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Defects in the Cell Cycle of Fibroblasts Obtained from Patients With Cystic Fibrosis (38305)

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Some years ago, Danes and Bearn (1) discovered that the fibroblasts obtained from the cutaneous biopsy of patients diagnosed as cystic fibrotic were more metachromatic than similar cells derived from patients with conditions other than cystic fibrosis. In our studies of the population doubling times of diploid human fibroblasts in culture (2), we reported that the fibroblasts derived from cutaneous biopsies of three different patients with cystic fibrosis had significantly longer population doubling times than did either normal diploid human fibroblasts or cells derived from patients with acid mucopolysaccharidoses (Hunter's and Hurler's disease). Further, we have reported (3) that cystic fibrosis-derived cutaneous fibroblasts do not make normal amounts of collagen hydroxyproline when exposed *in vitro* to ascorbic acid and radioactive proline.

The findings, then, of metachromasia, prolonged population doubling time and failure of collagen synthesis on the part of fibroblasts derived from the skin of patients with cystic fibrosis suggest in general that these cells are not normal in their *in vitro* behavior. We have pointed out that there is no evidence *in vivo* to support the proposition that the fibroblasts of patients with cystic fibrosis are in any way inadequate or different from normal (4). We presume, then, that the autosomal recessive defect in these patients manifests itself in their cutaneous fibroblasts because of the inadequate *in vitro* medium in which they are normally cultivated. Therefore, it might appear that cells from cystic fibrosis patients are sufficiently "sick" *in vitro* so as to have lost both their ability to make collagen or divide at the proper rate and hence accumulate supernormal amounts of metachromatic granules.

This paper analyzes the cell cycle of cystic fibrosis-derived fibroblasts and statistically demonstrates their failure to support a normal population doubling time *in vitro* and indicates that this is due largely to a failure of the cystic fibrosis-derived cells to enter the cell cycle in normal numbers.

Materials and Methods. Fibroblasts were routinely derived from the cutaneous biopsy of the skin from the inferior surface of the forearm. These biopsies were cultured in Eagle's Minimum Essential Medium (MEM), supplemented with glutamine and antibiotics in the usual fashion, and containing 20% fetal calf serum. After a period of 2–3 weeks, those fibroblasts which had grown out from these cutaneous biopsies were harvested via trypsinization, replanted, and cultivated in Leighton tubes. These replicate cell cultures were counted, using an inverted microscope and a Whipple eyepiece. From the slope of the plot of the log of the number of cells against the number of hours of cultivation, the population doubling time or generation time of these cells was determined (2). Patients were diagnosed in the Cystic Fibrosis Clinic at this hospital in accordance with established clinical criteria (4).

The duration of G_2 and S phases of the cell cycle were determined in hours for both normal and cystic fibrosis-derived fibroblasts, firstly using the microspectrophotometric and autoradiographic procedures of Mak (5), and secondly from an analysis of the kinetics of metaphase labeling, as determined by autoradiography using the procedures of Stanner and Till (6). Finally, the percentage of cells entering into S phase was determined by the autoradiographic

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procedure of Stanners and Till (6) during the log phase of cell growth *in vitro*, during which the cells had been exposed to a 60-min pulse of 0.5 μCi of H^3-thymidine.

The plating efficiency of these cells was determined by introducing into a Leighton tube a known number of cells, as determined by hemocytometry, and after 24 hr the supernatant containing the cells which had not adhered to the glass was removed and forced through a millipore filter (0.450 nm). The number of cells in this millipore filter was determined by fixing the millipore filter and cells in formalin and then staining and counting microscopically (7).

Six cystic fibrosis-derived cutaneous fibroblasts were obtained from the American Type Culture Collection (ATCC) (12301 Parklawn Drive, Rockville, MD 20852), and these cell lines, together with the samples from explants of six different patients with cystic fibrosis, were used in the following study.

Experimental results. The plot of the log of the cell number in Leighton tubes, as determined using a Whipple eyepiece, against time is shown in Fig. 1, in which two cell types, one WI-38 (derived from embryonic lung) and the other

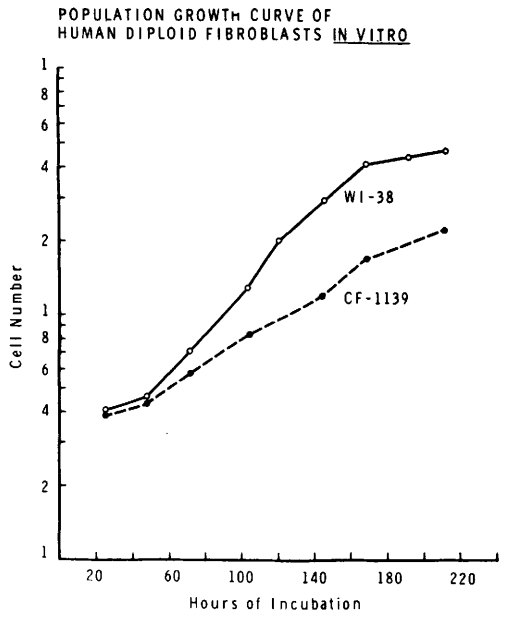

FIG. 1. Plot of the log of fibroblast cell number per mm^2 versus incubation time (hr) for normal WI-38 and cystic fibrosis-derived cutaneous fibroblasts after seeding 4×10^4 cells per Leighton tube into MEM supplemented with 10% calf serum, glutamine, and antibiotics.

CF-1139 (derived from the cystic fibrosis fibroblast collection of ATCC), were analyzed for population doubling time. From the slope of these two lines, the population doubling time may be determined.

The data of Table I summarize the mean population doubling or generation time for fibroblasts from eight normal cutaneous biopsies, one cell line obtained from a Hunter's, one from a Hurler's, four from patients with dwarfism, six from juvenile diabetics, and 12 patients with cystic fibrosis, six of which are from cell lines available from ATCC. The eight normal and 12 cystic fibrosis-derived cutaneous fibroblasts were also subjected to analysis of cell cycle in terms of the duration of their G_2 and S phase portions of the cell cycle. These data clearly indicate that there is a very considerable delay in the population doubling time or generation time of fibroblasts derived from the cutaneous biopsy of patients with cystic fibrosis which is statistically different from the generation times of cutaneous fibroblasts derived from the biopsy of patients with diseases other than cystic fibrosis. Further, the duration of both the G_2 and S phases of the cell cycle for normal and cystic fibrosis-derived fibroblasts were essentially the same. Thus, either the duration of G_1 was enormously long (i.e., 22 hr for the normal and 45 hr for the cystic fibrosis fibroblasts), or a significantly smaller proportion of these cells during log phase were recruited into the actual cell cycle.

One possibility might be that the plating efficiency of these fibroblasts derived from patients with cystic fibrosis was significantly less than that obtained from the cells of patients with other diseases, or normals. This was experimentally tested and it was found that the plating efficiency of all these cell types, regardless of disease entity, was essentially 90–95%.

The percent of labeled cells which could be determined by autoradiography (5, 6) was determined for CF-1139 cell line and WI-38 cell line during log phase growth, as shown in Fig. 2. At each time period, triplicate samples of cultured fibroblasts were exposed for 60 min to 0.5 μCi of H^3-thymidine per ml. Approximately 2000 cells from each of three separate Leighton tubes were counted, and the proportion of labeled cells was determined. The mean percentage of labeled cells was expressed in Fig. 2 with the extreme range indicated by the vertical lines. These data indicate very clearly that for the

first few days after seeding and hence during log phase growth, approximately 20% of the cells in these cultures were labeled with H^3-thymidine, whereas only about 8% of the cells from the cystic fibrosis-derived cell strain 1139 were labeled during the same period of time.

These cystic fibrosis-derived cell lines were cultivated routinely in 10% calf serum and MEM, as described above. Experiments were conducted in which these cells were cultivated in Medium 199, a much more nutritionally complete medium, containing either 10% or 20% calf or fetal calf serum. The population doubling time and the percent of cells recruited into the cell cycle for cystic fibrosis-derived cells or for WI-38 fibroblasts were in no way different when cultivated in these differing media supplemented with different amounts of either calf or fetal calf serum.

Discussion. The data of Fig. 1 and Table I indicate clearly that the population doubling time, or "generation time," of fibroblasts derived from patients with cystic fibrosis was significantly longer than those for fibroblasts obtained from either the skin of normal patients or of patients suffering from diseases other than cystic fibrosis. The most rapidly proliferating cell population from cystic fibrosis patients was still slower in its population doubling time than was the most slowly growing population of fibroblasts derived from non-cystic fibrosis patients. That is, there was no statistical overlap between these two derived cell populations.

The data of Table I indicate clearly that the duration of the S and G_2 phases of the cell cycle did not differ for cystic fibrosis-derived cells from that demonstrated by the fibroblasts derived from the skin of either normal or non-cystic fibrosis but diseased patients. Further, the plating efficiency of established cell lines for cystic fibrosis-derived fibroblasts from the American Type Culture Collection and from normal cutaneous biopsies was identical.

TABLE I. The Mean (and Standard Deviation) Generation Times and Cell Cycle of Fibroblasts Obtained from Cutaneous Explants from a Number of Children with and without Cystic Fibrosis.

	Generation time (hr)	G_2	S
8 normal (8–13 yr)	34 ± 3	5 – 7	7 – 9
1 Hunter's (12 yr)	35 ± 1.5	—	—
1 Hurler's (11 yr)	33 ± 0.7	—	—
4 dwarfism (6–12 yr)	30 ± 2	—	—
6 diabetics (8–13 yr)	33 ± 2	—	—
12 cystic fibrosis (6–15 yr)	58 ± 6[a]	7 – 9	8 – 10

[a] Significantly larger than normal ($P < 0.05$).

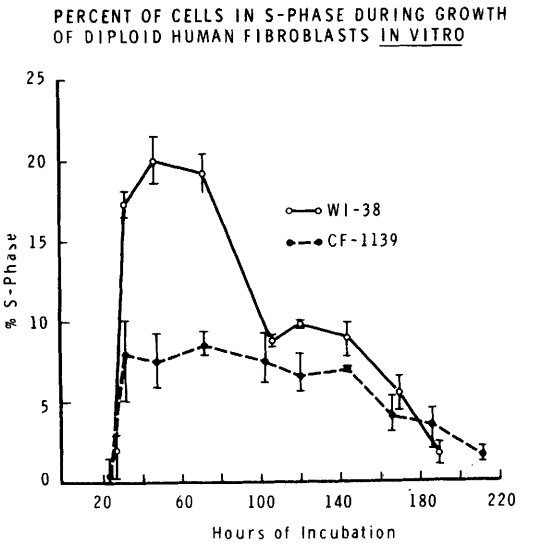

FIG. 2. Percent of fibroblasts labeled after a 60-min pulse of 0.5 μCi of H^3-thymidine at various times during log phase growth *in vitro* in 10% calf serum and MEM. The range for the three replicate means, each of 2000 cells, are indicated by horizontal lines. Upper curve, WI-38, lower curve, CF-1139.

The proportion of cells which could be recruited into the cell cycle, as determined from autoradiographic studies of H^3-thymidine incorporation, was about 20% for the normal fibroblasts *in vitro*; for the cystic fibrosis-derived fibroblasts, this proportionality dropped to about 8%. Further, this inability to recruit the usual numbers of cells into the cell cycle, which would lead to a significantly decreased *population* doubling time, was not altered by improving the nutritional quality of the *in vitro* medium used for the cultivation of these cells. That is, increasing the serum concentration from 10% to 20% and shifting the medium from MEM to Medium 199 had no effect upon either population doubling time or the percentage of cells in S phase during the log phase growth of these cultures *in vitro*.

These data lead us to conclude, then, that for reasons presumably related to the autosomal recessive character of the cystic fibrosis genes, fibroblasts from patients with cystic fibrosis tend

to have a significantly smaller number of cells recruited into the cell cycle at any given time, which in turn, causes a significantly decreased population doubling time or generation time of these cells *in vivo*.

We have no explanation for the significant difference in the behavior of these cells from patients with cystic fibrosis *vis-a-vis* either normal or disease control fibroblasts, save to conclude that it proceeds from an intrinsic property of these cells themselves. We originally (3) suggested that the genetic defect in these cells, arcane though it was, might, upon detailed examination, provide insight into the mechanism of cystic fibrosis pathophysiology *in vivo*. This paper has attempted to refine these defects in these cells in terms of the recruitment of these cells into the cell cycle. The significance of this observation remains to be elaborated by further studies.

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