Functional Failures of Cultured Human Diploid Fibroblasts After Continued Population Doublings[1] (35571)

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Recently a large number of investigators has been studying the expression of metabolic disease in human fibroblasts (1–5). These studies have often required growing large populations of cells from the relatively small number of fibroblasts found in a dermal biopsy. One of us, [Hayflick (6)], has shown that normal human diploid fibroblasts of embryonic origin have a limited lifetime in that the cell population is capable of doubling *in vitro* only 40–60 times. This finding suggests a possible complication in the continual use of a cell strain derived from a single explant for biochemical or other studies of the various metabolic diseases expressed in these cells.

Normal human diploid fibroblasts of embryonic lung origin (Strain WI-38) can be induced by oxyphenylbutazone to produce collagenolytic and proteolytic enzymes in culture, presumably by the de-repression of an operon (7, 8). Collagenolytic activity can also be induced in the skin of young rats, but not in old rats (9), by administration of oxyphenylbutazone, cortisol, or indomethacin (10, 11).

For these reasons, we decided to study the effects of increasing number of population doublings upon the ability of WI-38 to synthesize collagen and to induce collagenolytic enzyme activity when exposed to oxyphenylbutazone.

Materials and Methods. WI-38 fibroblasts were grown in confluent monolayer cultures 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. When they had become confluent, 12.5 μg/ml of vitamin C was added to the medium of each culture; and, after standing for 7 days (and two medium changes) to allow collagen synthesis, these monolayers were harvested mechanically, rinsed twice with serum free medium and re-suspended in medium were then incubated with 20 μg/ml of oxyphenylbutazone for 4 hr; those in HCl were hydrolyzed for 8.5 hr at 100°. These hydrolysates were then analyzed for hydroxyproline chemically (12) while the incubated cells were then sonicated and along with the medium were in turn re-incubated for 16 hr at pH 5.5 and 32° with a suspension of 10 mg/ml of insoluble native collagen (from 450-g rats) which had been swollen in 0.1 *M* acetate buffer pH 5.5 in the cold for 5–7 days. These incubation mixtures were then centrifuged for 1 hr at 15,000*g* in the cold; the clear supernatants were recovered and hydrolyzed in 4 *N* HCl and their hydroxyproline was determined (12) and converted into collagen concentration by calculation (7).

In this fashion, the amount of collagenolytic activity found in these cultures after 4 hr of induction by oxyphenylbutazone could be determined in terms of the micrograms of soluble, peptide bound hydroxyproline re-

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leased from 10 mg of insoluble collagen/4 $\times$ 10^6 cells (13).

Finally, similar cultures were prepared in which the monolayers were exposed to medium containing either 0 or 12.5 μg/ml of added vitamin C and 2 μCi/4 $\times$ 10^6 cells of ^{3}H-proline (5 μCi/μmole). After 7 days and two medium changes, these monolayers were also harvested mechanically, rinsed, and their hydroxyproline was separated from proline by a modification of the Prockop and Udenfriend procedure (12). The counts per 30 min of the hydroxyproline were recorded (it took this long for the samples to be essentially three times background) and the counts per minute of ^{3}H-hydroxyproline per 10^6 cells were calculated.

Cell strains of WI-38 were obtained which by calculation had reached 13 to 44 population doublings by the time they were harvested in culture. All of these cultures were treated in triplicate and the results represent the mean of three separate samples of the same cell strain after a given number of population doublings.

Results. Chemically, no significant amounts of hydroxyproline could be demonstrated in any cultures which had not received 12.5 μg/ml of Vitamin C. Further, no collagenolytic activity could be demonstrated in these cultures unless the cells had been exposed for 4 hr to oxyphenylbutazone as has been shown previously (13, 14).

After drug induction, collagenolytic activity could be demonstrated in these cultures at pH 5.5, except when the cells had doubled 39 or 44 times. Even after 37 doublings, these cells demonstrated significantly less collagenolytic activity than did cells which had doubled 35 times as shown in Table I.

Although a small amount of presumed ^{3}H-hydroxyproline could be found in cultures which had not received vitamin C (about 280 counts/30 min/4 $\times$ 10^6 cells), those cultures which had been treated with ascorbate contained more than twice this amount of radioactive proline (680 counts/30 min/4 $\times$ 10^6 cells); except when these cells had doubled 44 times. These latter cell monolayers increased their concentration of tritiated hydroxyproline only to 360 counts/30 min/4 $\times$ 10^6 cells.

TABLE I. Collagen Synthesis and Collagenolytic Enzyme Induction in Diploid Human Fibroblast Populations After Various Numbers of Doublings.

Doubling no.	Collagenolytic activity[a]	Collagen synthesis[b] ^{3}H-Hypro	^{3}H-Hypro + vitamin C	Hypro[c]
13	45	—	—	40
20	38	72	171	40
22	38	—	—	40
25	38	—	—	40
26	38	66	168	40
30	29	—	—	40
32	29	—	—	40
33	29	75	171	40
35	21	—	—	40
37	8[d]	—	—	28[d]
39	0[d]	—	—	0[d]
44	0[d]	66	90	0[d]

[a] Hydroxyproline solublized (μg/4 $\times$ 10^6 cells) from insoluble collagen after 4-hr induction by 20 μg/ml of oxyphenylbutazone.

[b] Counts per 30 min/10^6 cells above background (with or without 12.5 μg/ml of vitamin C added).

[c] Collagen (μg/10^6 cells) after 7 days in monolayer with 12.5 μg of vitamin C added (calculated from hydroxyproline determination).

[d] Significantly different from 20 to 33 doublings.

When incubated with ascorbate, these cells made amounts of hydroxyproline which could be detected chemically, but only up to the 37th doubling. At 44 doublings, the addition of ascorbate to these cells resulted in only a 30% increase in radioactive hydroxyproline. This would be below the level of detectability by the chemical method.

Finally, the doubling times of cell strains which had doubled 20 and 39 times were determined (15) and their karyology was also examined (16). Both sets of cells had 34–38-hr generation times and an essentially normal number of chromosomes, even after 39 doublings.

Conclusions and Summary. Cell populations which were karyologically normal and which were capable of a normal rate of cell division apparently lost much of their ability to synthesize collagen and to induce collagenolytic activity after approximately 37 to 39 doublings. This loss of at least two fibroblastic functions prior to either the loss of the ability to divide or to the appearance of significant changes in the karyology of these cells suggest that other cell functions might also be altered as human diploid cells near the end of their *in vitro* life time (Phase III). Fewer cell doublings can be expected from fibroblasts derived from adult tissue (6). Consequently caution should be exercised when interpreting the results of metabolic studies on embryonic cells that have doubled many times or cells grown out of explants from adult tissues which had been allowed to double even a relatively small number of times.

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