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Age Dependency of the Metabolic Conversion of Polyamines Into Amino Acids in IMR-90 Human Embryonic Lung Diploid Fibroblasts

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When radioactive polyamines (putrescine or spermidine) were incubated with mammalian cells in tissue culture, the radioactivity was incorporated into cellular proteins via two different metabolic pathways; one is metabolic labeling of an 18,000-dalton protein via hypusine formation, and the other is general protein synthesis employing radioactive amino acids derived from biodegradation of polyamines via GABA shunt and Krebs cycle. Aminoguanidine, a potent inhibitor of diamine oxidase, blocked the metabolic conversion of polyamines to amino acids but had no effect on the metabolic labeling of the 18,000-dalton protein. We have investigated these two polyamine-associated biochemical events in IMR-90 human diploid fibroblasts as a function of their population doubling level (PDL). We found that (1) the metabolic labeling of the 18,000-dalton protein was about two-fold greater in young cells (PDL = 22) than that in old cells (PDL = 48), and (2) the metabolic labeling of other cellular proteins, employing amino acids derived from putrescine via polyamine catabolic pathway, was more than six-fold greater in the old cells (PDL = 48) than in the young cells (PDL = 22). Since the rate of protein synthesis was about 1.4-fold higher in the young cells as compared to the old cells, our data indicated that the activity of catabolic conversion of putrescine (or spermidine) to amino acids in old IMR-90 cells was about eight-fold greater than that in young cells. This remarkable increase of polyamine catabolism and the slight decrease of metabolic labeling of the 18,000-dalton protein were also observed in cell strains derived from patients with premature aging disease.

Normal human diploid fibroblasts have a limited doubling potential in tissue culture (Hayflick, 1965, 1979). This phenomenon, initially described by Hayflick and Moorhead (1961), is in contrast with tumor cell or transformed cell lines which appear to have undiminished dividing potential in vitro. Human embryonic diploid fibroblasts express 50 ± 10 doublings in tissue culture (Hayflick, 1965; Nichols et al., 1977). Progeria fibroblasts derived from patients with Hutchinson-Gilford syndrome or other premature aging diseases exhibit a much curtailed life span as compared to fibroblasts from age-matched controls (Goldstein, 1979). These studies together with the observation that the in vitro life span of human diploid fibroblasts is inversely related to the age of the donor (Hayflick, 1965; Martin et al., 1970; Martin, 1977) have made human diploid fibroblasts a useful in vitro model for the study of biochemical basis of cellular aging.

Polyamines are organic cations widely distributed in living organisms. Abundant literature evidence has shown that these organic cations are closely associated with proliferation (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 polyamine accumulation generally precede DNA synthesis (Heby et al., 1975; Russell and Stambrook, 1975; Boynton et al., 1976). Inhibition of ODC induction and polyamine biosynthesis by specific inhibitors such as α-difluoromethyl ornithine and methylglyoxal bis-(guanylhydrazone) invariably leads to reduced DNA synthesis and growth cessation (Mamont et al., 1978; Prakash et al., 1980; Poso and Pegg, 1982). Recently we have found a significant decrease of serum-induced ODC induction and putrescine accumulation during aging of IMR-90 human fibroblasts (Chen and Chang, manuscript submitted). Previously, Duffy and Kremzner (1977) also reported a reduction of ODC activity in old WI-38 human fibroblasts after serial dilution. In light of these results, together with the consideration of the importance of polyamines in growth regulation, it seems possible that the loss of doubling potential during the aging of cultured human fibroblasts may be associated with

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specific changes of some aspects of polyamine metabolism.

We (Chen and Liu, 1981; Chen, 1983) and others (Park et al., 1981: Cooper et al., 1983) have shown that radioactive polyamines can be incorporated into proteins in cultured cells via two different metabolic pathways: (1) putrescine (or spermidine) can be incorporated into an 18,000-dalton protein via hypusine formation, and (2) putrescine can be converted to amino acids in via GABA shunt and Krebs cycle and used for general protein synthesis. The metabolic labeling of the 18,000-dalton protein represents a posttranslational modification of a unique cellular protein by polyamines (Park et al., 1981, 1984; Chen, 1983). The modification involves the attachment of butylamino group from spermidine to lysine residue and formation of hypusine moiety (Park et al., 1984). The reaction appears to be growth related, and occurs ubiquitously in various mammalian cells (Cooper et al., 1982; Chen, 1983). The catabolic conversion of polyamines to amino acids via GABA shunt and Krebs cycle has been demonstrated in some mammalian tissues and cells (Seiler and Al-Therib, 1974; Sobue and Nakajima, 1977; Antrup and Seiler, 1980). Since the amino acids formed can be utilized for general protein synthesis, the specific radioactivity of labeled cellular proteins provides an indirect measurement of the rate of polyamine catabolism.

In this study, we have examined these two polyamine-associated metabolic events as a function of population doubling level (PDL) of the IMR-90 human lung fibroblasts. Our results demonstrated a two-fold decrease of labeling of the 18,000-dalton protein and a more than eight-fold increase of catabolic conversion of putrescine to amino acids in aging IMR-90 human fibroblasts. Similar changes were also observed in two strains of progeria cells derived from patients with Hutchinson-Gilford syndrome.

MATERIALS AND METHODS

Materials

[1,4-^{14}C]Putrescine·2HCl (122 mCi/mmol), [5,8-^{14}C] spermidine 3HCl (20 Ci/mmol), and [^{35}S]methionine (1435 Ci/mmol) were purchased from Amersham, Arlington Heights, IL. L-[4,5-^{3}H(N)]leucine (56.5 Ci/mmol) and Enhancer were obtained from New England Nuclear, Boston. Dulbecco's modified Eagle medium, fetal bovine serum, trypsin-EDTA solution, and Earle's balanced salt solution were from Gibco, Grand Island, NY. Aminoguanidine, phenylmethylsulfonyl fluoride were from Sigma, St. Louis.

Cell cultures

IMR-90 human embryonic lung fibroblasts at very low passage number (passage number 5, PDL = 12) and progeria skin fibroblasts strain AG1177A and strain AG1178 were obtained from the Institute for Medical Research, Camden, NJ. Cells were grown in Dulbecco's modified Eagle medium (with 4,500 mg glucose per liter without pyruvate) supplemented with 10% fetal bovine serum and maintained at 37°C in a Forma water-jacketed CO_2 incubator (95% air and 5% CO_2). The low-passage IMR-90 seed cultures were expanded through subculturing using trypsinization techniques to obtain cultures at various PDL. The apparent PDL number of an IMR-90 cell culture was determined by the accumulated number of doublings. The number of doublings for each subcultivation (passage) was estimated from split ratio as described by Nolan and Packer (1974). Under our experimental conditions IMR-90 cultures routinely achieved a PDL value of 52 $\pm$ 2 before entering phase III. This phase is defined as the passage at which cultures after a 1:2 split do not become confluent after 7 days. In addition to PDL, the age of IMR-90 cell cultures was also monitored by measuring the incorporation of thymidine. The increase of PDL was consistently accompanied by a proportional decrease of DNA synthesis (data not shown). The progeria cells were used when less than 50% of their in vitro lifespan was consumed.

Metabolic labeling with radioactive polyamines

Unless otherwise indicated, cells at an early stationary phase of growth were used for all experiments. For studying the metabolic incorporation of radioactive polyamines, confluent monolayers of IMR-90 cells were washed once with fresh Dulbecco's medium and reincubated in fresh Dulbecco's medium containing 6% fetal bovine serum. Carrier-free radioactive polyamines and various chemicals were added to the medium at appropriate concentrations as indicated in figure legends. Metabolic labeling was carried out at 37°C in a Forma water-jacketed CO_2 incubator. At designated time intervals, the incubation medium was decanted, and cells were washed three times with cold phosphate buffered saline (pH 7.4). Cells were then harvested and homogenized in a Tris buffer (20 mM, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride. Aliquots of cell homogenates were used for analysis of the incorporation of radioactivity into trichloroacetic acid-insoluble material and for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the method of Lowry et al. (1951). SDS-PAGE was performed according to the procedure of Laemmli (1970). Fluorograms were prepared according to the method of Bonner and Lasky (1974) except that Enhancer was used.

Rate of protein synthesis

Protein synthesis in IMR-90 cells at various PDL was measured under two experimental conditions: (1) stringent condition, in which cells were incubated with [^{3}H]leucine (12.5 μCi/ml) in Earle's balanced salt solution at 37°C in a CO_2 incubator for various time intervals, and (2) growth-stimulated condition, in which cells were incubated with [^{35}S]methionine (50 μCi/ml) in a fresh, methionine-free Dulbecco's medium containing 6% fetal bovine serum at 37°C in a CO_2 incubator for 2 hr. Cells were then harvested and processed for gel electrophoresis and fluorography as described above.

RESULTS

Confluent cultures of IMR-90 cells at different PDL were incubated with [^{14}C]putrescine in a serum-containing fresh Dulbecco's medium for various time intervals. At the end of incubation, the total cellular uptake of radioactivity and the incorporation of radioactivity into acid-insoluble material were determined and compared. Results shown in Figure 1A indicated that the total uptake of [^{14}C]putrescine over a period of 24 hr in IMR-

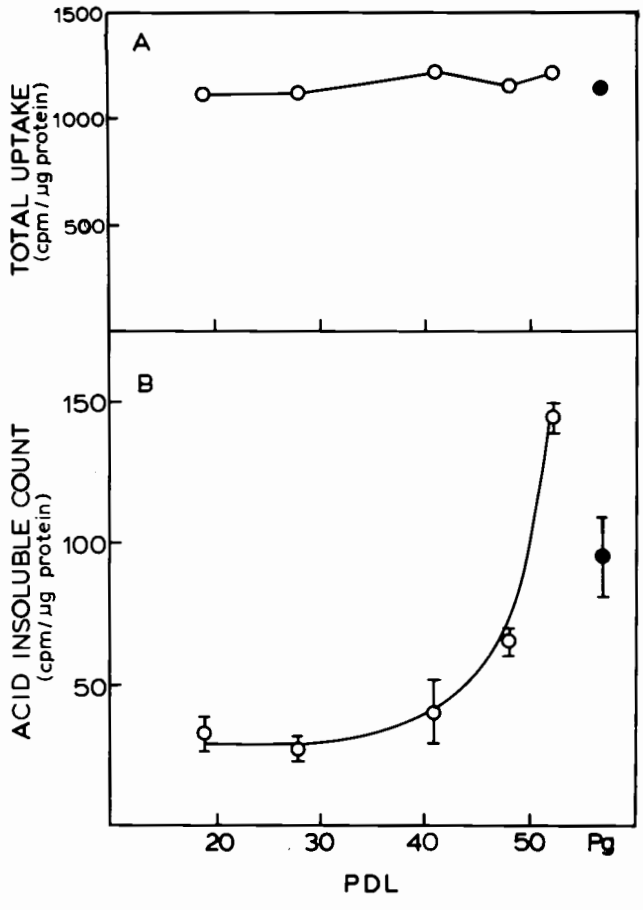

Fig. 1. Total uptake of [^{14}C]putrescine (A) and acid-insoluble radioactivity (B) in IMR-90 human fibroblasts as a function of their PDL. Confluent cultures of IMR-90 cells at various PDL were incubated with [^{14}C]putrescine (0.5 μCi/ml) in fresh Dulbecco's medium containing 6% fetal bovine serum for 24 hr. Cells were washed, harvested, and homogenized by sonication. Aliquots of cell homogenate were used to determine the protein concentrations, total radioactivity, and acid-insoluble radioactivity as described in "Methods." Result of two separate experiments were presented. Each point represents duplicate samples. Closed circles represents data obtained employing both AG1177A and AG1178 cell strains.

90 cells was independent of the cell age (i.e., PDL). In contrast, the incorporation of radioactivity into acid-insoluble material showed a sharp increase in aging diploid cells. The difference in the acid-insoluble counts between old cells (PDL = 52) and young cells (PDL = 18–28) was five-fold on per mg protein basis (Fig. 1B). Since the old cells contain more protein per cell (Schneider and Shorr, 1975), the difference could more than ten-fold on per cell on per mg DNA basis. The specific radioactivity of acid-insoluble material in two progeria cell strains (AG1177A and AG1178) was comparable to that in old cells with PDL > 45 (Fig. 1B).

Radioactive putrescine can be incorporated into cellular proteins via two different metabolic pathways: (1) posttranslational modification of an 18,000-dalton protein via hypusine formation (Park et al., 1981; Chen, 1983), and (2) general protein synthesis employing radioactive amino acids metabolically derived from putrescine (Chen and Liu, 1981). To determine whether either one or both pathways may contribute to an increase of acid-insoluble radioactivity in old cells, we analyzed the the acid-insoluble material by SDS-PAGE and fluorography. We have previously shown that aminoguanidine, a potent inhibitor of diamine oxidase, inhibits the formation of GABA from putrescine, but has no effect on the metabolic labeling of the 18,000-dalton protein in mouse neuroblastoma cells (Chen and Liu, 1981). Thus it can be used to distinguish these two metabolic pathways. Figure 2 shows a fluorogram of protein patterns of IMR-90 cells at PDL 19, 35, and 48 and AG1178 progeria cells metabolically labeled by [^{14}C]putrescine in the presence and absence of aminoguanidine. In the absence of aminoguanidine (Fig. 2, FBS), the radioactive labeling pattern closely resembled the pattern of Coomassie blue-stained proteins (data not shown) or the labeling protein pattern employing radioactive amino acids as precursors (see Fig. 5), indicating that the labeling was due to general protein synthesis. The labeling intensities of general cellular proteins increased as a function of PDL (PDL: 19, 35, 48). The labeling intensity of progeria cells (PDL: Pg), was also significantly higher than that of the young (PDL = 18) or middle-aged cells (PDL = 35). The metabolic labeling of the 18,000-dalton protein in IMR-90 cells at various PDL was performed in the presence of aminoguanidine (Fig. 2, FBS + AG). As the PDL value of the cultures increased from 19 to 48, the labeling intensity of this protein decreased slightly. Quantitation of the radioactivity incorporated into various labeled protein bands by counting the excised protein bands revealed that (1) an inverse linear relationship between the metabolic labeling of the 18,000-dalton protein and the PDL values of IMR-90 cells, and the labeling intensity in the old cells was about two-fold lower than that of the young cells (Fig. 3A), and (2) metabolic labeling of general cellular pro-

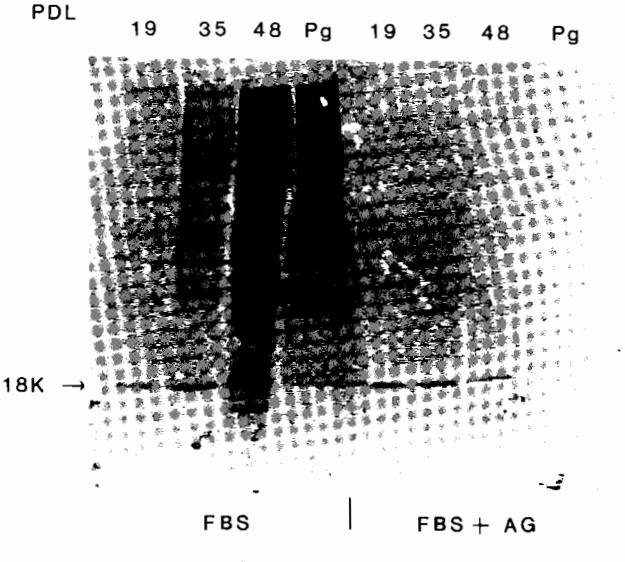

Fig. 2. Fluorogram of [^{14}C]putrescine-labeled protein pattern of human fibroblast cell strains. IMR-90 cells at three different PDL (19, 35, and 48) and a progeria cell strain AG1178 (Pg) were metabolically labeled by [^{14}C]putrescine (1 μCi/ml) for 36 hr in fresh Dulbecco's medium supplemented with 6% fetal bovine serum (FBS) or 6% fetal bovine serum and 10^{-4} aminoguanidine (FBS + AG). Each lane contained 50 μg of cellular proteins.

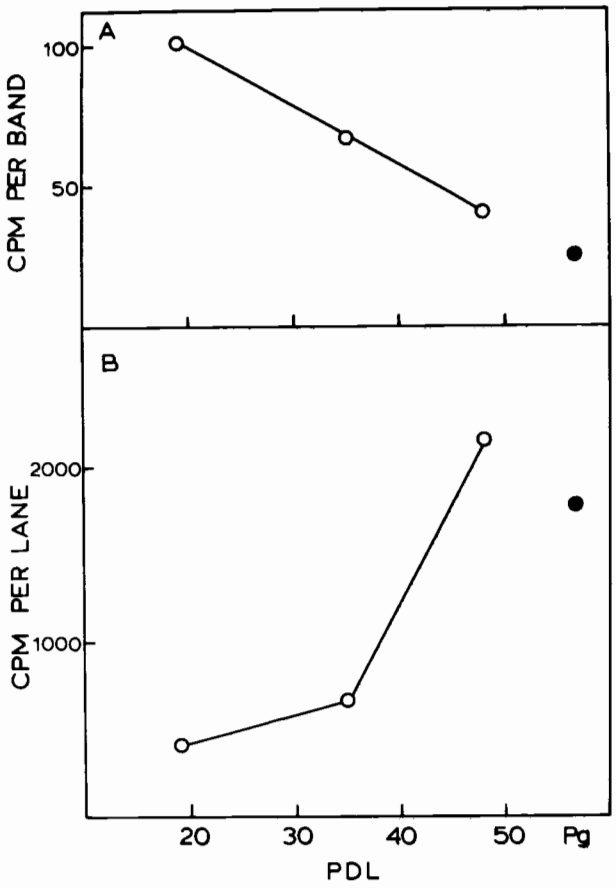

Fig. 3. Labeling intensity of the 18,000-dalton protein (A) and labeling intensity of general cellular proteins (B) in IMR-90 cells as a function of their PDL. Results represent an average of data from two gels. The error was less than 10%.

teins increased as a function of PDL, and the amount of labeling in the old cells was 5.1-fold higher than that in the young cells (Fig. 3B). It should be noted that the data in both Figures 2 and 3 were presented on per mg protein basis. If expressed on per cell on per mg DNA basis, the difference in the labeling of the 18,000-dalton protein between young and old cells would not be too significant because the protein content in the old cells is two-fold higher than that in the young cells (Schneider and Shorr, 1975). In contrast, the difference in metabolic labeling of general cellular proteins between young and old cells would be greater than ten-fold on per cell or per mg DNA basis. Since the metabolic labeling of the 18,000-dalton protein accounted for less than 5% of acid-insoluble radioactivity in IMR-90 cells (Fig. 2), the increase of acid-insoluble radioactivity in the old cells (Fig. 1B) was primarily due to an increase of the biodegradation of putrescine to amino acids and subsequent general protein synthesis.

To examine whether possible difference in the fluctuation of polyamine pool size may contribute to the differences in the specific radioactivity of cellular polyamines and thus cause differences in the labeling intensities of cellular proteins between young and old cells, we examined the effect of α-difluromethyl orithine, a potent enzyme-activated irreversible inhibitor of ornithine decarboxylase (Mamont et al., 1978), on the metabolic labeling by [^{14}C]putrescine (Fig. 4). Our results indicated that although α-difluromethyl ornithine inhibited ODC induction and polyamine biosynthesis in both young and old cells (data not shown), it did not affect the metabolic labeling pattern and intensity by [^{14}C]putrescine in both young and old cells (Fig. 4). Previous studies in animal tissues and cultured cells have shown that putrescine has a fast turnover rate and is converted to spermidine and spermine with a half-life of approximately 4 hr (Russell et al., 1970). Thus, it is possible that difference in the rate of biosynthesis of spermidine from [^{14}C]putrescine may contribute to the difference in the metabolic labeling of young and old cells by [^{14}C]putrescine. To test this possibility, [^{14}C]spermidine was used directly as a precursor to metabolically label IMR-90 cells at three different PDL. Results shown in Figure 4 indicated that (1) the carbon skeleton of spermidine could be channeled into amino acids pool, and (2) the labeling intensity of cellular proteins by [^{14}C]spermidine also showed an age-dependent increase. The degradation of spermidine to putrescine (putrescine cycling) has been demonstrated in a mouse and rat brain tissues (Holtta, 1977; Antrup and Seiler, 1980). Our results indicated that active putrescine cycling also existed in human fibroblasts.

The increased metabolic labeling of cellular proteins using [^{14}C]amino acids derived from [^{14}C]putrescine in the old IMR-90 cells could be due to (1) an increased metabolic conversion of putrescine to amino acids (i.e., biodegradation of putrescine), (2) a reduction of endogenous polyamine pools, (3) a reduction of endogenous amino acid pools, (4) an increased rate of protein synthesis, or any combination of these factors in the old cells. In a preliminary study, we found no significant differ-

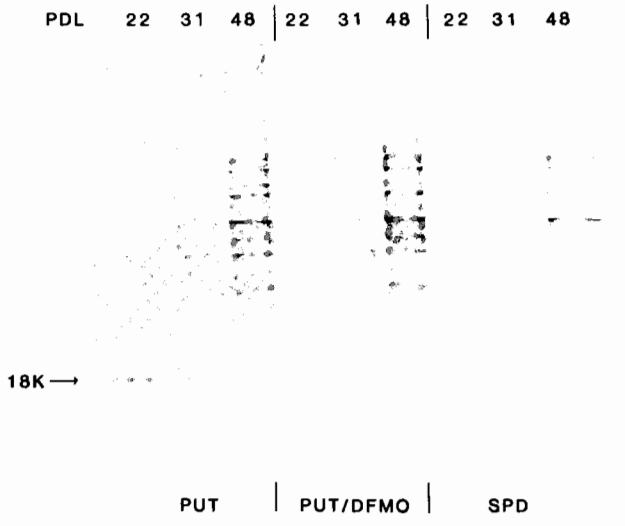

Fig. 4. Fluorogram of polyamine-labeled cellular proteins in IMR-90 human fibroblasts. IMR-90 cells at PDL 22, 31, and 48 were labeled by [^{14}C]putrescine (1.5 μCi/ml) in the absence (PUT) and in the presence of 1 mM α-difluromethyl ornithine (PUT/DFMO) or were labeled by [^{14}C]spermidine (SPD: 3 μCi/ml). The incubation was carried out for 24 hr as described in "Methods." Each lane contained 50 μg of cellular proteins.

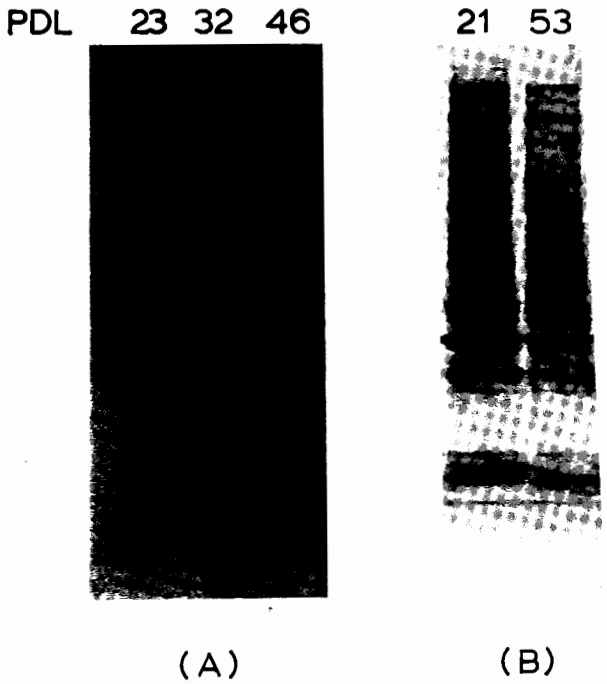

Fig. 5. Fluorograms of [^{3}H]leucine- and [^{35}S]methionine-labeled protein pattern in IMR-90 cells. A. IMR-90 cells at various PDL (23, 32, and 46) were incubated with [^{3}H]leucine (12.5 μCi/ml) in Earle's balanced salt solution for 2 hr. Cells were harvested and processed for gel electrophoresis and fluorography. B. Cells were incubated with [^{35}S]methionine (50 μCi/ml) in methionine-free fresh Dulbecco's medium containing 6% fetal bovine serum for 2 hr. Cells were then harvested and processed for protein determination, gel electrophoresis, and fluorography. Each lane contained 50 μg of cellular proteins.

ence in polyamine contents in confluent young and old IMR-90 cells (Chen and Chang, submitted). To differentiate other possibilities, we have compared the rate of protein synthesis in young and old cells employing exogenously added [^{3}H]leucine or [^{35}S]methionine. We found that the rates of protein synthesis in young and old cells were comparable when they were measured under stringent conditions (Fig. 5A) but differed by 30–40% when they were measured under growth-stimulated conditions (Fig. 5B). These data suggested that the remarkable increase of metabolic labeling of cellular proteins in the old cells could not be related to differences in amino acid pools or in the rate of protein synthesis and most likely was due to an age-dependent increase of biodegradation of putrescine.

DISCUSSION

The data presented in this study demonstrated a significant increase in the metabolic incorporation of exogenously added [^{14}C]putrescine into acid-insoluble cellular material in IMR-90 human fibroblasts (Fig. 1B). When Figure 1A and B are compared, it can be noted that 15% of total radioactivity in the old cells, but only 3% in the young cells, appeared in acid-insoluble fraction. The difference in the specific activity of the acid-insoluble counts between young and old cells was five-fold on per mg protein basis and can be as great as 10- to 15-fold on per cell or per mg DNA basis. It is also of interest to note that the sharp increase in acid-insoluble counts did not occur until cells reached a PDL value greater than 45, suggesting some fundamental changes in polyamine metabolism when cells became old. Two strains of progeria cells, AG1177A and AG1178, derived from patients with premature aging disease, exhibited similar high acid-insoluble radioactivity. These data suggested that this phenomenon is truly age dependent and is not an artifact that is due to culturing conditions. However, it should be noted that the IMR-90 cell strain and the two progeria strains are of different tissue origins.

Further analysis indicated that more than 80% of acid-insoluble radioactivity was due to the incorporation of radioactivity, derived from [^{14}C]putrescine, into cellular proteins (Fig. 2), similar to what we have reported in other cultured mammalian cell lines (Chen, 1983). The two metabolic pathways that lead to the incorporation of radioactivity into acid-insoluble material are (1) metabolic labeling of the 18,000-dalton protein, and (2) general protein synthesis employing radioactive amino acids derived from putrescine degradation. These two pathways were distinguished by aminoguanidine and analyzed by PAGE (Fig. 2). Although the activities of both events change as a function of the PDL of IMR-90 cells (Figs. 2, 3), the direction and magnitude of changes were distinctively different. It is also clear from Figures 2 and 3 that the significant increase of acid-insoluble radioactivity in the old cells (Fig. 1B) was primarily due to general protein synthesis when using radioactivity derived from [^{14}C]putrescine.

The intensity of the metabolic labeling of the 18,000-dalton protein appeared to be inversely proportional to the PDL values of the cells only when the specific activity is calculated on per mg protein basis (Fig. 3A). Cooper et al. (1983) have shown that the 18,000-dalton protein in Chinese hamster ovary cells is identical with eIF-4D in reticulocyte and have suggested that it may have a role in protein synthesis. The precise function of eIF-4D remains to be elucidated. Moldave (1985) recently suggested that it may be an "interphase" factor rather than an initiation factor. Since the difference in the labeling of the 18,000-dalton protein between young and old cells becomes less significant if the specific radioactivity is calculated on per cell or per mg DNA basis, this metabolic event may simply reflect the reduced growth rate of old cells rather than biochemical event closely associated with aging. In contrast, the metabolic labeling of general cellular proteins, which is due to metabolic conversion of putrescine to amino acids, increased significantly when cells reached high PDL values (Fig. 3B). This increase could be attributed to an increase of conversion of putrescine to amino acids via GABA formation (i.e., polyamine degradation), an increase of rate of protein synthesis in the old cells, or changes of pool size of amino acids and/or polyamines. Our results (Fig. 5) indicated that the rate of protein synthesis in the old cells was either comparable to or slower than that in the young cells (Fig. 5). Similar results have also been reported by others (Cristofalo et al., 1970; Wang et al 1970). Our studies also excluded possible changes of amino acid pools and polyamine pools during cellular aging as a contributing factor for the elevated metabolic labeling in old cells. Thus, we concluded that the dramatic increase of metabolic labeling of cellular proteins

by [^{14}C]putrescine or [^{14}C]spermidine was, most likely, due to increased catabolism of polyamines in old human diploid fibroblasts.

Most of the studies on polyamines emphasize the biosynthesis rather than biodegradation of polyamines. Thus, the physiological significance of polyamine degradation is largely unclear. Many enzyme systems are involved in the conversion of putrescine and spermidine to amino acids (Antrup and Seiler, 1980; Tabor and Tabor, 1984). They include diamine oxidase, monoamine oxidase, acetylase, aldehyde dehydrogenase, and enzymes in GABA shunt and Krebs cycle. Further analysis will be needed to identify which enzyme system(s) is responsible for the increased polyamine catabolism in the aging human fibroblasts. Goldstein et al. (1982) studied energy metabolism in cultured human fibroblasts during aging in vitro and found no difference in the activity of Krebs cycle between young and old cells. Thus, it seems that the cause(s) for the difference in polyamine catabolism between young and old cells is most likely due to changes of amine oxidases and/or aldehyde dehydrogenases. In this connection, it may be of interest to note that Tabor (1954) and Buffoni (1966) have reported that diamine oxidase activity increases with age in some tissues in several animal studies. Using a large control population, a positive correlation between age of donor and level of monoamine oxidase activity in cultured human fibroblast has also been reported (Breakfield et al., 1980).

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