Changes of Serum-Induced Ornithine Decarboxylase Activity and Putrescine Content During Aging of IMR-90 Human Diploid Fibroblasts

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The roles of ornithine decarboxylase (ODC, EC 4.1.1.17) and polyamines in cellular aging were investigated by examining serum-induced changes of these parameters in quiescent IMR-90 human diploid fibroblasts as a function of their population doubling level (PDL) and in human progeria fibroblasts. Serum stimulation caused increases of ODC and DNA synthesis in IMR-90 human diploid fibroblasts, with maximal values occurring, respectively, 10 hr and 22 hr after serum stimulation. Both serum-induced ODC activity and DNA synthesis in IMR-90 cells were found to be inversely related to their PDL. Maximal ODC activity and DNA synthesis in young cells (PDL = ~18–22) were, respectively, five-fold and six-fold greater than that in old cells (PDL = ~50–55), which in turn were comparable or slightly higher than that in progeria fibroblasts. Polyamine contents (putrescine, spermidine, and spermine) in quiescent IMR-90 cells did not show significant PDL-dependency. The putrescine and spermine contents in quiescent progeria cells were comparable to those in quiescent IMR-90 cells. The spermidine content in quiescent progeria cells, however, was extremely low, less than half of that in quiescent IMR-90 cells. Serum stimulation caused a marked increase in putrescine content in young cells but not in old cells or in progeria cells. The spermidine and the spermine content in IMR-90 cells, either young or old, and in progeria cells did not change significantly after serum stimulation. Our study indicated that aging of IMR-90 human diploid fibroblasts was accompanied by specific changes of polyamine metabolism, namely, the serum-induced ODC activity and putrescine accumulation. These changes were also observed in progeria fibroblasts derived from patients with Hutchinson-Gilford syndrome.

Human embryonic diploid fibroblasts undergo 50 ± 10 doublings in tissue culture and then cease to divide (reviewed by Hayflick, 1979; Cristofalo and Stanulis-Praeger, 1982). This phenomenon, initially described by Hayflick and Moorhead (1961), is to be contrasted with tumor cell or transformed cell lines, which have unlimited dividing potential in vitro. Progeria fibroblasts derived from patients with Hutchinson-Gilford syndrome or other premature aging diseases exhibit a much shorter life span as compared to normal fibroblasts from age-matched controls (reviewed by Goldstein, 1979). These results together with the observation that the in vitro life span of human diploid fibroblasts is inversely related to the age of donor (Hayflick, 1965; Martin et al., 1970; Martin, 1977) have made human diploid fibroblast a useful in vitro model for studying the biochemical basis of cellular aging.

Polyamines (putrescine, spermidine, and spermine) are naturally occurring organic cations ubiquitously distributed in living organisms. Abundant evidence has been reported in literature indicating the importance of polyamines in growth regulation (for reviews see Janne et al., 1977; Pegg and McCann, 1982; Tabor and Tabor, 1984). The induction of ornithine decarboxylase (ODC, EC. 4.1.1.17), the rate-controlling enzyme for polyamine biosynthesis, and the subsequent accumulation of polyamines generally precede DNA synthesis (Heby et al., 1975; Russell and Stambrook, 1975; Boynton et al., 1976). Inhibition of polyamine biosynthesis by specific inhibitors such as α-difluoromethyl ornithine (Mamont et al., 1978; Prakash et al., 1980; Poso and Pegg, 1982) and methylglyoxal bis-(guanylhydrozone) (Boynton et al., 1976) invariably leads to reduced DNA synthesis and cessation of cell growth. Despite extensive studies of the regulation and function of polyamines in cell proliferation and cell differentiation (reviewed by Cohen, 1971; Bachrach, 1973; Tabor and Tabor, 1976, 1984), the possible involvement of polyamines in cellular aging has

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received very little attention. Studies in animal tissues have indicated changes in polyamine content with increased age of the animal (reviewed by Scalabrino and Ferioli, 1984), but possible changes of polyamine contents in human diploid fibrobasts during aging have not been previously investigated. Duffy and Kremzner (1977) have reported that the onset of the in vitro cellular senescence of WI-38 human fibroblasts is associated with reduced ODC activity in response to the stimulus of fresh culture medium. These studies, together with the consideration of the importance of polyamines in growth regulation, prompted us to initiate a systematic study of the regulation of polymaine metabolism in IMR-90 human diploid fibroblasts as a function of the PDL over their entire life span in culture. In addition, to ensure that changes of polyamine metabolism in IMR-90 cells after serial passage in vitro are truly age dependent, we also examined polymaine metabolism in progeria cell strains derived from patients suffering from Hutchinson-Gilford syndrome, a premature aging disease that has certain aspects in common with normal aging (Goldstein, 1979).

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagles medium, fetal bovine serum, dithiothreitol, trypsin-ethylenediamine tetraacetic acid (EDTA) solution, and gentamycin were purchased from Gibco, Grand Island, NY, L-[1-^{14}C]ornithine monohydrochloride (59 mCi/mmol) was obtained from Amersham, Arlington Heights, IL. Putrescine, spermidine, spermine, L-ornithine, 5-dimethyl amino-1-naphthalene sulfonyl chloride (dansyl chloride), and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., St. Louis. High pressure liquid chromatography (HPLC) grade solvents were obtained from Baker Chem. Co. Other chemicals were of standard reagent grade.

Cell culture

Low passage (PDL = 12, passage number 5) IMR-90 human embryonic lung fibroblasts, and the AG1178 and AG3198 progeria cell strains were obtained from the Institute for Medical Research, Camden, NJ. The low passage cultures of IMR-90 cells were expanded through subculturing at 1:4 or 1:2 split ratio to obtain cultures of higher population doubling level (PDL) (Nolan and Packer, 1974). Cells were grown in Dulbecco's modified Eagles medium (with 4,500 mg glucose per liter without sodium pyruvate) supplemented with 10% fetal bovine serum at 37°C in a water-jacketed CO_2 incubator (95% air, 5% CO_2). The apparent PDL of an IMR-90 human fibroblast culture was determined by the cumulative number of cell doubling; the number of doublings for each subcultivation (passage) was estimated from the split ratio (Nolan and Packer, 1974). Except when indicated, confluent monolayer cultures were used for all experiments described in this study. The progeria cells were used when less than 50% of the in vitro life span was consumed. They were also used at confluent state.

Growth stimulation

Confluent cultures of human fibroblasts were serum-deprived in fresh Dulbecco's medium for 24 hr to ensure that cells were in a quiescent state. Growth stimulation was initiated by refurbishing the quiescent cultures with fresh Dulbecco's medium supplemented with 10% fetal bovine serum. At various times after serum stimulation, cells were harvested in a 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA, 50 μM pyridoxal phosphate, and 5mM dithiothreitol. The cell homogenates were used for determining ODC activity and polyamine content.

Assay of ODC activity

ODC activity was determined using the cytosol fraction obtained from cell homogenate according to procedures previously described (Chen et al., 1976). One unit of ODC activity is defined as 1 nmol CO_2 evolved per 60 min. Protein concentration was determined by a modified Lowry method using bovine serum albumin as the standard (Ross and Shatz, 1973).

Quantitation of cellular polyamine content

Polyamines were dansylated by the method of Seiler and Wiechman (1970). The dansylated polyamines were separated by high pressure liquid chromatography (Beckman) using a reverse-phase column (RP-18, 7 μm ODS column), and quantitated by a Schoeffel spectrofluoromonitor (Model FS970) as previously described (Chen et al., 1982).

DNA synthesis

The incorporation of [^{3}H]thymidine into acid-insoluble material was used to estimate the rate of DNA synthesis. At indicated time intervals after serum stimulation, [^{3}H]thymidine (2 μCi/ml) was added to the cultures, and the metabolic incorporation was carried out at 37°C in a CO_2 incubator for 1 hr. Cells were then washed three times with cold phosphate-buffered saline (pH 7.2) and harvested in a 10 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA and 50 μg/ml phenylmethylsulfonyl fluoride. The amount of radioactivity incorporated into acid-insoluble material present in homogenates was determined according to procedures previously described (Chen and Canellakis, 1977).

RESULTS

Figure 1 shows the time course of the induction of ODC activity and DNA synthesis in IMR-90 human diploid fibroblasts and AG3198 progeria human fibroblasts. IMR-90 cell cultures at PDL of 22 (young), 36 (middle age), and 54 (old) were used in this experiment. Results indicated that the magnitudes of the serum-induced ODC induction and DNA synthesis in IMR-90 cells were inversely related to the PDL of the cultures. Maximal ODC activity and the rate of DNA synthesis in young cells (PDL = 22) were five- and six-fold higher than the respective values in old cells (PDL = 54). The increases of both ODC activity and rate of DNA synthesis in progeria cells were insignificant as compared to that in normal diploid fibroblasts.

Analysis of the kinetics of the serum-induced increase in ODC activity and DNA synthesis indicated little difference between young and old IMR-90 cells and between normal diploid fibroblasts (IMR-90) and progeria fibroblasts (AG3198). In all cases studied, the increase of ODC activity was detectable 3 hr after serum stimulation, reaching a maximal value ~8–10 hr after serum stimulation. DNA synthesis in both normal and progeria fibroblasts, however, showed a lag period of about

13 hr, characteristic of synchronized cultures. Maximal increases of DNA synthesis in these cells occurred at about 22 hr after serum stimulation.

Results from seven separate experiments demonstrated an inverse linear relationship between serum-induced maximal ODC activity and PDL of the cell culture (data not shown). Thus, four- to five-fold decrease of serum-induced ODC activity was consistently observed during aging of IMR-90 cells. The serum-induced ODC activity in progeria cells was either comparable to or less than that in old IMR-90 cells.

ODC is the rate-controlling enzyme for the biosynthesis of polyamines. In view of this consideration and to gain a better understanding of the possible role of polyamines in cellular aging, we determined the basal levels of putrescine, spermidine, and spermine in quiescent IMR-90 cells as a function of their PDL (Fig. 2). In addition, we determined the time course of the effect of serum stimulation on polyamine contents in both quiescent young (PDL = 22) and old (PDL = 52) IMR-90 diploid fibroblasts and in a progeria cells strain (AG3198) after serum stimulation (Fig. 3).

The results summarized in Figure 2 indicated that the polyamine contents (putrescine, spermidine, and spermine) in quiescent cultures of the IMR-90 cells did not vary significantly as a function of PDL; although it may be noted that in old cells (PDL > 50) the spermidine and the spermine contents were slightly (10–20%) lower than that in young cells. The spermidine/spermine ratio in quiescent IMR-90 cells remained relatively constant with a value of 0.94 $\pm$ 0.06 over their entire life span. Both the spermidine and spermine content in quiescent IMR-90 cells were in the range of 6–10 nmol per mg protein. The putrescine content, however, was much lower and only accounted for less then 5% of total polyamines. The putrescine and the spermine content in quiescent progeria cells were comparable to that in IMR-90 cells. The spermidine level in progeria cells, however,

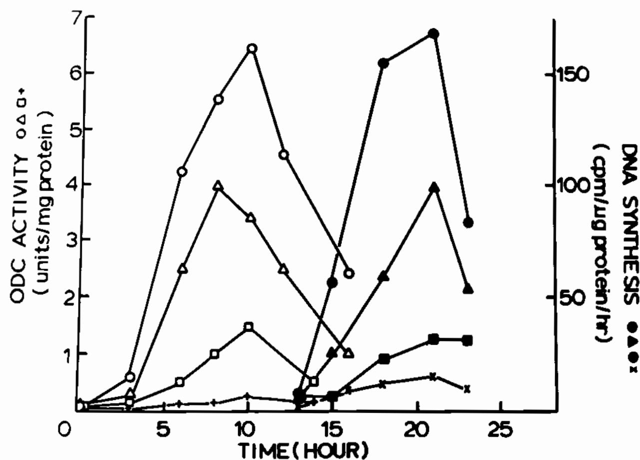

Fig. 1. Time course of serum-induced ODC activity and DNA synthesis in IMR-90 human diploid fibroblasts. Quiescent confluent cultures of IMR-90 cells at PDL = 22 (-○-, -●-), PDL = 36 (-Δ-, -▲-), and PDL = 54 (-□-, -■-), and of progeria (AG3198) cells (-+-, -×-) were serum stimulated as described in "Materials and Methods". At designated time points after serum stimulation, cells were used for ODC assay (○, Δ, □, and +) or measurement of [^{3}H]thymidine incorporation (●, ▲, ■, and ×). Each data point represents an average of duplicate assay with an error less than 10%.

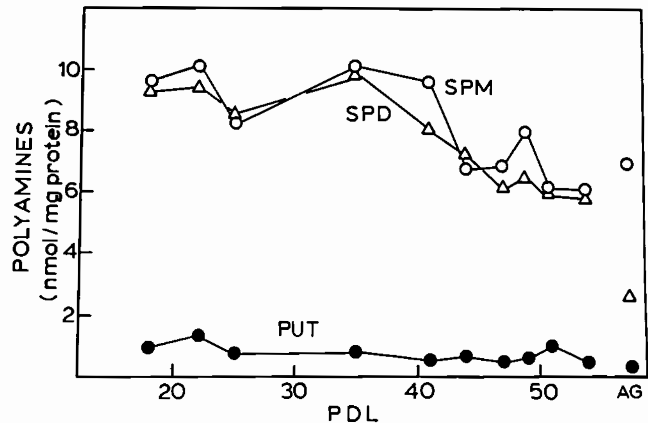

Fig. 2. Polyamine content in IMR-90 human diploid fibroblasts at various PDL and in progeria cells (AG3198). Individual polyamine content (putrescine, spermidine, and spermine) in the confluent cultures of IMR-90 cells at different PDL was determined by HPLC analysis as described in "Materials and Methods." Each data point represents an average of duplicate samples. PUT, putrescine; SPD, spermidine; SPM, spermine; AG, progeria cells.

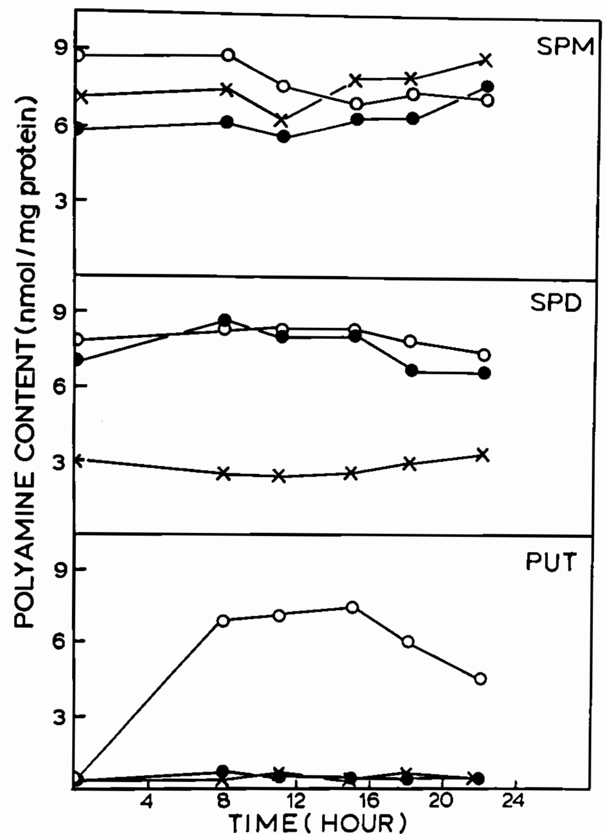

Fig. 3. Time course of changes of polyamine content in quiescent IMR-90 cells and progeria cells (AG3198) after serum stimulation. Confluent cultures of IMR-90 cells at two different PDL (PDL = 22, -○-; PDL = 52, -●-) and progeria cells (AG3198 -×-) were serum deprived for 24 hr and then stimulated with fresh Dulbecco's medium containing 10% fetal bovine serum. Polyamine content was determined at various time points after serum-stimulation. PUT, putrescine; SPD, spermidine; SPM, spermine.

was extremely low as compared to IMR-90 cells, and consequently the spermidine/spermine ratio in progeria cells was only 0.37, distinctly different from that of the IMR-90 cells. The difference in spermidine content between normal fibroblasts and progeria fibroblasts suggests that additional changes in polyamine metabolism (e.g., difference in spermidine synthase activity) may have occurred in progeria cells.

Figure 3 shows that serum stimulation caused a striking increase of putrescine content in young IMR-90 cells but not in old IMR-90 cells. No putrescine accumulation in progeria cells was detected either after serum stimulation. The increase of putrescine content in young IMR-90 cells reached a maximal value 8 hr after serum stimulation and remained elevated until 16 hr after serum stimulation. The time course of putrescine accumulation appeared to correlate with that of ODC induction in young IMR-90 cells. A five-fold increase of putrescine content in response to serum stimulation in low passage WI-38 human diploid fibroblasts has been reported by Heby et al., (1975). They also found that after serum stimulation putrescine level remains elevated for an extended peirod of time in WI-38 cells. In contrast, the spermidine and the spermine content in young and old IMR-90 cells and in progeria cells did not show a significant fluctuation after serum stimulation.

DISCUSSION

Monolayer cultures of quiescent human fibroblasts can be stimulated to enter the proliferative cell cycle by the addition of fetal bovine serum to the cultures or by replenishing the serum-deprived medium with fresh serum-containing growth medium (Wiebel and Baserga, 1969; Rovera and Baserga, 1973; Heby et al., 1975). In this study, we have employed quiescent confluent IMR-90 human diploid fibroblasts at various PDL and human progeria diploid fibroblast to examine possible age-associated changes in polyamine metabolism in these cells after serum stimulation.

Our results indicated that serum-stimulated induction of ODC activity and putrescine accumulation in young IMR-90 cells (PDL = ~ 18–22) were, respectively, approximately four- to five-fold and eight-fold greater than that in old IMR-90 cells (PDL $\geqslant$ 50) (Figs. 1 and 3). The spermidine and spermine content in young IMR-90 cells, however, did not differ significantly from that in old IMR-90 cells after serum stimulation (Fig. 3). The difference in putrescine content between young and old IMR-90 cells appeared to be only associated with growth stimulation. When the basal cellular content of polyamines in quiescent IMR-90 cells was determined, no significant changes were observed as the PDL of the cultures increased (Fig. 2). Similar to results in the old IMR-90 cells, serum stimulation caused only a minimal induction of ODC activity and no detectable putrescine accumulation in progeria cells (Figs. 1 and 3). The spermidine content in progeria cells was, however, extremely low when compared to that in either young or old IMR-90 cells. The calculated spermidine/spermine ratio was 0.94 for IMR-90 cells and was found to be independent of PDL, whereas the spermidine/spermine ratio for progeria cells was only 0.37. It has been suggested that high spermidine/spermine ratio is typical for tissues undergoing rapid growth or hypertrophy, and low spermidine/spermine ratio is generally typical of highly differentiated tissues with low biosynthetic activity (Janne et al., 1964; Russell and Durie, 1978). Since the spermidine/spermine ratio in IMR-90 cells was independent of PDL, this parameter could not be related to cellular aging. Thus, the low value of the spermidine/spermine ratio in progeria cells suggests that, in addition to age-related changes in polyamine metabolism (i.e., serum-induced ODC activity and putrescine accumulation), there may be other changes in polyamine metabolism associated with premature aging diseases.

The reduction of serum-induced ODC activity and putrescine accumulation in old IMR-90 cells and in progeria cells correlated well with the reduction of DNA synthesis in these cells (Figs. 1, 3). Heby et al. (1975) have shown that by varying the composition of the stimulating medium, the magnitude of putrescine accumulation correlated with the rate of DNA synthesis in young WI-38 human diploid fibroblasts (PDL = 24–28). Poso and Pegg (1982) have shown that the administration of a α-difluoromethyl ornithine (400 mg/kg) to rats following partial hepatectomy reduces putrescine level to less than 2 nmol/g and causes a 70% inhibition of DNA synthesis. They also showed that the inhibitory action of α-difluoromethyl ornithine can be reversed by exogenously added putrescine. In view of these observations, it seems likely that the induction of ODC and putrescine accumulation in IMR-90 cells after serum stimulation may be involved in the initiation of DNA synthesis and that decreases of these responses may contribute to the reduced DNA synthesis in old IMR-90 cells and in progeria cells. The molecular mechanism for the alteration of serum-induced ODC activity in IMR-90 cells during aging is currently under investigation.

Polyamine metabolism involves many metabolic pathways, and polyamines themselves interact with many biological molecules (e.g., DNA, RNA, and proteins) (see Tabor and Tabor, 1976, 1984). Thus, it is possible that changes of other aspects of polyamine metabolism may also play a role in aging of human diploid fibroblasts. In this connection, we have recently found that the catabolic conversion of putrescine to amino acids via the GABA shunt and Krebs cycle in IMR-90 cells increased by more than eight-fold during aging (Chen and Chang, 1986).

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