Progeria: a Cell Culture Study on Aging

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A B S T R A C T Progeria is an autosomal recessive disorder showing precocious senility. The cultured skin fibroblast from both the homozygous affected individual and the heterozygous parents can be distinguished from normals by decreased cell growth in culture. Mitotic activity, DNA synthesis, and cloning efficiency are markedly reduced.

INTRODUCTION

Hayflick and coworkers first demonstrated that the human diploid cell has a finite life-span in culture and suggested that this restricted growth potential reflected senescence at the cellular level (1–3).

Thus, it appeared that the life-span of the cultured skin fibroblast is a function of the genotype of the donor (1–6). If so, cells derived from patients with genetic disorders might reveal growth differences in culture. One such syndrome, progeria, is an autosomal recessive disorder characterized clinically by precocious senility during the first decade of life and death from complications of coronary artery arteriosclerosis within the first two decades of life (7, 8).

The purpose of this paper is to report studies on cultured skin fibroblasts from both the homozygous affected individual and the obligatory heterozygote for progeria to study the influence of this cell genotype on cell growth in culture.

METHODS

Skin biopsies were obtained from three affected children (two males, one female) with the clinical phenotype associated with progeria, three obligatory heterozygotes, parents of two of the affected children, four age- and sex-matched normal unrelated children, and four age- and sex-matched

adults. All biopsies were split thickness (2 × 3 mm) specimens taken from the inner, upper aspect of the right arm.

The cell cultures were established using standard culture procedures (9). Each biopsy was cut in half and attached to the floor of the plastic Petri dish by a sharp knife. 3 ml of culture medium (Eagle's minimum essential medium containing 20% by volume of human AB serum and 5% beef embryo extract ultrafiltrate [10]) was added, and the Petri dish was placed in an atmosphere of 5% CO_2 in air at 37°C for 2 wk. After this initial period, the medium was then changed weekly. After several weeks (usually 4) during which time the fibroblasts migrated from the original explant and divided, each culture was trypsinized and established as a monolayer culture. Between the fourth and sixth subcultures, the following culture activities were studied.

Cell growth. Petri dish cultures (diameter 5 mm) were seeded with 1×10^5 cells in 5 ml of medium which gave a nonconfluent monolayer. Total cell number for each culture was determined every 96 hr for a period of 2 wk. Cell counts were performed with an electronic particle counter (Coulter) or a hemocytometer. Growth curves were plotted as total cell number per culture.

Mitotic activity. Approximately 10 cells in 100 μl of nutrient medium were seeded into 10 small depressions in Falcon plastic microdrop plates. The number of mitotic figures per depression was counted microscopically every 24 hr for a period of 1 wk. By this method of scoring, the cells in prophase could not be determined. No attempt was made to count the total number of cells per culture. The time at which a confluent monolayer was established was recorded.

DNA synthesis. Petri dish cultures (diameter 5 mm) were established with 1×10^5 cells in 5 ml of medium. After 12 hr of control growth, the medium was changed to one containing 2 μCi tritiated thymidine (specific activity 6.7 Ci/mM) per 3 ml of medium. After a pulse of 24 hr, the medium was removed, and the monolayer was washed with warmed balanced salt solution. The cells were then trypsinized using 10 ml of trypsin (0.25%). 1 ml was used for a cell count, and the remaining 9 ml was processed according to the method of Hughes and Caspary (11). The cell suspension was centrifuged (800 rpm for 5 min at 4°C), and the pellet was washed three times with chilled buffer (0.9% NaCl in 0.01 M KH_2PO_4–$NaHPO_4$, pH 7.4) and precipitated with chilled 5% trichloroacetic acid. The precipitate was then dehydrated in cold methanol, centrifuged,
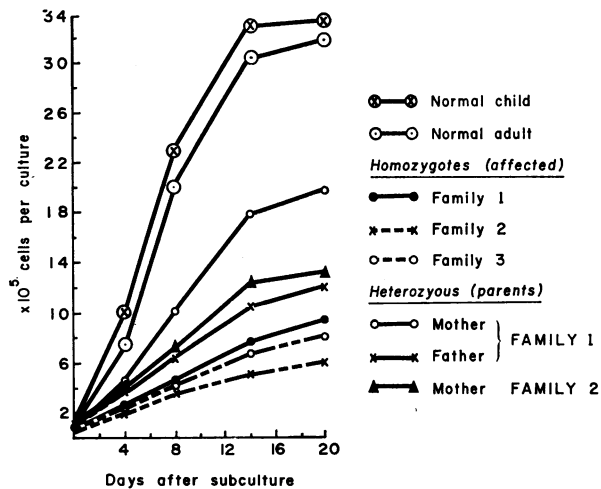
FIGURE 1 Growth of cultured skin fibroblasts from homozygous affected and heterozygous parents from three families with progeria, one normal adult, and one normal child. All cultures were in the fourth subculture by trypsinization.

and dried in a vacuum. The pellet was dissolved in 0.5 ml Hyamine (Packard Instrument Co., Inc., Downersgrove, Ill.) overnight and transferred to scintillation vials containing 6 ml of scintillation fluid (11). The radioactivity was expressed as counts per minute per cell.

Cloning efficiency. Cells in the fourth subculture were cloned by the dilution method of Puck, Marcus, and Cieciura (12). To avoid error from cell clumping, individual cells were identified 24 hr after seeding. 2 wk later, the medium was removed, and the culture was washed with warm balanced salt solution, fixed with methanol, and stained with toluidine blue O (13). The areas identified as containing individual cells were examined microscopically under low power; if more than 10 cells were present, it was scored as a clone. Cloning efficiency was expressed as the percentage of inoculated cells which formed clones.

RESULTS

No information was obtained about cellular growth within individual explants. Observations started with
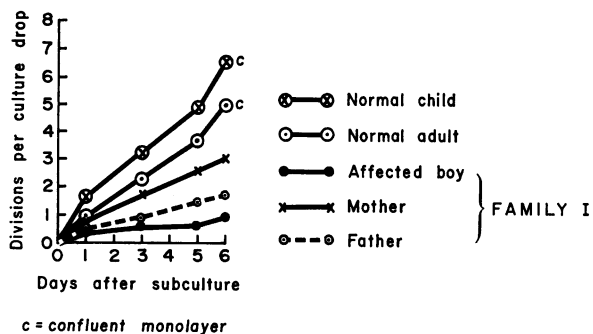

FIGURE 2 Mitotic activity of cultured skin fibroblasts from one family with progeria and a normal adult and child. All cultures were in the fourth subculture by trypsinization.

migration of cells from these explants. Cell migration occurred from all biopsy specimens from the three patients with progeria and three heterozygous parents so that cell lines could be obtained. Establishment of the initial cell lines from the original biopsy specimens from the progeria patients took approximately twice as long as those from normal controls and obligatory heterozygotes.

At each subculture, approximately 90% of the plated cells attached to the plastic in all cultures. At the fourth subculture, cell lines from all three patients and three heterozygotes were successfully stored in liquid nitrogen for future studies. Cultures have been reestablished from this source without difficulty.

Cell growth. In cultures from normal individuals (Fig. 1), cell number increased relatively constantly until confluency was attained; then the increase in cell number markedly decreased.

In cultures derived from all three patients with progeria, the increase in cell number was so gradual that any change in growth with confluency had to be detected by monitoring DNA synthesis. The cultures from the heterozygous parents showed activity intermediate between that of the patients and normals.

Mitotic activity. The cells from the progeria patients showed a marked reduction in the number of mitotic figures present in a microculture as compared with that of matched controls (Fig. 2). Cells from heterozygotes showed a reduction in mitotic figures as compared with age-matched controls but not to the same degree as their affected offspring.

DNA synthesis. When subculture number (No. 5)

TABLE I

Uptake of Thymidine-³H by Cultured Skin Fibroblasts from Families with Progeria and Normal Age- and Sex-Matched Controls during a 24 hr Pulse Period

Subjects studied	Thymidine-³H incorporation	Age-sex matched controls
	cpm × 10⁻²/cell	
Family 1		
Affected homozygote	0.55 ±0.03	4.22 ±0.31
Heterozygous father	1.66 ±0.12	3.41 ±0.52
Heterozygous mother	2.38 ±0.22	3.59 ±0.22
Family 2		
Affected homozygote	0.56 ±0.02	4.93 ±0.26
Heterozygous mother	2.05 ±0.26	3.07 ±0.32
Family 3		
Affected homozygote	0.50 ±0.04	3.92 ±0.24

Thymidine-³H added 12 hr after subculture. All cultures in fifth subculture.

Progeria: a Cell Culture Study on Aging 2001

and cell concentration (1×10^5 cells) were standardized, the rate of incorporation of thymidine-^{3}H in cultures from progeria patients was 1/10 that observed in cultures from matched control children (Table I). Maximum incorporation in both groups occurred when the cell population was nonconfluent and decreased when confluency was reached (Table II).

Cultures from the three heterozygotes showed less incorporation compared with cultures from matched controls but more than cultures from their affected children.

All fibroblasts irrespective of source showed contact inhibition using thymidine-^{3}H incorporation as an index of cell duplication (Table II).

Cloning efficiency. The cloning efficiency (Table III) of cultures from the three affected children was markedly less than for the normal children. The cloning efficiency of the heterozygous parents was lower than that of the matched cultures from normal adults. These differences were statistically significant (Table IV). In family 1, although the cultures from both parents showed reduced cloning efficiency, the cultures derived from the mother showed greater cloning efficiency than those from the father (Table III).

DISCUSSION

The limited life-span of the human diploid cell in culture was considered by Hayflick to be a manifestation of aging at the cellular level (1–4). The culture characteristics Hayflick (3) described for senescence (phase III) were (*a*) an increased generation time, (*b*) gradual cessation of mitotic activity, (*c*) accumulation of cellular debris, and (*d*) total degeneration of the culture.

Fibroblasts from homozygotes and heterozygotes for

TABLE II

The Influence of Cell Confluency on the Uptake of Thymidine-^{3}H by Cultured Skin Fibroblasts from a Homozygous Affected Patient with Progeria (Family 1) and a Normal Age-Matched Child

Subjects studied	Total cell number/ Petri dish	Thymidine-^{3}H incorporation
		$cpm \times 10^{-1}/cell$
Normal child	217,680	3.90
	367,290	4.30
	402,120	1.70
	678,000	0.56
Affected child	195,600	0.87
	289,170	0.70
	376,500	0.34
	459,960	0.14

Thymidine-^{3}H added to cultures 12 hr after subculture for a 24 hr pulse period. All cultures in fifth subculture.

TABLE III

Cloning Efficiencies of Cultured Skin Fibroblasts from Three Families with Progeria and Eight Normals. Cultures Cloned in Fourth Subculture

Subjects	No. of individuals studied	Cloning efficiency
		% ± SEM
Normals		
Adults:		
Male	2	9.1 ±1.0
Female	2	8.8 ±1.2
Children:		
Male	2	12.2 ±1.4
Female	2	11.4 ±0.8
Affected homozygotes		
Family 1	1	0
Family 2	1	2.3 ±0.4
Family 3	1	1.5 ±0.2
Heterozygous parents		
Family 1		
Father		2.7 ±0.5
Mother		5.6 ±0.2
Family 2		
Mother		6.0 ±0.8

progeria were studied in culture to determine if such cells would show any of these characteristics. In studying certain parameters of cell growth (increase in total cell number [Fig. 1], presence of mitotic figures in microdrop cultures [Fig. 2], DNA synthesis [Tables I and II], and cloning efficiency [Tables III and IV]), decreased growth was found in cultures from both homozygotes and heterozygotes. These measurements merely demonstrated that in a culture of progeric cells, there was less growth as compared with that in normal cultures. These studies did not establish that decreased growth was due to an increased generation time of the individual cell. As suggested by Merz and Ross (14),

TABLE IV

Statistical Analyses: Cloning Efficiencies of Cultured Fibroblasts from Three Homozygotes and Three Heterozygotes for Progeria, Four Normal Children, and Four Normal Adults

Subjects	No. of observations	t test	P
Normal child: homozygous child	22	27.4358	<0.001
Normal female adult: heterozygous female adult	13	7.6923	<0.001
Normal male adult: heterozygous male adult	12	18.2857	<0.001

such decreased growth could have reflected an increase in the number of nondividing cells throughout the entire life-span of the culture.

Goldstein (15) reported that skin fibroblast cultures derived from one 9 yr old boy with progeria could not be subcultured more than twice whereas age-matched controls could be subcultured 20–30 times. Martin, Sprague, and Epstein (5), using similar culture techniques, were unable to confirm this observation as cultures from their patient showed a life-span within normal limits (although ranked 23rd of 26 in the age group studied). The present studies on three homozygotes and three heterozygotes agreed with the findings of Martin et al. (5).

Physical signs of precocious senility are seen in another human syndrome, Werner's syndrome (8). Epstein and coworkers (16, 17) reported that cultured skin fibroblasts from patients with Werner's syndrome could not be subcultured more than five times (10 divisions in culture) and showed degenerative changes in the cytoplasm (PAS-positive perinuclear inclusions). Thus the cultured cells from Werner's syndrome meet the criteria for senescence (phase III of Hayflick [3]). As the cultured cells from both the homozygotes and heterozygotes for progeria were repeatedly subcultured and did not show cytoplasmic changes (accumulation of cellular debris), the characteristics of the cultured fibroblasts from these two syndromes appeared to be different. Since fibroblast cultures have not been established from both syndromes in the present study, the possibility has not been excluded that these differences were due to techniques of culture.

Survival under standard culture conditions is dependent on cell multiplication. That the progeric cell has a decreased ability to survive under these conditions is supported by the present studies (Figs. 1 and 2, Tables I–IV). As previously suggested (5) homozygosity for a single gene mutation may have a striking effect on the longevity of cultured somatic cells. If culture conditions are imposed upon the progeric cell which require rapid growth activity, the conclusion will be reached that the progeric cell cannot be maintained in culture.

ACKNOWLEDGMENTS

REFERENCES

1. Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. Exp. Cell. Res. 25: 585.
2. Hayflick, L., S. A. Plotkin, T. W. Norton, and H. Koprowski. 1962. Preparation of poliovirus vaccines in a human fetal diploid cell strain. Amer. J. Hyg. 75: 240.
3. Hayflick, L. 1965. The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. 37: 614.
4. Hayflick, L. 1966. Cell culture and the aging phenomenon. In Topics in the Biology of Aging. P. L. Krohn, editor. Interscience Publishers, New York. 83.
5. Martin, G. M., C. A. Sprague, and C. J. Epstein. 1970. Replicative life-span of cultivated human cells. Lab. Invest. 23: 86.
6. Goldstein, S., J. W. Littlefield, and J. S. Soeldner. 1969. Diabetes mellitus and aging: diminished plating efficiency of cultured human fibroblasts. Proc. Nat. Acad. Sci. U. S. A. 64: 155.
7. Gabr, M., N. Hashem, M. Hashem, A. Fahmi, and M. Safouh. 1960. Progeria, a pathologic study. J. Pediat. 57: 70.
8. Epstein, C. J., G. M. Martin, A. L. Schultz, and A. G. Motulsky. 1966. Werner's syndrome. Medicine (Baltimore). 45: 177.
9. Danes, B. S., and A. G. Bearn. 1969. Cystic fibrosis: a study in cell culture. J. Exp. Med. 129: 775.
10. Chu, E. H. Y., and N. H. Giles. 1959. Human chromosome complements in normal somatic cells in culture. Amer. J. Hum. Genet. 11: 63.
11. Hughes, D., and E. A. Caspary. 1970. Lymphocyte transformation in vitro measured by tritiated thymidine uptake. Int. Arch. Allergy Appl. Immunol. 37: 506.
12. Puck, T. T., P. I. Marcus, and S. J. Cieciura. 1956. Clonal growth of mammalian cells in vitro. J. Exp. Med. 103: 273.
13. Danes, B. S., and A. G. Bearn. 1966. Hurler's syndrome: a genetic study in cell culture. J. Exp. Med. 123: 1.
14. Merz, G. S., and J. D. Ross. 1969. Viability of human diploid cells as a function of in vitro age. J. Cell Physiol. 74: 219.
15. Goldstein, S. 1969. Lifespan of cultured cells in progeria. Lancet. 1: 424.
16. Martin, G. M., S. M. Gartler, C. J. Epstein, and A. G. Motulsky. 1965. Diminished lifespan of cultured cells in Werner's syndrome. Fed. Proc. 24: 678.
17. Epstein, C. J., G. M. Martin, and A. G. Motulsky. 1965. Werner's syndrome: caricature of aging. A genetic model for the study of degenerative diseases. Trans. Ass. Amer. Physicians Philadelphia. 78: 73.