EFFECTS OF β INTERFERON ON HUMAN FIBROBLASTS AT DIFFERENT POPULATION DOUBLING LEVELS

Proliferation, Cell Volume, Thymidine Uptake, and DNA Synthesis

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Prolonged cultivation of human diploid fibroblasts in vitro is associated with a loss of proliferative capacity, which has been considered to be a manifestation of senescence at the cellular level (1). Cultures of senescent populations exhibit a marked decrease in the percentage of S phase cells. Most of the cells are blocked in G0/G1 although some appear to be blocked in G2 (2–5). In vitro aging is associated with increases in cell volume and nuclear area (6) and with a decrease in cell locomotion (7, 8). Martin and co-workers (9) observed changes in cytoskeletal elements (stress fibers) in the cytoplasm of older cells, which more recent work has shown to represent an increased organization of microfilaments into bundles (10).

A similar array of alterations in cell structure and function has been described for fibroblasts treated with interferons. There is a progressive increase in the cell population doubling time of interferon-treated cell populations (11, 12), which appears to result from a block in or prolongation of G0/G1 or G2 or both (13–16). Interferon treatment of human fibroblasts also results in an increase in cell volume and nuclear area (17). The locomotion of interferon-treated cells is decreased and there is an increase in the number of actin-containing microfilament bundles (17, 18).

The possibility has been considered that the reaction pathway underlying the phenotypic changes observed during in vitro aging of fibroblasts and after interferon treatment may be a common one (15, 17). If so, the effects of interferon and senescence on the proliferative capacity of cells may be additive, i.e., interferon may be expected to cause the same percent increase in doubling time at varying population doubling levels. We have found that the population growth rate, modal cell volume, and thymidine uptake are affected to a similar extent at middle and high population doubling levels, but that DNA replication is affected differently depending on the in vitro age of the cultures.

Materials and Methods

Cells and Culture Conditions. The human diploid fibroblast cell line 76-109, kindly provided by Dr. George M. Martin, Department of Pathology, University of Washington, Seattle, was originally isolated from the foreskin of a 4-d-old newborn (19). The cells were

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subcultured once a week using a solution of 0.13% trypsin (1-300; ICN Nutritional Biochemicals, Cleveland, OH)-0.025% Na_2EDTA (trypsin-versene) and seeding at a concentration of 4×10^3 cells/cm^2 in Dulbecco-Vogt medium supplemented with 16% fetal calf serum (FCS).[1] The population doubling level (PDL) at each passage was calculated from [log(cell number at passage) − log (cell number seeded)]/log 2. For experimentation, cells were seeded at a density of 1.6×10^3 cells/cm^2 in tissue culture flasks or culture dishes.

Interferon Treatment. Two preparations of partially purified human β interferon were used; one was provided by Dr. J. Horoszewicz, Roswell Park Memorial Institute, Buffalo, NY (sp act, 5.2×10^7 U/mg protein) and the other by Dr. W. Carter, Temple University, PA (sp act, 1×10^7 U/mg protein). Interferon preparations were titered by the semimicrotitration procedure of Havell and Vilček (20) on monolayers of either 76-109 cells (PDL 49) or FS4 cells (passaged as described above but in medium plus 10% FCS). Titers are expressed as units/ml (U/ml) using human fibroblast interferon reference standard 69/19 for comparison.

Interferon was added directly to cell cultures at a final concentration of 1,000 U/ml 24 h after seeding. An equivalent amount of bovine serum albumin (2 μg/ml final concentration) was added to other cultures as a control for the interferon received from Dr. Horoszewicz. Plasmanate at a final concentration of 2 μg/ml (Cutter Laboratories, Inc., Berkeley, CA) and propylene glycol (at a final concentration of 0.05%) was added as a control for the interferon from Dr. W. Carter. The source of interferon had no effect on the results of experiments.

Cell Proliferation. For measurement of population doubling times, cultures in 25-cm^2 flasks were photographed at 24-h intervals as previously described (21) and cell numbers were determined from projected negatives. Population doubling time was calculated from the slope of the linear regression curve of cell number vs. time of treatment over a 72-h period.

Cell Volume. At 72 h after the beginning of interferon treatment, cultures were harvested by incubating in trypsin-versene for 2–3 min at 37°C. The trypsin was neutralized by the addition of FCS and the cells were resuspended in phosphate-buffered saline with Ca^{+2} and Mg^{+2} omitted (PBS def). Cell volume analysis was performed with a Coulter channelyzer model ZBI (Coulter Electronics, Inc., Hialeah, FL), courtesy of Dr. Arthur Balin, as previously described (17). Volume measurements were calibrated with polystyrene beads of 19.2 μm diam.

Thymidine Incorporation into Soluble Pools and DNA. At varying times after the beginning of interferon treatment, [^{3}H]thymidine (78 Ci/mmol; New England Nuclear, Boston, MA) was added directly to cultures at a final concentration of 2 μCi/ml for a 30-min pulse at 37°C. Within each experiment, triplicate 35-mm culture dishes were used. At the end of the labeling period, monolayers were washed four times with cold PBS def and solubilized in 1% sodium dodecyl sulphate at 4°C. The suspension was mixed with an equal volume of 10% trichloroacetic acid (TCA) and duplicate 10-μl aliquots from each culture dish were then taken for measurements of total incorporation. TCA-precipitable material was collected onto GF/C filters. The filters were washed three times with 5% TCA, twice with ethanol, dried, and then counted in a toluene-based scintillant (22). The difference between total cell-associated radioactivity per sample and TCA-precipitable radioactivity was recorded as radioactivity incorporated into the acid-soluble fraction. Uptake into TCA soluble and TCA-precipitable material is expressed as cpm per 10^4 cells. Cell number was determined from plating efficiency and cell multiplication over the 72-h period as measured on separate culture flasks.

Autoradiography of [^{3}H]Thymidine-labeled Cells. Cells were seeded at a concentration of 1.7×10^3 per mm^2 in 60-mm dishes containing four 18-mm diam coverglasses. 72 h after interferon treatment, cells were labeled with [^{3}H]thymidine as described above and the coverglasses were fixed in three changes of a solution of (3:1) methanol-glacial acetic

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; PBS def, phosphate-buffered saline with Ca^{+2} and Mg^{+2} omitted; PDL, population doubling level; FCS, fetal calf serum; TCA, trichloroacetic acid.

acid for 15 min at 26°C and stained for 20 min in 2% acetoorcein. The coverglasses were mounted on slides, dipped in Kodak NTB2 emulsion, and incubated in the dark at −20°C for 2 wk. The slides were developed in Kodak D19 developer. Two coverglasses were used per variable. Nuclei with ≥30 silver grains were considered to be labeled; background was 0–6 grains per nucleus. The percent of labeled nuclei was calculated from evaluation of 500 cells per variable for each experiment.

Results

Population Doubling Times. Cells at high population doubling levels (PDL 55–70) showed a mean population doubling time that was twice as long as the doubling time of cultures at middle PDL, i.e., 30–40 (Table I). For cells at both PDL ranges, interferon treatment resulted in a 40–44% increase in population doubling time. Thus, despite the fact that the high PDL cells were dividing less frequently than the younger cells, the two cell populations were equally sensitive to the antiproliferative action of interferon.

Cell Volume. With increasing PDL, the cell populations exhibited a shift to larger cell volumes, which was evidenced mainly by skewing of the cell volume distributions towards higher values (Fig. 1). This is in agreement with the findings of Mitsui and Schneider (6) who described an age-dependent increase in cell volume. Table II shows modal cell volumes of populations after 3 d of β interferon treatment. When the ratios for modal cell volumes, interferon treated vs. control, were first calculated for each experiment and then pooled, interferon treatment caused a 29.3 ± 11.3% increase at PDL 30–40 and a 23.7 ± 6.34% increase at PDL 55–70. The interferon-induced increases in volume of young vs. old cells were not significantly different by the t test ($P > 0.5$).

Thymidine Uptake and Incorporation into DNA. Aging of cultures was associated with a 22.3% increase in the uptake of [^{3}H]thymidine into the TCA-soluble pool during a 30-min period of labeling and a 42.7% decrease in the incorporation of [^{3}H]thymidine into TCA-precipitable material expressed per 10^4 cells (Table III*A*). To separate the effects on uptake of [^{3}H]thymidine into TCA-soluble pools from the effects on DNA synthesis, we have calculated the ratio of radioactivity in TCA-precipitable material to that in total cell-associated material (22). By this measure, the increase in PDL from 30–40 to 55–70 was associated with a 55% decrease in DNA synthesis.

Interferon treatment resulted in decreases in both the uptake of labeled precursor into the TCA-soluble pool and incorporation into DNA (Table III*A*).When values for [^{3}H]thymidine labeling of the TCA-soluble material in

TABLE I

*Prolongation of Population Doubling Time by Interferon Treatment of Fibroblast Cultures at Middle and High PDL**

PDL	Treatment	Population doubling time
30–40	Control	24.1 ± 1.7
30–40	Interferon	33.7 ± 9.0
55–70	Control	57.0 ± 13.1
55–70	Interferon	82.2 ± 21.1

* Mean results of five experiments ± standard deviation.

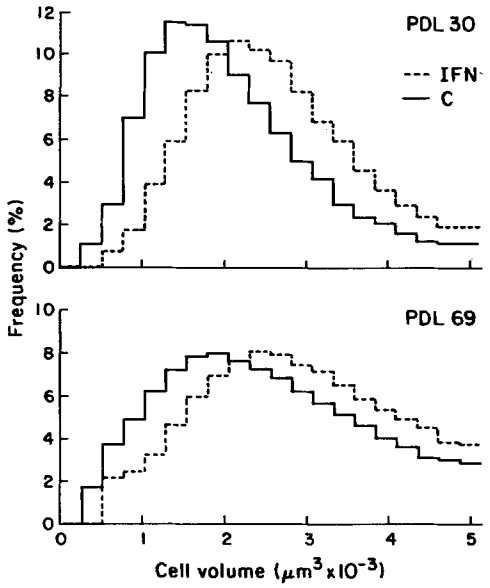

FIGURE 1. Effect of β interferon treatment on the cell volume distribution of 76-109 cells at PDL 30 and PDL 69. At 72 h after the beginning of treatment, electronic volume analysis was performed on control (——) and interferon-treated (1,000 U/ml) (---) 76-109 cells using a Coulter channelyzer model ZBI.

TABLE II

*Increases in Cell Volume of Interferon-treated Middle and High PDL Fibroblasts**

PDL	Treatment	Modal volume
30–40	Control	1,888.3 ± 221.1
30–40	Interferon	2,422.2 ± 97.8
55–70	Control	2,057.6 ± 106.3
55–70	Interferon	2,536.2 ± 40.2

* Mean results of three experiments expressed as μm^3 ± standard deviation.

interferon-treated relative to control cultures are calculated for each experiment and then pooled (Table III*B*), interferon treatment is found to decrease uptake of [^{3}H]thymidine into the TCA-soluble pool by 22.0% and 14.8% at middle and high PDL, respectively. This difference in the interferon effect on thymidine uptake at the two PDL was not significant by the *t* test ($P > 0.5$). At PDL 30–40, interferon treatment had no effect on DNA synthesis when calculated as the ratios of the TCA-precipitable to total cpm. However, at PDL 55–70, interferon treatment was associated with a 23% decrease in this ratio, which was marginally significant by the *t* test ($P = 0.029$).

Percent Labeled Nuclei. The above results suggest that interferon inhibits DNA replication in high PDL fibroblasts, but not in middle PDL cells. This difference in interferon sensitivity was confirmed by using whole cell autoradiography to determine the percentage of cells synthesizing DNA during a 30-min labeling period at the end of 72 h of interferon treatment (Table IV). First, it

TABLE III

*A. Effects of Interferon Treatment on Uptake of [^{3}H]Thymidine into the TCA-soluble Pool and Incorporation into DNA in Fibroblasts at Middle and High PDL**

PDL	Treatment	[^{3}H]Thymidine uptake and incorporation		
		TCA-soluble fraction‡	TCA-precipitable fraction‡	TCA-precipitable/total radioactivity
		cpm	*cpm*	
30–40	Control	3,029 ± 941	969 ± 350	0.238 ± 0.051
30–40	Interferon	2,405 ± 602	807 ± 261	0.248 ± 0.049
55–70	Control	4,398 ± 1,337	556 ± 286	0.107 ± 0.000
55–70	Interferon	3,725 ± 1,196	375 ± 236	0.084 ± 0.041

*B. [^{3}H]Thymidine Labeling: Interferon/Control**

PDL	TCA-soluble fraction	TCA-precipitable/total radioactivity
30–40	0.780 ± 0.126	1.044 ± 0.058
55–70	0.852 ± 0.128	0.773 ± 0.190

* Mean results of six experiments ± standard deviation.
‡ Per 10^4 cells.

TABLE IV

*Effects of Interferon Treatment on the Percent of Labeled Nuclei in Middle and High PDL Cells**

PDL	Treatment	Percent labeled nuclei
30–40	Control	32.1 ± 2.1
30–40	Interferon	39.4 ± 0.6
55–70	Control	14.5 ± 2.2
55–70	Interferon	9.8 ± 1.7

* Cells were labeled with [^{3}H]thymidine (78 Ci/mmole, 2 μCi/ml) for 30 min. Mean results of three experiments ± standard deviation.

should be noted that there was a 55% decrease in the percent of labeled cells in high as compared with middle PDL cultures, which corresponds to the decrease observed in the ratio of TCA-precipitable to total radioactivity after [^{3}H]thymidine labeling. Interferon treatment did not decrease the percent of S phase cells in cultures at PDL 30–40, but it caused a 32% decrease in PDL 55–70 fibroblasts relative to controls. The ratio of percent labeled nuclei in interferon-treated cultures vs. controls was 1.24 ± 0.06 for cultures at PDL 30–40 and 0.68 ± 0.03 for cultures at PDL 55–70. The difference between the middle and high PDL populations was highly significant by the *t* test ($P < 0.001$). In these experiments, a cell was scored as labeled if it had ≥30 grains per nucleus. Interferon treatment of high PDL cultures did not increase the percent of nuclei with 10–30 grain per nucleus. In addition, the washing and fixation procedure used removed cell-associated radioactivity that was not incorporated into DNA (23 and unpublished observations). Thus, our results are not an artifact of the grain counting threshold, a decrease in the uptake of labeled thymidine, or a decrease in the specific

activity of the labeled DNA. Rather, they indicate a PDL-dependent effect of interferon on DNA replication.

Interferon treatment of middle PDL cells was consistently associated with an increase in the percent of labeled nuclei. This could be explained by an interferon-induced prolongation of the S phase. Previous studies in 3T3 cells, as well as mouse embryo and human fibroblasts, have indicated that the antiproliferative effect is associated with an increase in the length of G0/G1 or G2 phase (13–15). A prolongation of the S phase or a block in the S phase has been observed after interferon treatment of some transformed cell lines (24–27). In the present experiments, the percent of cells synthesizing DNA was significantly decreased in high PDL 70-109 fibroblasts after interferon treatment.

Discussion

While the aging of 76-109 cells in culture is associated with increases in population doubling time and cell volume, interferon treatment causes even further increases. In terms of population doubling time and cell volume, sensitivity to interferon is independent of the PDL of fibroblasts in culture. The finding that 76-109 cells at high PDL are as sensitive to the antiproliferative action of interferon as cells at low PDL is in agreement with previous observations of five human fibroblast cell lines (28) and with the findings that the antiviral action of interferon is not affected by the aging of cell populations in vitro (29). Our results also show that interferon treatment decreases the uptake of thymidine into the acid-soluble pool at both high and low PDL. However, interferon treatment appears to inhibit DNA replication in high but not in middle PDL cells.

It has been suggested that interferons act as cellular regulators in a manner analogous to polypeptide hormones (30). Previous investigations on hormone responsiveness during senescence in vitro have indicated that in the majority of cell-hormone systems the sensitivity of cells to stimulation by hormones appears to decrease with increasing in vitro age (for reviews see 31 and 32). This decrease has been correlated with decreases in the number of insulin receptors on human skin fibroblasts (33) and glucocorticoid hormone receptors on WI-38 cells (34–36). It has also been demonstrated that senescent cells bind less low density lipoprotein, which carries cholesterol to cells, than do young cells (37). Sensitivity to epidermal growth factor (EGF) stimulation decreases with cellular age, which is correlated with a decrease in the ability of the EGF receptor to be autophosphorylated at tyrosine residues, but is not associated with changes in the number of specific binding sites per unit of surface area or the kinetics of EGF binding (38, 39). Our findings have provided no evidence of a decrease in the sensitivity of cells to β interferon with increasing in vitro age.

A marked decrease in the percent of cells synthesizing DNA is characteristic of in vitro senescence (3). Blocks in G0/G1 and G2 have been associated with aging in vitro (2–5). Studies demonstrating that entry into S phase in immortal cell lines is inhibited by fusion to senescent cells (40, 41) suggest that an inhibitor of DNA synthesis accumulates in senescent cells. The pattern of protein synthesis is altered as the PDL increases (42), but a specific inhibitor has not yet been identified. The further decrease in the percent of cells synthesizing DNA,

observed when high PDL 76-109 cells are treated with interferon, may be related to an increased intracellular level of the putative inhibitor in the interferon-treated fibroblasts. Our data provide further support for the hypothesis that there is an overlap in the pathways involved in the establishment of the senescent state and the interferon-mediated antiproliferative state. Further studies to determine the interferon-sensitive step in DNA synthesis in older cells are now in progress.

Summary

Cellular aging had no effect on the ability of β interferon to increase cell volume and population doubling time in 76-109 cells, a line of human skin fibroblasts. However, DNA synthesis in cells at high population doubling levels (PDL 55–70) was inhibited after 72 h of β interferon treatment (1,000 U/ml) while no inhibition of DNA synthesis was observed in cells at middle population doubling levels (PDL 30–40).

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