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A MARKED INCREASE OF FUCOSYLATION OF GLYCOPROTEINS IN IMR-90 HUMAN DIPLOID FIBROBLASTS DURING SENESCENCE IN VITRO

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SUMMARY: Possible changes of glycoproteins in IMR-90 human embryonic lung fibroblasts during senescence in vitro were studied by the metabolic labeling technique using radioactive precursors for carbohydrate moieties of glycoproteins. IMR-90 fibroblasts at three different population doubling level (PDL) were incubated with [^{3}H]fucose and [^{3}H]glucosamine for various periods of time. The radioactively labeled glycoproteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. The results indicated a marked increase, by more than eight-fold on per mg protein basis, of labeling by [^{3}H]fucose in old IMR-90 fibroblasts (PDL = 45) as compared to young (PDL = 22) and middle-age (PDL = 30) IMR-90 fibroblasts. In contrast, no significant difference in [^{3}H]glucosamine labeling was observed in young and old IMR-90 cells.

Normal human diploid fibroblasts such as IMR-90 cells have a finite life span in tissue culture. Under normal culturing conditions, they express 50±10 doublings and then cease to divide [reviewed in 1,2]. The causes for the loss of dividing potential in senescent cells are unclear. It is thought that identification and characterization of specific age-dependent biochemical changes in normal human diploid fibroblasts may provide clues on the molecular basis of cellular aging [3]. In view of the importance of plasma membranes in cellular growth regulation [4,5], it is somewhat surprising that very few studies have been carried out concerning possible changes of membrane structures and functions associated with senescence of normal cells in culture [3]. Glycoproteins are integral components of plasma membranes and play crucial roles in many of membrane functions [4,5]. Courtois and Hughes [6] have examined surface glycoproteins in chick embryo fibroblasts as a function

Abbreviations used: PDL, population doubling level; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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of passage numbers by analyzing the trypsin digests of [^{3}H]glucosamine labeled surface proteins using gel filtration chromatographic technique. They reported that the incorporation of radioactivity into macromolecules is similar for cells regardless of their age. Their study, however, suffers from poor resolving power of gel filtration chromatography. In addition, analysis of tryptic digests revealed no information on the size of glycoproteins.

In this paper, we used two specific precursors of the carbohydrate moiety of glycoproteins to metabolically label IMR-90 human diploid fibroblasts at three different PDLs corresponding to young, middle-age, and old cells. The composition of labeled glycoproteins and the labeling intensities in these cells were analyzed and compared by SDS-PAGE and fluorography. Our results indicated that there was a marked increase of incorporation of [^{3}H]fucose into glycoproteins in old (PPL = 45) cells as compared to young (PDL = 22) and middle aged (PDL = 30) IMR-90 cells. In contrast, the labeling by [^{3}H]glucosamine in young and old cells appeared to be similar.

MATERIAL AND METHODS

Chemicals L-[6-^{3}H]fucose (6 Ci/mmol) and D-[6-^{3}H]glucosamine hydrochloride were purchased from Amersham, Arlington, IL. Tissue culture media and sera were from Gibco, Grand Island, NY. Enhancer was obtained from New England Nuclear, Boston, MA. Phenylmethylsulfonyl fluoride and α-L-fucosidase (bovine kidney) were purchased from Sigma Chem. Co., St. Louis, MO. All other chemicals were of standard reagent grade.

Cell Culture IMR-90 human embryonic lung fibroblasts at passage number 5 (PDL = 12) were obtained from the Institute for Medical Research, Camden, N.J. Cells were grown in Dulbecco's modified Eagle medium (with 4500 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 IMR-90 seed cultures (PDL = 12) were expanded through subcultivation using the trypsinization technique to obtain cultures at various PDL. The PDL number of an IMR-90 cell culture was determined by the accumulated number of doubling as previously described [7]. The "age" of the IMR-90 cells was arbitrarily divided into young, middle age, and old groups based on the total *in vitro* life span of IMR-90 cells grown in our laboratory (52±2). 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 [7].

Metabolic Labeling with Radioactive Precursors Confluent cultures of IMR-90 cells at various PDLs were washed once with fresh Dulbecco's medium and reincubated with radioactive precursors (5 μCi per ml) in fresh Dulbecco's medium with or without 6% fetal bovine serum. The incubation was carried out

at 37°C in the CO_2 incubator for 12 and 24 hrs. At the end of incubation, the medium was decanted, and cells were washed three times with cold phosphate buffered saline (pH 7.4). Cells were then harvested and sonicated in a Tris buffer (20 mM, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride. Aliquots of cell homogenates were used for protein determination [8] and SDS-PAGE [9]. Fluorograms were prepared according to the method of Bonner and Lasky [10] except that Enhancer was used.

Fucosidase treatment L-[^{3}H]Fucose labeled IMR-90 cells were washed three times with cold phosphate buffered saline (pH 7.0) and suspended in 75 mM sodium citrate (pH 6.00) containing 1 mM phenylmethylsulfonyl fluoride and 0.01% NaN_3. Cells were homogenized by brief sonication and then treated with α-L-fucosidase (1 unit/ml) at 37°C according to the procedure described by Carlson and Pierce [11].

RESULTS AND DISCUSSION

L-Fucose can be incorporated directly into animal cells without prior conversion into other carbohydrates or amino acids [12-14]. D-Glucosamine is the precursor of sialic acid, N-acetylgluosamine and N-acetylgalactosamine which are all constituents of glycoproteins and glycolipids [12]. Both [^{3}H]fucose and [^{3}H]glucosamine have been previously employed to metabolically label glycoproteins in animal cells such as HeLa cells [15,16], chick embryo fibroblasts [17] and mouse neuroblastoma cells [18]. In all these studies, [^{3}H]fucose and [^{3}H]gluosamine were found to label specifically the carbohydrate moieties of glycoproteins. In agreement with these reports, we found that both fucose and glucosamine were readily taken up by IMR-90 human fibroblasts and that the majority of cellular radioactivity could be recovered in the acid insoluble fraction (Table 1). SDS-polyacrylamide gel analysis (Fig. 1) indicated that labeled macromolecules could be stained by Coomassie Blue and appeared to be glycoproteins. Treatment of [^{3}H]fucose labeled cells with specific α-L-fucosidase for 25 hrs removed 40-60% of acid-insoluble radioactivity (data not shown), further suggesting that the labeling by [^{3}H]fucose was specific. We did not attempt any longer period of fucosidase treatment because it has been shown that α-L-fucosidase releases 50% of fucose from purified LH-β-glycopeptide after a 25 hr incubation period [11].

The metabolic labeling pattern of various glycoproteins by [^{3}H]fucose in young (PDL = 22), middle-age (PDL = 30) and old (PDL = 45) IMR-90 cells after 12 hrs and 24 hrs incubation period is shown in Fig. 1. At both time points, the labeling intensity of various glycoproteins in the old cells was

Table I Distribution of radioactivity from [^{3}H]fucose and [^{3}H]glucosamine in IMR-90 human diploid fibroblasts

	Fucose Incorporation		Glucosamine Incorporation	
	Total Uptake (cpm/mg protein)	Acid-Insoluble Counts (cpm/mg protein)	Total Uptake (cpm/mg protein)	Acid-Insoluble Counts (cpm/mg protein)
PDL				
22	46,000	38,000	310,000	310,000
30	43,000	40,000	420,000	400,000
45	110,000	108,000	450,000	450,000

[3H]Fucose or [3H]glucosamine was added to the confluent cultures of IMR-90 cells (5μCi/ml) for 12 hrs, cells were then washed and harvested for protein determination, acid-insoluble assay and radioactivity counting as described in Materials and Methods. Each data point represents an average of duplicate assay. Error is less than 10%.

significantly higher than that in the young and middle-age cells. Quantitative estimation based on densitometric tracing of the fluorogram and radioactive counting of the excised gel slices indicated that the amount of [^{3}H]fucose incorporated into glycoproteins in old IMR-90 cells was more than

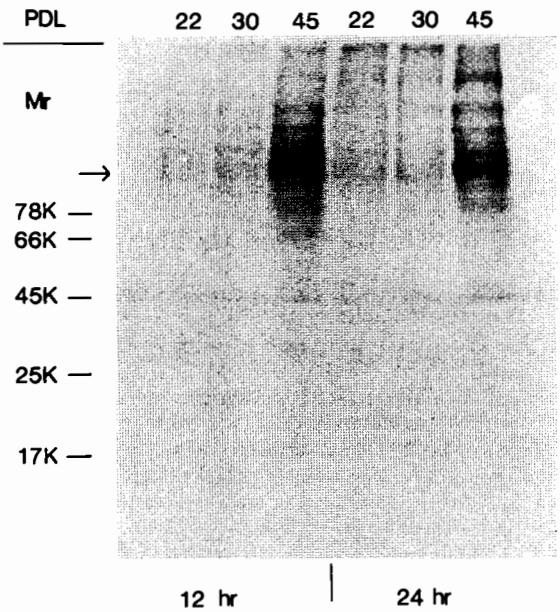

Figure 1. Fluorogram of [^{3}H]fucose labeled glycoproteins in IMR-90 human diploid fibroblasts at PDL 22, 30, and 45. The metabolic labeling was carried out in the presence of 6% fetal bovine serum for 12 hrs and 24 hrs as indicated. Each lane contained 50 μg of proteins. The detailed labeling condition, gel electrophoresis and fluorographic procedure were described in METHODS. Arrow indicates the 92,000-dalton protein band.

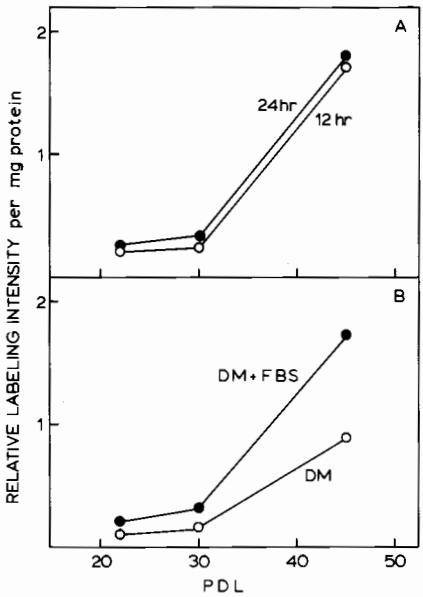

Figure 2. (A) Relative labeling intensity of fucose-containing glycoproteins in IMR-90 human fibroblasts as a function of their PDL. The labeling was carried out for 12 hrs (-O-) or 24 hrs (-●-). Labeling intensity was quantitated by densitometric tracing of the fluorograms. Similar results were obtained by counting the excised gel slices. Results represent an average of data from two gels. (B) Effect of fetal bovine serum on the labeling intensity of fucose containing glycoproteins in IMR-90 human fibroblasts. The labeling was carried out in the absence (-O-) or in the presence (-●-) of 6% fetal bovine serum for 12 hrs. DM: Dulbecco's medium; FBS: fetal bovine serum.

8-fold greater than that in young and middle-age cells (Fig. 2A). Since the old cells contain more protein per cell [19], the difference in labeling intensity of fucose-containing glycoproteins between young and old cells could be more than 16-fold on per cell or per mg DNA basis. Although we do not have direct evidence that the labeled fucose-containing glycoproteins shown in Fig. 1 are localized at cell surface, studies using other animal cell lines [15,16] suggest that most, if not all, of fucosyl glycoproteins are cell surface localized. In fact, radioactively labeled fucose-containing glycoproteins have been used as a surface marker for monitoring the purity of isolated plasma membranes [17,18].

Qualitatively, the [^{3}H]fucose labeling patterns in young, middle age, and old IMR-90 cells were similar (Fig. 1). More than 90% of labeled fucose-containing glycoproteins had an apparent molecular weight greater than 80,000. The most prominent one had an apparent molecular weight of 92,000. We have

previously reported that the most prominently labeled protein by [^{3}H]fucose in N-18 mouse neuroblastoma cells has an apparent molecular weight of 92,000 [18]. It will be of interest to examine whether these two fucose-containing glycoproteins share any other similarities.

The quantitative differences in [^{3}H]fucose labeling intensities between young and old IMR-90 cells could be due to many reasons such as dirrerence in activities of fucosyl transferases [20], difference in the total number of fucose-containing glycoproteins, or difference in fucose and GDP-fucose pools [21]. Further study will be needed to delineate the causes for this age-associated difference.

Nowakowski et al. [22] have shown that the incorporation of L-[^{3}H]fucose into plasma membranes of HeLa cells is maximal in late S phase of the cell cycle. The late S phase in quiescent IMR-90 cells occurred approximately 22~24 hrs after serum stimulation [23]. Since we did not find significant difference in [^{3}H]fucose incorporation at 12 hrs and 24 hrs time intervals (Fig. 2A), the [^{3}H]fucose labeling in IMR-90 cells may be cell cycle independent. Our study also indicated that the metabolic labeling of IMR-90 cells by [^{3}H]fucose was responsive to serum stimulation (Fig. 2B). The presence of fetal bovine serum (6%) increased [^{3}H]fucose incorporation by about 2-fold. Similar increases of incorporation occurred in cells at three different PDLs, suggesting that the stimulatory effect of fetal bovine serum was not related to the dividing potential of the cells.

To further examine whether the observed difference in [^{3}H]fucose labeling between young and old IMR-90 cells represents a general phenomenon and is true for other types of glycoproteins, we compared the metabolic labeling patterns in young and old cells using another radioactive precursor [^{3}H]glucosamine. The results shown in Fig. 3 revealed no major difference in either the labeling intensity or the labeling pattern by [^{3}H]glucosamine in young, middle-age, and old IMR-90 cells. In addition, the composition of labeled glucosamine-containing proteins appeared quite distinct from that of labeled fucose-containing proteins in IMR-90 (Fig. 3 vs Fig. 1).

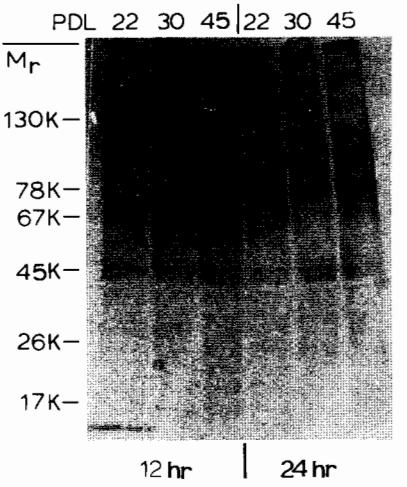

Figure 3. Fluorogram of [^{3}H]glucosamine labeled glycoproteins in IMR-90 human diploid fibroblasts at PDL 22, 30, and 45. The labeling was carried out in the presence of 6% fetal bovine serum for 12 hrs and 24 hrs. Each lane contained 50 μg of proteins.

Taken together our data indicated that the marked increase of fucosylation in IMR-90 human diploid fibroblasts was a specific metabolic event associated with senescence. Although the physiological functions of these fucose-containing proteins are largely unknown, the [^{3}H]fucose labeling may be used as a new marker of aging in human diploid fibroblasts. In addition, the availability of specific fucose (α1-3) binding lectin [24] may allow us to further explore the physiological significance of the observed differences in fucosylation between young and old cells.

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REFERENCES

1. Hayflick, L. (1979) J. Invest. Dermatol. 73, 8-14.
2. Cristofalo, V.J. and Stanulis-Praeger, B.M. (1982) in Advances in Cell Culture (Maramorosch, K. ed) Vol. 2, pp. 1-68, Academic Press, N.Y.
3. Hayflick, L. (1977) in Handbook of The Biology of Aging (eds. Finch, C.E. and Hayflick, L.) p. 159-188, van Nostrand Reinhold Co., N.Y.
4. Cook, G.M.W. and Stoddart, R.W. (1973) Surface Carbohydrates of Eukaryotic Cell, pp. 1-346, Academic Press, N.Y.
5. Nicolson, G.L. (1976) Biochim. Biophys. Acta 458, 1-72.

6. Courtois, Y. and Hughes, R.C. (1974) Eur. J. Biochem. 44, 131-138.
7. Chen, K.Y. and Chang, Z.F. (1986) J. Cell. Physiol. 128, 27-32.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. Laemmli, U.K. (1970) Nature 227, 680-685.
10. Bonner, W.M. and Lasky, R.A. (1974) Eur. J. Biochem. 46, 83-88.
11. Carlsen, R.B. and Pierce, J.G. (1972) J. Biol. Chem. 247, 23-32.
12. Kaufman, R.L. and Ginsberg, V. (1968) Exp. Cell. Res. 50, 127-131.
13. Coffey, J.W., Miller, O.N. and Sellinger, O. (1964) J. Biol. Chem. 239, 4011-4017.
14. Bekesi, J.G. and Winzler, R.J. (1967) J. Biol. Chem. 242, 3873-3879.
15. Atkinson, P.H. and Summers, D.F. (1971) J. Biol. Chem. 246, 5162-5175.
16. Bennet, G. and Leblond, C.P. (1970) J. Cell Biol. 46, 409-416.
17. Garvican, J.H. and Brown, G.L. (1977) Eur. J. Biochem. 76, 251-261.
18. Chen, K.Y. and Rinehart, C.A., Jr. (1982) Biochim. Biophys. Acta 685, 61-70.
19. Schneider, E.L. and Shorr, S. (1975) Cell 6, 179-184.
20. Bosmann, H.B., Hagopian, A., and Eylar, E.H. (1968) Arch. Biochem. Biophys. 128, 470-481.
21. Kornfeld, S., Kornfeld, R., and Ginsburg, V. (1965) Arch. Biochem. Biophys. 110, 1-7.
22. Nowakowski, M., Atkinson, P.H., and Summers, D.F. (1972) Biochim. Biophys. Acta 266, 154-160.
23. Chen, K.Y., Chang, Z.F., and Liu, A.Y-C. (1986) J. Cell. Physiol. 129, 142-146.
24. Prieels, J.P., Pizzo, S.V., Glasgow, L.R., Paulson, J.C., and Hill, R.L. (1978) Proc. Natl. Acad. Sci. USA 75, 2215-2219.