Replication Rate and Lifespan of Cultured Fibroblasts in Down's Syndrome[1] (36940)

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Since the original report of Hayflick and Moorhead, many laboratories have confirmed the observation that cultured human diploid fibroblasts have a limited *in vitro* lifespan (1–3). Subsequent studies have shown that this limitation is unrelated to external cellular environment, is inversely related to the age of the fibroblast donor, and varies with the tissue of origin (3, 4). Reduced *in vitro* fibroblast lifespan is a feature of Werner's syndrome, an autosomal recessively inherited disease which is associated with accelerated degenerative changes in many tissues (3).

Retarded pre-natal and post-natal growth are well-recognized clinical features of patients with Down's syndrome or trisomy-21 (5). At the tissue level, a pathological study of organs from newborns with this disorder revealed a generalized decrease in cell number (6). Finally, at a cellular level, the rate of DNA synthesis was diminished in trisomy-21 fibroblasts as measured by autoradiography and by DNA dilution (7, 8). Based on the likelihood that impaired cellular replication was a feature of this chromosomal disorder, a long-term study of cultured trisomy-21 fibroblasts was undertaken.

Materials and Methods. Establishment of cultures. Skin explants were obtained from the antecubital fossa during cardiac catheterization from 3 patients with Down's syndrome and 3 age-matched patients. Punch skin biopsies were taken from the gluteal region from an additional pair, a patient with Down's syndrome and her sibling (4T and 4N). All explants were minced into 1 mm × 1 mm sections and placed under 10 mm × 20 mm coverslips in Leighton tubes, 3 per tube.

Subcultivation. When approximately 50% of the coverslip surface was covered by cell growth, the fibroblasts were transferred to and further subcultivated in 25 cm^2 plastic flasks (Falcon Plastics Co.). (0.25% pronase was employed to harvest the cells from glass or plastic surfaces). At the initial passage, two separate cultures (A and B) were established, subcultured on alternate days, cultured in individually prepared batches of medium, and kept in different incubators. Cell number (N_f) was determined by hemocytometer counts at the harvest preceding each passage, and cultures were inoculated at 1.6×10^5 cells (N_0) in an effort to provide maximal sustained growth. Initially, the cultures were subcultured weekly. When cell replication rates diminished, fresh medium was added weekly, but the cells were not harvested until confluent. If, after 1 month, the fibroblasts did not reach confluency, they were harvested, counted, and reinoculated at 1.6×10^5 cells (N_0) per flask. When N_f after 1 month was at or less than N_0, the culture was considered to be "senescent."

Culture conditions. All cultures were grown in MEM medium (Gibco) with supplementary non-essential amino acids, glutamine, penicillin, streptomycin, and 10% fetal-calf serum in a closed 5% CO_2 environment at 37°.

Calculations. Cell-population doublings at each passage was calculated as $\log_2 (N_f/N_0)$. Cumulative cell population doublings (CPD) from the initial passage to senescence were therefore:

$$\text{CPD} = \sum_{n=1}^{P} \log_2 \left(\frac{N_f}{N_0} \right).$$

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The cell population doubling rate (R_{CPD}) was computed as

$$R_{CPD} = \log_2 \left(\frac{N_f}{N_0} \right) / t$$

where t is time in days.

Chromosome analysis was done on all cultures, confirming the clinical diagnoses.

Results. CPD for the 8 normal and 8 trisomy-21 cultures are listed in Table I. The difference in *in vitro* lifespan of the two groups as represented by mean CPD was 11.0 doublings. This difference is highly significant ($p < 0.005$). Although there was a slight difference in mean donor age between the two groups (7.75 for normals vs 4.75 for trisomy-21), the results of Martin *et al.* indicate that this would not account for the observed difference in CPD (3). The time between explanting and the first subculture was almost identical in normal (18.0 days) and trisomy-21 fibroblasts (18.7 days) indicating that initial cellular outgrowth was not responsible for this difference.

TABLE I. Cumulative Cell Population Doublings of Normal and Trisomy-21 Fibroblast Cultures.

Source	Culture no.	Donor age (yr)	Doublings (CPD)
Trisomy-21	1A	11	38.4
	B		45.7
	2A	9	40.7
	B		42.1
	3A	4	41.1
	B		42.0
	4A	7	39.3
	B		30.9
	$\bar{X}^a$	7.75	40.5
	SE		1.5
Normal	1A	3	52.8
	B		51.1
	2A	7	59.6
	B		59.7
	3A	3	50.7
	B		44.0
	4A	6	47.1
	B		47.1
	$\bar{X}^a$	4.75	51.5
	SE		2.0

[a] $t = 4.55$ ($p < 0.005$) for difference of CPD.

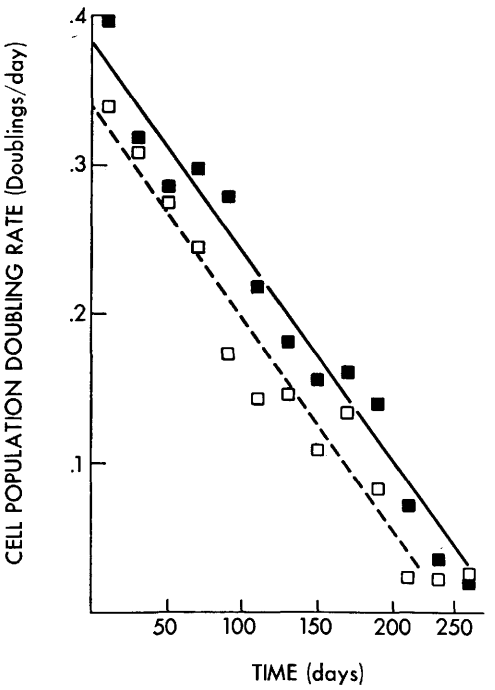

FIG 1. The mean cell population doubling rate (R_{CPD}) of normal fibroblasts ■—■ and trisomy-21 fibroblasts □—□ over 250 days in continuous cell culture. Covariance analysis reveals that the slopes are not significantly different but that the decreased R_{CPD} of trisomy-21 fibroblasts is highly significant ($p < 0.001$).

The rate of cell population doubling (R_{CPD}) in both normal and trisomy-21 fibroblasts decreased linearly with time (Fig. 1). There is no significant difference in the slope of the lines plotted by the method of least squares (—.001404 for normal cultures vs —.001423 for trisomy-21 cultures). Analysis of covariance revealed a highly significant decrease in the R_{CPD} for trisomy-21 fibroblasts ($p < 0.001$). Only in the final passages did the R_{CPD} become essentially equal. The majority of the individual A and B cultures derived from the same donors had similar R_{CPD} and CPD.

Discussion. Determination of the exact number of cumulative cell replications is limited by several difficulties. The first is in measuring the number of doublings that occur between explanting and the initial subculture. Secondly, 100% cellular attachment, which is used for calculation of CPD, is cer-

tainly not always present. Lastly, not all cells in a culture are replicating. Since the calculations begin with the first subculture, assume a 100% cell attachment, and reflect population, not individual doublings, the 51.5 ± 2.0 (mean ± standard error) CPD of the normal fibroblasts represents a gross underestimate of the total cellular replicative potential. Nevertheless, it approximates the values obtained for the human fetal-lung fibroblast strain, WI 38, under a variety of culture conditions (4).

The 40 ± 1.5 CPD of the trisomic fibroblasts represents a highly significant 20% reduction of their *in vitro* lifespan. In the two previously reported disorders in which *in vitro* lifespan is reduced, there was no indication of whether this was the result of earlier onset of senescence or rather a decrease in the rate of cellular replication (3, 9). Figure 1 demonstrates that the latter is the explanation for the diminished CPD in trisomic fibroblasts. The parallel linearity observed in Fig. 1 suggests that the same mechanism is responsible for diploid cell senescence in both normal and trisomy-21 fibroblasts, but that the latter cells start their *in vitro* lifespan with a lower replicative rate.

The observation of decreased R_{CPD} in these trisomy-21 fibroblasts agrees well with the increased cellular doubling time (DNA half-life) found by Kaback and Bernstein (8).

Therefore, Down's syndrome or trisomy-21 is characterized by retarded growth by both clinical and cellular parameters. This is probably not the result of specific genes located on the 21st chromosome pair, since clinically retarded growth is associated with other chromosomal disorders (5). Rather, the diminished *in vitro* lifespan and reduced cellular replication may be related to more generalized cellular effects secondary to the chromosomal aneuploidy.

Summary. Skin fibroblasts derived from patients with Down's syndrome (trisomy-21) have a significantly decreased number of cumulative cell population doublings (CPD) measured from the initial passage to senescence when compared with cultures from karyotypically normal age-matched controls (40 ± 1.5 vs 51.5 ± 2.0 CPD).

In both instances there is a linear decrease in the cell population doubling rate with increasing passage number. However, the trisomic fibroblasts start with and constantly maintain a lower rate, leading to an earlier cessation of replication.

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