PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 161, 449–451 (1979)

Effects of the Incorporation of *p*-Fluorophenylalanine on the *in Vitro* Lifespan of Human Diploid Cells (40571)

M. R. DUNCAN AND R. T. DELL'ORCO

Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Route 1, Ardmore, Oklahoma 73401

The error catastrophe theory of aging (1) attributes senescence to a cascade of erroneous protein synthesis initiated by errors occurring randomly in information-processing molecules. The detection of heat-labile glucose-6-phosphate dehydrogenase (G6PD) molecules in normal senescent human fibroblast-like cells (2), as well as in fibroblast-like cells obtained from donors afflicted with diseases characterized by premature aging (3–5), has been used to support this theory.

We recently reported that the accumulation of heat-labile G6PD with *in vitro* age in human diploid fibroblast-like cells may not be associated with errors in protein synthesis but rather with a shift in equilibrium to a more heat-sensitive molecular form of the enzyme (6). Pendergrass *et al.* found no heat-labile G6PD in senescent human cells, but reported that exposure of the cells to the proline analog azetidine carboxylic acid resulted in significant levels of heat-sensitive enzyme (7). These authors, however, did not study the effect of the analog-induced errors on the *in vitro* lifespan of the cells. Ryan *et al.* (8) studied the effects of the amino acid analogs *p*-fluorophenylalanine (pfpa) and ethionine on the *in vitro* lifespan of WI-38 cells. They found that exposure of these cells to "nontoxic" concentrations of the analogs did not significantly alter the lifespan. No attempt was made in these studies, however, to determine the level of errors produced by incorporation of the analogs.

Orgel (1) predicted that a brief exposure to an amino acid analog should, according to the error catastrophe theory, result in a self-perpetuating error cascade and lead to premature senescence. In the experiments described here, this specific prediction of the error theory was tested by exposing human diploid fibroblast-like cells to the amino acid analog pfpa for a short portion of their *in vitro* lifespan. The effects of the exposure of this analog on the heat sensitivity of cellular G6PD and on the number of population doublings achieved by the exposed cells were determined.

Materials and methods. Normal human diploid fibroblast-like cells obtained from newborn foreskin material and designated CF-3 were used in these experiments. These cells were cultivated in McCoy's medium 7a (9) and have a characteristic *in vitro* lifespan of 66 ± 10 population doublings (PD). Cells were disrupted by sonication and heat-labile G6PD assays were performed according to Holliday and Tarrant (2). Electrophoresis of cell homogenates was performed as described by Dietz and Lubrano (10) except that the acrylamide concentration was increased to 10%. G6PD activity in the gels was stained by the method of Richards and Hilf (11).

To study incorporation of pfpa into cellular G6PD, cells were grown to confluency in 6 days in medium containing 1.0 μCi/ml of ^{3}H-pfpa. Cells were disrupted by sonication, the 30,000 *g* supernatant subjected to electrophoresis, and the gels stained to demonstrate G6PD activity. The gels were scanned at 610 nm in a Quick Scan Jr. densitometer (Helena Laboratories Corp., Beaumont, Tx.) and then frozen and sectioned into 1.0-mm slices. Each slice was dissolved in 0.5 ml of 30% H_2O_2 within a scintillation vial, Aquasol (New England Nuclear) was added, and radioactivity determined by counting in a LS8000 liquid scintillation counter (Beckman Instruments, Inc.).

To study the effects of pfpa incorporation on the level of heat-labile G6PD and on the *in vitro* lifespan of cells, control cultures were grown to confluence in 6 days in growth medium while identical cultures were grown in growth medium containing 175 μM pfpa. On the sixth day the percentage heat-labile G6PD in control and experimental cells was determined. One control culture was subcultured for the remainder of its *in vitro* lifespan. Fresh growth medium without pfpa was

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added to one experimental flask and this culture was maintained for an additional 7 days, during which time the cells became confluent. The experimental culture was then subcultured for the remainder of its lifespan at a split ratio identical to that used for the control cells. Four cultures from two freezer stocks were utilized in these experiments. pfpa was purchased from Calbiochem and ^{3}H-pfpa (2 Ci/mmol) was purchased from Amersham/Searle Corp.

Results. Table I compares the percentage heat-labile G6PD present in control cells to that found in cells grown for 6 days in the presence of 175 μM pfpa. At each population doubling level (PDL) tested, exposure to pfpa resulted in a dramatic increase in heat-sensitive G6PD levels. The values from pfpa-treated cultures in these experiments approximated the percentage heat-labile G6PD determined previously insenescent CF-3 cultures (6).

It was possible that the increased heat lability of G6PD in pfpa-treated cells was not a direct result of analog incorporation into the enzyme. Homogenates from pfpa-treated and control cultures were subjected to electrophoresis, and the G6PD activity specifically stained to determine if the analog had induced a shift to the more heat-sensitive form of the enzyme. Figure 1 demonstrates that the profiles of G6PD activity from treated and control cultures were similar. Additionally, cultures were grown in the presence of ^{3}H-pfpa, the homogenates subjected to electrophoresis and the gels stained for enzymatic activity. The gels were then sliced and assayed for radioactivity. Figure 2 demonstrates that the radiolabeled analog was

TABLE I. PERCENTAGE HEAT-LABILE G6PD IN PFPA-TREATED AND CONTROL CULTURES

Population doubling level	Percentage heat-labile G6PD[a]	
	Control	pfpa treated
16	5	20
23	3	24
23	5	19
29	7	17

[a] Values are the percentage heat-labile G6PD from cultures of either control cells (control) or cells exposed to 175 μM *p*-fluorophenylalanine for 6 days (pfpa treated). Cultures were exposed to pfpa at the population doubling level indicated and heat-labile G6PD was assayed as described under Materials and Methods.

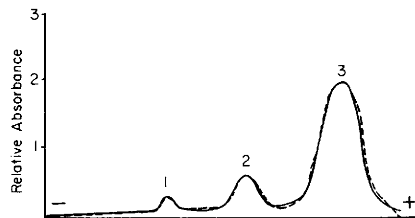

FIG. 1. Densitometer tracings of polyacrylamide gels stained to demonstrate G6PD activity. Extracts from control cells (——) and from cells exposed to 175 μM *p*-fluorophenylalanine for 6 days (----) were subjected to electrophoresis as described under Materials and Methods and yielded similar profiles of activity. Tentative identification of peak 1 as a nonspecific hexose phosphate dehydrogenase and peaks 2 and 3 as the tetrameric and dimeric forms, respectively, of G6PD has been previously discussed (6). Cultures were population doubling 29.

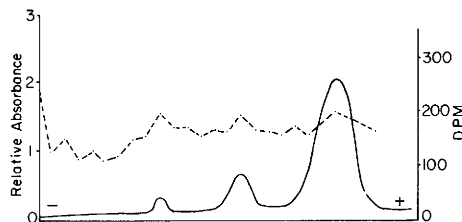

FIG. 2. Densitometer tracing (——) and radioactivity profile (●) of a polyacrylamide gel stained to demonstrate G6PD activity and fractionated for the determination of radioactivity. Electrophoresis of an extract of population doubling 29 cells, which were grown for 6 days in the presence of *p*-[^{3}H]fluorophenylalanine, was performed as described under Materials and Methods. Corresponding G6PD activity and radioactivity peaks indicated incorporation of ^{3}H-pfpa into G6PD molecules.

distributed throughout the gel, indicating incorporation into many proteins present; however, peaks of radioactivity corresponded with those areas of the gel containing G6PD activity. This experiment provided further evidence that pfpa was incorporated into G6PD molecules.

Table II illustrates the number of PD achieved by analog-treated and control cultures. In each case the treated cultures reached PDLs within at least 3% of control cultures. In these experiments cultures were considered "phased out" when they failed to reach confluence within 2 weeks of subculture at a split ratio of 1:2.

Discussion. These data confirm and extend

the work of Ryan *et al.* (8). We have shown that exposure of low PDL cultures to pfpa resulted in the rapid accumulation of heat-labile G6PD molecules to levels observed in senescent cells (6). The electrophoretic profiles of G6PD activities from treated cells were similar to those from control cells indicating that the observed increase in heat lability was caused by analog incorporation as opposed to a shift in equilibrium between the molecular forms of the enzyme (see Fig. 1). Further, our data indicated that cultures which accumulated such levels of analog incorporation errors did not exhibit shortened lifespans.

It is possible that the cellular response to analog incorporation differs from the response to misincorporation of a natural amino acid. Shakespeare and Buchanan (12), however, showed that low PDL fibroblast-like cells grown in the presence of pfpa exhibited an increased rate of protein degradation similar to that observed in senescent cells. These authors thus suggested that the analog-treated younger cells responded to artificial errors in a similar manner as older cells responded to natural errors (12).

Holliday recently reported that pfpa concentrations must be below a certain threshold level in order to significantly shorten the lifespan of mice (13). Also, it is possible that during the exposure time of our experiments analog-incorporation errors did not occur in critical information-processing proteins. Ryan *et al.* (8) showed, however, that very low concentrations of pfpa and ethionine had no effect on lifespan even when continuously supplied to cultured human cells.

It is emphasized that our experiments were designed to test the specific prediction of the error catastrophe theory (1) that a pulse exposure of amino acid analogs should induce premature senescence. Even though a pulse of pfpa resulted in increased heat-labile G6PD, these experiments failed to demonstrate such premature senescence in cultured human cells.

TABLE II. TOTAL POPULATION DOUBLINGS ACHIEVED BY PFPA-TREATED AND CONTROL CULTURES

Population doubling level[a]	Population doublings achieved[b]	
	Control	pfpa treated
16	76	75
23	76	76
23	75	73
29	73	74

[a] Population doubling level of individual cultures at the time of the 6-day exposure to *p*-fluorophenylalanine.

[b] The number of population doublings achieved during the *in vitro* lifespan of control cells (control) and cells exposed to 175 μM p-fluorophenylalanine for 6 days (pfpa treated). After exposure to the analog at the indicated population doubling levels, cells were subcultured in the absence of the analog at split ratios identical to those used for controls for the remainder of their lifespan.

Summary. Normal human diploid fibroblast-like cells were grown for 6 days in the presence of 175 μM *p*-fluorophenylalanine. Treated cultures exhibited elevated levels of heat-labile glucose-6-phosphate dehydrogenase as compared to controls. When fresh growth medium without the analog was added to treated cultures, however, these cells achieved as many population doublings as untreated controls. Thus, the prediction of the error catastrophe theory of aging that a pulse of amino acid analog should induce premature senescence could not be demonstrated in cultured human cells.

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Received February 5, 1979. P.S.E.B.M. 1979, Vol. 161.