Functional Failures of "Fibrocystic" Fibroblasts[1] (35053)

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Danes and Bearn (1) made the original observation that the cultured fibroblasts derived from patients with fibrocystic disease contained supernormal amounts of metachromasia. Matalon and Dorfman (2) confirmed these observations and showed that these cells also contained supernormal amounts of glycosaminoglycans. This finding of an increased fibroblastic content of acid mucopolysaccharides and metachromasia with fibrocystic disease is similar to the findings of Frantantoni *et al.* (3) for Hunter's and Hurler's diseases; these workers also showed that there was decreased catabolism of acid mucopolysaccharides in these fibroblasts. All this in spite of the fact that cystic fibrotic patients give no evidence *in vivo* of decreased fibroblastic function (4).

Therefore, we explored the ability of diploid human fibroblasts from patients with fibrocystic disease to make collagen and induce collagenolytic activity when exposed to oxyphenylbutazone (5). The results of these studies are described below.

Materials and Methods. Fibroblast lines were grown out of cutaneous biopsy explants from six normal and six patients with fibrocystic disease (diagnosed by Dr. Paul Di Sant'Agnese, NIH) as well as from one patient each with Hunter's and Hurler's disease by Dr. Elizabeth Neufeld, NIAMD, NIH. These cells and five samples of embryonic lung fibroblast lines [WI-38] (courtesy of Dr. L. Hayflick) were grown in our laboratory into confluent monolayers in 32-oz pharmacy bottles in Eagle's minimal essential medium (MEM) (changed every 3–4 days) containing 10% fetal calf serum, 2 m*M* glutamine and 90 units/ml, each, of streptomycin and penicillin. After reaching confluency, 0, 12.5, or 50 μg/ml of ascorbic acid was added to the medium and the cultures were allowed to stand for 7 days with one medium change to permit collagen synthesis to take place. The various population doublings of the cells used were calculated to be less than 25 at the time each culture studied reached confluency. These monolayers were harvested mechanically, rinsed twice in serum-free medium and resuspended in serum-free medium to a concentration of 4×10^6 cells/ml as determined in a hemocytometer. All cell cultures were then incubated for 4 hr in triplicate either with or without 20 μg/ml of oxyphenylbutazone. After this time the cell suspensions were sonicated in their medium and portions of these homogenates were then incubated with 10 mg/ml of insoluble native collagen from the skin of 400-g rats (which had been swollen in the cold in buffer [pH 5.5] for 1 week) for 16 hr at pH 5.5 and 32° (5, 3). No apparent bacterial or fungal growth could be demonstrated in the swollen substrate suspension either during the time of standing in the cold or during incubation with the penicillin–streptomycin containing MEM after enzyme induction. That the insoluble collagen was native rather than denatured was indicated by the fact the exposure of this substrate to 1 mg/ml of trypsin at pH 7.5 had no solublizing effect upon the hydroxyproline content of the collagen after 16 hr incubation at 32°.

The incubation mixtures were centrifuged for 1 hr at 15,000*g* in the cold at zero and 16 hr after incubation; and the clear supernatants were removed and hydrolyzed in 4 *N*

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HCl for 8.5 hr at 100°. These hydrolysates were analyzed in triplicate for their hydroxyproline content (7).

In this fashion, the amount of collagenolytic activity at pH 5.5 could be determined in terms of the micrograms of soluble peptide bound hydroxyproline released from 10 mg of insoluble collagen/10^6 cells.

Other portions of these mechanically removed monolayers were hydrolyzed directly in 4 *N* HCl and the total amount of peptide-bound hydroxyproline in the confluent cell monolayer was determined (7) in triplicate as a measure of collagen synthesis by these monolayers during 7 days confluency in the presence of ascorbic acid. These results were translated into micrograms of collagen per 10^6 cells by the fact that normal collagen contains 1 μmole of hydroxyproline/mg(8).

Other similar fibroblast cultures were also exposed for 7 days after reaching confluency to 1 μCi/10^6 cells of 3,4-^{3}H-proline (5 μCi/μmole) in the presence of 0, 12.5, 50, or 100 μg of ascorbate/ml. After harvesting the cell monolayers mechanically, the insoluble material was rinsed twice with serum-free medium and hydrolyzed in acid as described above. The hydroxyproline from these hydrolysates was separated chemically from proline according to the procedure of LeRoy *et al.* (9) and the counts per 30 min/10^6 cells were determined in a Nuclear Chicago liquid scintillation counter.

Finally, all cell lines were examined karyologically and were found to be normal with respect to chromosomal number, breaks, gaps, and morphology (10).

Results. The chemical concentrations of collagen per 10^6 cells in monolayers of variously derived fibroblasts at various concentrations of ascorbic acid is shown in Table I.

These data indicate firstly that no significant amounts of hydroxyproline can be determined chemically in these monolayers without adding ascorbate to the medium; secondly that monolayers of fibroblasts derived

TABLE I. The Effects of Ascorbic Acid and Oxyphenylbutazone upon the Collagen Synthesis and the Induction of Collagenolytic Activity, Respectively, by Diploid Human Fibroblasts Obtained from Skin Biopsies of Normal and Fibrocystic Patients and from Two Other Patients with Either Hunter's or Hurler's Disease, as Well as Cells Derived from Embryonic Lung (WI-38).

	Collagen synthesis; ascorbate (μg/ml)				Collagenolytic activity (μg hypro solubilized/10^6 cells)	
Fibroblasts	Control	12.5	50	100	Control	+ Drug (20 μg/ml)
1) Normal (6)						
Chemical hypro[c]	0	*38* ± 4[b]	*100* ± 10	—	0	*7.0* ± 1
Radioactive hypro[d]	59 ± 6	*155* ± 12	*188* ± 7	*220* ± 25	—	—
2) Fibrocystic (6)						
Chemical hypro[c]	0	0	—	—	0	*3.5* ± 1[a]
Radioactive hypro[d]	85 ±10[a]	105 ± 5[a]	*122* ± 5[a]	*133* ± 10[a]	—	—
3) WI-38 (5)						
Chemical hypro[c]	0	*40* ± 4	*100* ± 12	—	0	*7.0* ± 1
Radioactive hypro[d]	72 ± 7	*166* ± 12	*185* ± 10	*220* ± 20	—	—
4) Abnormals (chemical hypro)[c]						
Hunter's (1)	0	*40*	—	—	0	6.0
Hurler's (1)	0	*40*	—	—	0	8.0

[a] Significantly different from the appropriate normal ($p < .05$).

[b] Italicized numerals are significantly greater than control ($p < .05$).

[c] μg of hydroxyproline per 10^6 cells.

[d] Counts of hydroxyproline per 30 min/10^6 cells.

from patients which had been diagnosed as fibrocystic could not demonstrate any hydroxyproline chemically even with ascorbic acid added to the medium (12.5 μg/ml).

Separate experiments indicated that the chemical method was not sensitive to amounts of hydroxyproline in cells monolayers less than 2.5 μg/ml or about 20 μg of collagen/10^6 cells.

Fibroblasts from five separate samples of WI-38 and from the skin biopsies of patients with either Hunter's or Hurler's disease were capable of making significant amounts of hydroxyproline chemically in the presence of 12.5 μg/ml of ascorbic acid.

Finally, all non-"fibrocystic" fibroblasts could demonstrate a collagenolytic activity of about 7 μg/10^6 cells after 4-hr exposure in serum-free medium to 20 μg/ml of oxyphenylbutazone: the "fibrocystis" fibroblasts all demonstrated a drug-dependent appearance of about one-half of this amount of collagenolytic activity.

The counts of tritiated hydroxyproline per 30 min made from tritiated proline by 10^6 cells and not extractable into the medium are also recorded in Table I. These values show that while normal cells did contain a small amount of radioactive hydroxyproline in the absence of added ascorbate, the counts per 30 min of this material in the monolayer was increased about 160% when 12.5 μg of ascorbic acid was added to the medium; 220% with 50 μg/ml; and about 290% with 100 μg/ml of ascorbate. "Fibrocystic" fibroblast monolayers contained more radioactive hydroxyproline in the absence of ascorbate than did normal cells, but this radioactivity increased only by about 18% with 12.5 μg/ml of ascorbate; 43% with 50 μg/ml; and 56% with 100 μg/ml of ascorbate. Thus, the addition of ascorbic acid to these "fibrocystic" fibroblast monolayers increased their ability to synthesize insoluble hydroxyproline from proline only 1/5 to 1/8 as well as could be shown for normal fibroblasts.

Discussion and Conclusions. Fibroblasts derived from six patients with diagnosed fibrocystic disease differed significantly from fibroblasts derived from either normal patients or from patients with Hunter's or Hurler's disease. The "fibrocystic" fibroblasts could induce only as much collagenolytic activity as could normal cells and the addition of ascorbate to these cells increased their ability to make collagen *in vitro* only 13 to 20% as well as did the addition of similar amounts of ascorbic acid to normal cells.

These functional failures of "fibrocystic" fibroblasts were not demonstrated by cells from one patient each with either Hunter's or Hurler's disease.

If one patient each is statistically representative for these acid mucopolysaccharidoses, then these failures of two fibroblastic functions may be unique to fibrocystic disease rather than being associated with general defects glycosaminoglycan catabolism. Chemically, these materials are altered differently in fibrocystic disease than in Hunter's or Hurler's disease (2).

The mechanism of the decreased ability of "fibrocystic" fibroblasts to both synthesize collagen and induce collagenolytic activity *in vitro* is unknown. None of these cells had undergone large numbers of population doublings (less than 25) at the time they were studied. Perhaps "fibrocystic" fibroblasts have difficulty in passing either inducing drug or ascorbate through their membranes, but these compounds are vastly different structually and certainly we have no evidence for these membranes being impermeable to these materials.

The major question in interpreting these results is that there is no evidence that *in vivo* fibrocystic disease is associated with any defect in collagen synthesis or fibroblast functioning (4). Our tentative explanation is that the cells from these patients are "sick" and are not able to tolerate the inadequacies of *in vitro* culture conditions nearly as well as can fibroblasts derived from nonfibrocystic patients. Hopefully the reason(s) for the inability of "fibrocystic" fibroblasts to function as effectively as normal cells in culture may prove to be relevant to the disease at the molecular level.

Summary. Diploid human fibroblasts from patients with diagnosed fibrocystic disease

differed markedly in their ability *in vitro* to synthesize collagen and induce collagenolytic activity from cells derived from either normal human skin, embryonic lung, or from the skin of two patients with acid mucopolysaccharidoses.

Increasing amounts of ascorbic acid caused normal fibroblasts to increase their synthesis of collagen by about 150 to 300%: "Fibrocystic" fibroblasts increased their synthesis of collagen by only about 20 to 50%. The "fibrocystic" fibroblasts *in vitro* also induced about ½ as much collagenolytic activity with oxyphenylbutazone as did non-"fibrocystic" fibroblasts.

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