblasts were maintained in media containing radioactive leucine, two radioactive protein peaks were obtained upon DEAE-cellulose chromatography that are represented by broken lines. The first radioactive peak inhibited oyster cilia.

FIG. 2. Minutes required for cessation of ciliary activity after exposure to DEAE-cellulose fractions of media from fibroblasts derived from cystic fibrosis patients, heterozygotes, and normal subjects with the oyster cilia assay (1).

were maintained in media lacking fetal-calf serum and containing L-[U-¹⁴C]leucine (234.7 Ci/mol). Calf and fetal-calf serum were omitted from the media in these experiments to prevent fractionation of bovine IgG with the ciliary inhibitor. Fibroblasts cultures as described above (9) were washed in media (Gibco) that lacked leucine and fetal-calf serum. The same cells were then maintained for 24-48 hr in media in which 3 μ Ci/plate of L-[¹⁴C]leucine was added. Two types of control media were studied; those that contained radioactive leucine but no cells and those that contained radioactive leucine and cystic fibrosis cells maintained at 0°.

Gel Filtration. Molecular weight estimates of the radioactive proteins contained in the fraction from DEAE-cellulose that inhibited ciliary activity were performed on a Sephadex G-200 column, 0.9 $\times$ 61 cm, equilibrated in 0.2 M propionic acid. The column, described by Andrews (12), was calibrated by determination of elution volumes of three proteins, with molecular weights ranging from 17,500 to 160,000.

Autoradiography. Antisera against gammaglobulin fractions isolated by starch block electrophoresis of sera from cystic fibrosis patients were produced in rabbits. Antiserum to human serum proteins was obtained from Behring Diagnostics. Immunoelectrophoresis was done in agar gel according to the microtechnique of Scheidegger, as modified by Hirschfeld (13). The two-dimensional diffusion system on agar gel, as developed by Ouchterlony (14), was used. Unlabeled carrier of IgG fractions was added to the antigen wells to facilitate the development of immunoprecipitin lines for autoradiography. After immunoelectrophoresis and immunodiffusion were performed the slides were dried and stained. Autoradiography was done with Kodak Royal X Pan film. Film was exposed 7-40 days before development.

RESULTS

Detection of the ciliary inhibitor from media of fibroblast cultures

Fibroblast media subjected to ion-exchange chromatography on DEAE-cellulose yielded two protein peaks at pH 8.5. The elution position of the major peak that inhibited ciliary activity corresponded to the elution of the ciliary inhibitor

from serum of cystic fibrosis patients and heterozygotes (4). The minor peak appeared to be comprised of low molecular weight material that was lost during dialysis and therefore was not examined for ciliary inhibition. A comparison of the elution patterns of the ciliary inhibitor derived from cystic-fibrosis fibroblast media and from serum is shown in Fig. 1. The same elution pattern was also observed during chromatography of media from fibroblasts derived from heterozygotes and normal individuals. The fractions eluted during chromatography of media of fibroblasts derived from 18 patients, 10 heterozygotes, and 11 normal individuals were tested by the oyster cilia assay. The time required for these samples to cause cessation of ciliary activity is presented in Fig. 2. Fractions from media of 15 of 18 cystic fibrosis fibroblast cultures and 8 of 10 heterozygote cultures inhibited ciliary activity before 40 min. No inhibition was noted with fractions from media of 11 normal fibroblast cultures.

Attempts were made to label the ciliary inhibitor by culturing the fibroblasts in medium containing radioactive leucine. The radioactive media from fibroblasts derived from two patients, two heterozygotes, and two normal individuals were studied. The medium from each group was chromatographed at pH 8.5. A major radioactive peak was followed by a minor radioactive fraction that was lost during dialysis. The first protein fraction eluted during chromatography of the media from the cells derived from patients and heterozygous carriers was in the same chromatographic fraction as the ciliary inhibitor present in the serum of patients and heterozygotes. Media from fibroblasts derived from cystic fibrosis patients, heterozygotes, and normal individuals had two radioactive peaks in the identical positions as shown in Fig. 1. Each major fraction was concentrated under reduced pressure to a volume of 1 ml. Both fractions from cystic fibrosis media and heterozygous media inhibited oyster ciliary activity within 25 min. However, even after further concentration, the same fraction from media of normal cells failed to inhibit ciliary activity. Aliquots of the three radioactive fractions were dialyzed extensively against 5 M guanidinium chloride (pH 7.75) in order to eliminate any adsorbed L-[¹⁴C]leucine. Amino-acid analysis of an unhydrolyzed aliquot of the fraction from cystic fibrosis media confirmed the absence of free amino acids.

Incorporation of L-[¹⁴C]leucine into protein suggested that the fraction containing the ciliary inhibitor was synthesized, at least in part, by the fibroblasts. When the media that contained radioactive leucine but no cells or the media maintained at 0° that contained radioactive leucine and cells derived from cystic fibrosis patients were chromatographed, no protein peak or radioactivity was detected in the fraction analogous to the ciliary inhibitor. The same fractions from both of the two control media failed to inhibit oyster ciliary activity.

Gel filtration

In gel-filtration experiments with a calibrated Sephadex G-200 column, three radioactive peaks were obtained from DEAE-cellulose fractions of fibroblast media from cultures derived from normal individuals and cystic fibrosis patients. One peak was eluted in a volume corresponding to a molecular weight range of approximately 140,000–160,000. The second peak was eluted in a fraction whose molecular weight corresponded to about 20,000, while the third fraction corresponded to a

molecular weight of 13,000 or lower. Although ciliary inhibition was never observed in the DEAE-cellulose fraction of normal media, the fibroblasts derived from both normal individuals and cystic fibrosis patients synthesized proteins of these three molecular weights that were recovered from the radioactive media by DEAE-cellulose chromatography and gel filtration.

Autoradiography

The possibility that the ciliary inhibitor associated with fibroblasts derived from cystic fibrosis patients and heterozygotes might be an immunoglobulin or related fragments of an immunoglobulin was investigated by the techniques of double radioimmunoassay and immunoelectrophoresis.

When fibroblasts derived from cystic fibrosis patients were maintained in radioactive media from which fetal-calf serum had been omitted, the ciliary inhibiting fraction (0.4 mg/ml) did not form a radioactive precipitin line with antisera directed against serum IgG or against serum proteins.

DISCUSSION

The results reported in this study provide experimental evidence for the presence of a ciliary inhibitor in media of some fibroblast cultures derived from cystic fibrosis patients and heterozygotes that occurs in the same chromatographic fraction as an inhibitor present in the serum of these two genotypes. The presence of the inhibitor in media of some cultured cystic fibrosis cells was first suggested by the observation of Danes and Bearn (8). This evidence is somewhat strengthened by the results obtained from testing the DEAE-cellulose fractions from media of fibroblast cells. In these studies, fractions from media of fibroblasts derived from 15 of 18 cystic fibrosis patients and 8 of 10 heterozygotes inhibited oyster cilia, while all fractions derived from fibroblasts of 11 normal individuals permitted the cilia to be active for 40 min or longer. It is not clear why the media fractions from three cystic fibrosis patients and two heterozygotes failed to inhibit ciliary activity. Cellular metachromasia had indicated previously that morphological heterogeneity existed in cultured cystic fibrosis fibroblasts (6). Danes and Bearn (8) suggested that the demonstration of inhibitory activity in some, but not all, sera and used culture media supported the concept of genetic heterogeneity within the clinical entity known as cystic fibrosis. The presence of a radioactive protein fraction that chromatographed with the ciliary inhibitor from media of cystic fibroblasts maintained in radioactive leucine suggested that the ciliary inhibitor may be synthesized by fibroblasts. An alternative, although less likely explanation, is that the ciliary inhibitor was already present in fibroblasts and was isolated with one or more radioactive proteins during fractionation. It was of some interest that a radioactive protein fraction could be isolated from media of normal cells by the same fractionation procedure, but that the radioactive fraction in media from normal fibroblasts did not inhibit ciliary activity.

Immunological comparison of the cystic-fibrosis ciliary inhibitor from media and serum indicated that whereas the serum inhibitor is inseparable from IgG with the methods used, the fibroblast inhibitor has no apparent immunological reaction with antisera directed against IgG. The identification of the cystic-fibrosis ciliary inhibitor in fibroblast media

will greatly facilitate its characterization since, for the first time, the ciliary inhibitor appears to have been fractionated in the absence of IgG.

Molecular weight estimations of the DEAE-cellulose fraction containing the ciliary inhibitor from fibroblast media indicated the presence of three radioactive protein peaks, having molecular weights of approximately 150,000, 20,000, and 13,000. The three molecular-weight species were detected by radioactivity and were in concentrations too low to test on oyster cilia. Further studies will be necessary to determine the effects of the three classes of proteins on ciliary activity. If one or more of these fractions demonstrates ciliary inhibition, it will be of great interest to contrast its properties to the analogous fraction from media of fibroblasts derived from heterozygous and normal individuals. Although the DEAE-cellulose fraction from radioactive media of fibroblasts derived from normal individuals did not inhibit ciliary activity, gel filtration of this fraction also demonstrated three radioactive protein peaks having molecular weights of approximately 150,000, 20,000, and 13,000.

The component in the serum and fibroblast media of cells derived from cystic fibrosis patients and heterozygotes that is responsible for the response of oyster cilia may be an altered protein or a protein carrying a bound compound that is the result of a mutated gene, and may be responsible for the defects in membrane transport, pulmonary function, and pancreatic enzyme secretion observed in patients with cystic fibrosis.

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