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Cellular Aging and Expression of Fibronectin

Tsutomu KUMAZAKI

Department of Biochemistry and Biophysics, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Hiroshima 734, Japan

ABSTRACT

Several changes in the functional characteristics of fibronectin (FN) have been noted as cells become senescent in culture. In a previous report we showed that the steady state level of FN mRNA increases significantly during the process of *in vitro* cellular aging in a fibroblast strain. Because a phenomenon observed in one cell strain may not be the case in other cell strains, we extended the previous study and confirmed that this is a common phenomenon in at least two fibroblast strains of different origin. The greatest change in the proportion of cells expressing high levels of FN occurs near the end of a culture's proliferative potential. The proportion of cells unable to synthesize DNA follows a similar pattern. We also found that increasing cell size correlates closely with higher levels of FN expression. Thus, there is a clear correlation between increased FN mRNA content and *in vitro* cellular senescence. In order to see if this phenomenon could also be observed in cells aged *in vivo*, we analyzed cells aged *in vivo*. We found that fibroblasts from donors of higher age show lower labeling index, express a higher level of FN and come to have a larger cell area, similar to cells aged *in vitro*. This strongly suggests that a fibroblast *in vivo* ages with aging of the individual in like manner to that observed in *in vitro* aging cells, that is, by exhausting division potential. This supports studies using *in vitro* aging cells as a model for cellular aging *in vivo*.

Key words: *Cellular aging, Fibroblast, Fibronectin, In situ hybridization*

Replicative senescence is well established for normal human diploid fibroblasts grown in tissue culture and has been proposed as an *in vitro* model for aging *in vivo*[5,6]. Negative correlation between *in vitro* division potentials of fibroblasts and donor age also supports the effectiveness of fibroblasts as a model for studies of *in vivo* cellular aging[9,12]. Morphological changes observed in senescent cells consist of, for example, an increase in overall size and loss of the spindle shape and ordered array of fibroblasts[2,5,10]. In addition, the senescent cells are unable to synthesize DNA as shown by a decrease in the labeling index from >75% in young cell populations to <5% in senescent populations[3,10]. Some differences in biochemical parameters between young and senescent cells have been reported[16]. However, the mechanisms of cellular aging have not yet been clarified.

Extracellular matrix is composed of FN, collagens, glycosaminoglycans such as heparan sulfate and other macromolecules. Among these, one of the major components of extracellular matrix is FN which plays an important role in cell adhesion, spreading and movement. FN produced by old cells is reported to be 5 to 10 kilodaltons larger than that of young cells[17]. A qualitative difference of FN molecules from young and old cells is shown as diminished affinity to type I and II collagens[1]. FN secreted from old cells is less capable than that secreted from young of mediating cell adhesion and spreading[1,17].

Despite the potential importance of FN in cellular aging, a paucity of information exists with regard to the control of FN expression and deposition into the extracellular matrix during this process. In this study, we show that as a culture reaches the end of its *in vitro* life span virtually all the cells in two strains of fibroblasts from lung and skin accumulate increased levels of FN mRNA. Furthermore, we find that cells from older donor express a higher level of FN mRNA. These findings indicate that the changes shown in aging of *in vitro* cultured cells mimic those in *in vivo* cells, and thus support studies using *in vitro* aging cells as a model of *in vivo* cellular aging.

MATERIALS AND METHODS

Cells and culture conditions

TIG-1 (human normal diploid fetal lung fibroblast) and TIG-3S (human fetal skin fibroblast) were originally isolated at Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan. HCA2 (human neonatal foreskin fibroblast) was isolated in Smith laboratory, U.S.A[8]. GRC173, GRC161, GRC168, GRC196 and GRC191 were isolated by Schneider and Mitsui from skins of 29, 49, 67, 83 and 88 year old donor, respectively, at National Institute on Aging, Baltimore, U.S.A[12]. These fibroblasts were

cultured in minimum essential medium supplemented with 10% fetal bovine serum.

Preparation of cells for *in situ* hybridization

Cells of 0.14—4 × 10^4 were grown on a glass coverslip in a 12 well plate or 35mm dish for 3—4 days with one change of medium. For fixation, cells were quickly rinsed twice with calcium/magnesium-free phosphate-buffered saline (PBS), pH7.4 and fixed with 4% paraformaldehyde (Merck) in PBS supplemented with 5mM $MgCl_2$ for 15 minutes at room temparature. Cells were stored in 70% ethanol at -20°C until used.

Preparation of probes

A 2.4 kb FN cDNA fragment cloned into pT7T3 18U vector (Pharmacia), was cut with *Hin* dIII for antisense probe or with *Sma* I for sense probe. The linearized template DNA (0.2 μg) was transcribed with T3 or T7 RNA polymerase (BRL) and [α-^{35}S] UTP. After 1.8 hr of incubation at 37°C, non-isotopic UTP was added to final concentration of 0.5 mM, and incubated further. After a total of 2 hrs incubation, RNase-free DNase I (20 units, Pharmacia) was added and incubated for a further 30 min to digest the template. The resulting cRNA was digested in 40 mM $NaHCO_3$ and 60 mM Na_2CO_3 for 1 hr at 60°C to produce a probe with a mean size of 140 bases and used for *in situ* hybridization.

In situ hybridization

The procedure of *in situ* hybridization used in this study has been described previously[8]. Briefly, with some modifications, coverslips with appropriate cells were removed from -20°C freezer, treated serially with PBS plus 5 mM $MgCl_2$, 0.1 M glycine in 0.2 M Tris-HCl (pH7.4), and 2X SSC in 50% formamide. Cells were then hybridized with ^{35}S-labeled sense or antisense riboprobe for FN in 50% formamide, 20 mM Tris-HCl (pH8), 5 mM EDTA, 0.3 M NaCl, 10% dextran sulfate, 1X Denhardt's solution, 10 mM sodium pyrophosphate, 120 mM dithiothreitol, 10 mM vanadyl ribonucleoside complex, 150—200 μg/ml tRNA. 4—6 × 10^5 cpm/20 μl/coverslip of radioactive probe was used. After incubation of 20—24 hrs at 50°C, coverslips were washed with 2X SSC in 50% formamide plus 0.5% 2-mercaptoethanol for 1 hr at 60°C, and treated with 4 μg/ml of RNase A (Pharmacia) in 0.5 M NaCl, 10 mM Tris-HCl (pH8) and 5 mM EDTA for 30 min at 37°C. Coverslips were then dipped in NTB2 (Kodak) or NR-M2 (Konika) nuclear track emulsion and stored for autoradiography at 4°C. After 7—16 days, samples were developed in D19 (Kodak), fixed and counter-stained with haematoxylin and eosin. In order to quantify the degree of FN expression, the number of grains above a cell and the cell area were determined for 100 cells in each specimen as described previously[8]. Background was determined as previously[8] and subtracted from these numerical data.

Labeling index

Cells were grown on a coverslip as mentioned above and incubated with 1 μCi of [^{3}H] thymidine (ICN, 6.7 Ci/mmol). After 24 hrs incubation, cells were fixed and processed directly to emulsion-autoradiography as described above.

RESULTS

In vitro aging

We carried out *in situ* hybridization analysis to see the modulation of level of FN mRNA expression during *in vitro* cellular aging at the individual cell level. As shown in Fig. 1A, the labeling index of TIG-1 cells, which were derived from fetal lung, at population doubling level (PDL) 29, 43 and 61 decreased with increasing PDL. This shows that these cells were getting old *in vitro* and that most of cells of PDL61 became not to synthesize DNA, which meant that cells of PDL61 were senescent. *In situ* hybridization analysis for these cells showed that cells at PDL 61 expressed FN mRNA higher than younger cells (PDL29 and 43). To quantify the level of expression of FN mRNA, the number of

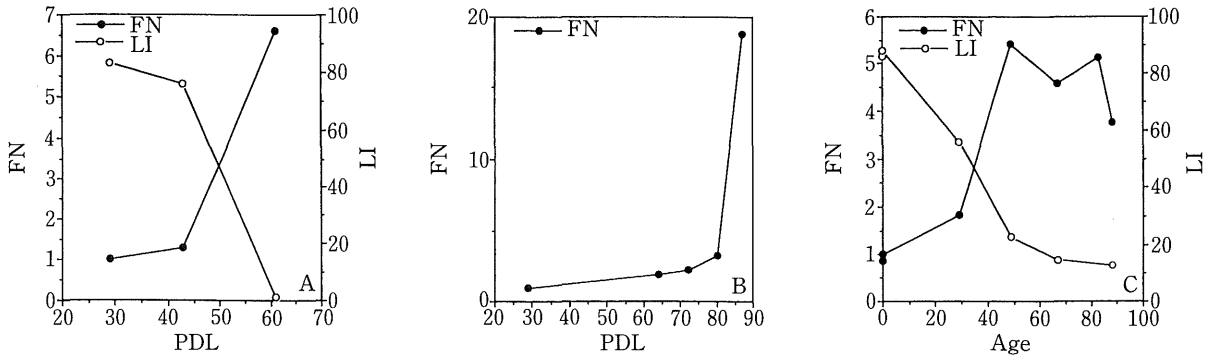

Fig. 1. Increase of FN expression and decrease of labeling index during cellular aging.
A. FN, ratio of mean grain number per cell of TIG-1 fibroblasts. The value of the youngest cell population was taken as 1.0. Shown as a function of PDL. LI, labeling index of the population of cells.
B. Ratio of mean grain number per cell of HCA2 fibroblasts.
C. FN, ratio of mean grain number per cell of fibroblasts from donors of different ages. The value of TIG-3S was taken as 1.0. Shown as a function of donor age. LI, labeling index of the population of cells.

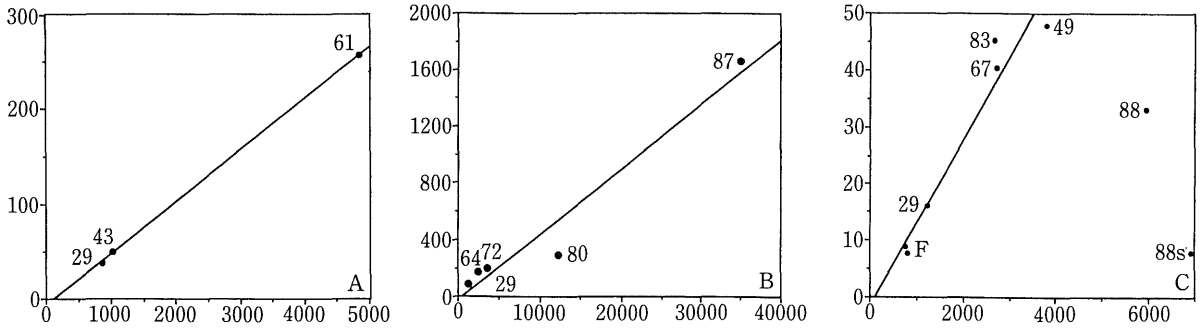

Fig. 2. Relationship between the mean of the FN mRNA content and the mean of cell area. Each point was obtained from 100 cells at each PDL or donor age.
A. Analysis for TIG-1. The PDL is indicated in the figure near the appropriate point. Correlation coefficient was 1.00.
B. Analysis for HCA2. Correlation coefficient was 0.96.
C. Analysis for fibroblasts from donors of different ages. The age of donor is indicated in the figure. F means fetues. Correlation coefficient was 0.94. (When the data for 88 year old donor was included for the least squeres analysis, correlation coefficient was 0.46.) The data probed with a sense strand for cells from 88 year old donor is shown as 88s in the figure (negative control).
Ordinate: mean grain number per cell; abscissa: mean cell area (μm^2).

silver grain above a cell was determined for 100 cells of each PDL. The mean of values for 100 cells was plotted as a function of the PDL (Fig. 1A). The figure clearly shows that the level of FN mRNA expression became higher when cells became senescent. The pattern of the increase was similar to that of Northern analysis (data not shown).

The increase of the level of FN expression was also observed in another fibroblast strain, HCA2, which was derived from neonatal foreskin. The pattern of the increase of FN expression is shown in Fig. 1B and is similar to that of TIG-1 (Fig. 1A). Thus, the increase of level of FN mRNA expression is a common characteristic of *in vitro* cellular aging of fibroblasts from different origins of lung and skin. The increase of FN protein with aging has also been observed[8].

The pattern of increase of cell area was very similar to that of FN mRNA expression (data not shown). Thus, a correlation between level of FN expression and cell area was suggested. When means of level of FN expression and cell area were plotted (Fig. 2A and B), a clear correlation was observed in both fibroblast strains. Thus, when fibroblasts senesce *in vitro*, virtually all the cells express increased levels of FN mRNA and also show increased cell area.

In vivo aging

We carried out experiments on cells from donors of different ages to verify whether the observation in cells aged *in vitro* is the case in cells aged *in vivo*. Skin and lung fibroblasts of fetus (PDL18 and 20, respectively) and skin fibroblasts from 29, 49, 67, 83 and 88 year old donors (PDL16, 12, 21, 30 and 23, respectively) were analyzed by *in situ* hybridization. Their labeling indices decreased with increasing donor age (Fig. 1C). This tendency to decrease is similar to that observed in cells aged *in vitro* and indicates that cells from the older donor are less active for DNA replication. The level of FN expression was higher in cells from old donors than in those from young donors, though some variation was observed.

When the means of level of FN expression and cell area were graphed (Fig. 2C), the correlation between them was also observed. This result suggests that cells *in vivo* age by a very similar manner to *in vitro* cells, that is, by exhausting division potential. Thus, these findings support studies on *in vitro* aging cells as a way of elucidating the mechanisms of cellular aging of cells *in vivo*.

DISCUSSION

Previous studies have shown significant changes in FN expression at both levels of mRNA and protein during *in vitro* cellular aging[7,8]. Northern hybridization analysis can only show mean levels of mRNA expression of approximately 10^6 cells. However, previous studies demonstrated clonal heterogeneity in populations of human diploid fibroblasts such that not all the cells in a culture become senescent at the same time[13]. Previous studies also determined that cellular senescence was not due to a simple single step process, but rather relied upon an intrinsic timing mechanism to determine the number of population doublings remaining in the *in vitro* life span[14]. We utilized *in situ* hybridization techniques to determine whether the increase in FN mRNA occurred gradually in all the cells of the population or precipitously in a fraction of cells as they became nondividing.

In situ hybridization analysis for TIG-1, which was derived from fetal lung, aged *in vitro* showed that cells at PDL 61 expressed FN mRNA higher than younger cells (PDL29 and 43). The increase of the level of FN expression was also observed in

another fibroblast strain, HCA2, which was derived from neonatal foreskin. Thus, the increase of level of FN mRNA expression is a common characteristic of *in vitro* cellular aging of fibroblasts from different origins of lung and skin. Furthermore, the data at a cell level from *in situ* hybridization analysis showed that virtually all the cells expressed increased levels of FN mRNA and increased cell area, when fibroblasts senesced *in vitro*.

A negative correlation between *in vitro* life span of fibroblasts and donor age has been reported[9,12]. This suggests that a fibroblast *in vivo* ages with aging of the individual. It is very important to verify whether the increase of FN expression is also observed in cells aged *in vivo* or whether it is only the case of cells aged *in vitro*, because it cannot be an *in vitro* model of *in vivo* cellular aging if it is not the case in cells aged *in vivo*. We carried out experiments on cells from donors of different age to clarify the problem. The level of FN expression was higher in cells from old donors than in those from young donors, though some variation was observed. Because these cells were not derived from a single donor, there were differences in their genetic backgrounds. The variation of the data may be caused by the genetic background. In spite of the existence of variation, the result suggests that cells *in vivo* age by a very similar manner to *in vitro* cells, that is, by exhausting division potential. Thus, these findings support studies on *in vitro* aging cells as a way of elucidating the mechanisms of cellular aging of cells *in vivo*.

Previous studies have shown that cellular senescence is a dominant trait in somatic cell hybrids between normal and immortal cells and between two different immortal cells[11]. This observation has led to the proposal that cellular aging is the result of an active, genetically programmed process[15]. The mechanisms that control the timing when gene expression must be changed during cellular aging are not yet known. The FN gene is a logical choice for future investigation of molecular mechanisms of cellular senescence in connection with gene expression. FN is an abundant protein in fibroblasts, the level of expression of its mRNA is significantly increased with cellular aging. Moreover, the promoter region of this gene has already been cloned and partially characterized[4]. Whether or not increased FN production is found to play a causal role in cellular aging, the elucidation of the mechanism(s) responsible for the change in expression will be important for a better understanding of the process of cellular senescence.

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REFERENCES

1. **Chandrasekhar, S., Kleinman, H.K. and Hassell, J.R.** 1983. Interaction of link protein with collagen. J. Biol. Chem. **258:** 6226–6231.
2. **Cristofalo, V.J. and Kritchevsky, D.** 1969. Cell size and nucleic acid content in the diploid human cell line WI-38 during aging. Med. Exp. Int. J. Exp. Med. **19:** 313–320.
3. **Cristofalo, V.J. and Sharf, B.B.** 1973. Cellular senescence and DNA synthesis. Thymidine incorporation as a measure of population age in human diploid cells. Exp. Cell Res. **76:** 419–427.
4. **Dean, D.C., Bowlus, C.L. and Bourgeois, S.** 1987. Cloning and analysis of the promoter region of the human fibronectin gene. Proc. Natl. Acad. Sci. USA **84:** 1876–1880.
5. **Hayflick, L. and Moorhead, P.S.** 1961. The serial cultivation of human diploid cell strains. Exp. Cell Res. **25:** 585–621.
6. **Hayflick, L.** 1965. The limited *in vitro* lifetime of human diploid fibroblasts. Exp. Cell Res. **37:** 614–636.
7. **Kleinsek, D.A.** 1989. Selection of mRNAs expressed during cellular senescence *in vitro*. Age **12:** 55–60.
8. **Kumazaki, T., Robetorye, R.S., Robetorye, S.C. and Smith, J.R.** 1991. Fibronectin expression increases during *in vitro* cellular senescence: correlation with increased cell area. Exp. Cell Res. **195:** 13–19.
9. **Martin, G.M., Sprague, C.A. and Epstein, C.J.** 1970. Replicative life-span of cultivated human cells. Lab. Invest. **23:** 86–92.
10. **Matsumura, T., Pfendt, E.A. and Hayflick, L.** 1979. DNA synthesis in the human diploid cell strain WI-38 during *in vitro* aging: an autoradiography study. J. Gerontol. **34:** 323–327.
11. **Pereira-Smith, O.M. and Smith, J.R.** 1988. Genetic analysis of indefinite division in human cells: Identification of four complementation groups. Proc. Natl. Acad. Sci. USA **85:** 6042–6046.
12. **Schneider, E.L. and Mitsui, Y.** 1976. The relationship between *in vitro* cellular aging and *in vivo* human age. Proc. Natl. Acad. Sci. USA **73:** 3584–3588.
13. **Smith, J.R. and Hayflick, L.** 1974. Variations in the lifespan of clones derived from human diploid fibroblast cultures. J. Cell Biol. **62:** 48–53.
14. **Smith, J.R. and Whitney, R.G.** 1980. Intraclonal variation in proliferative potential of human diploid fibroblasts: stochastic mechanism for cellular aging. Science **207:** 82–84.
15. **Smith, J.R. and Lincoln, D.W. 2nd.** 1984. Aging of cells in culture. Int. Rev. Cytol. **89:** 151–177.
16. **Smith, J.R. and Pereira-Smith, O.M.** 1985. Lung-derived fibroblast-like human cells in culture, p.375–423. *In* V.J. Cristofalo (ed.), CRC Handbook of Cell Biology, CRC Press, Boca Roton, Florida.
17. **Sorrentino, J.A. and Millis, A.J.T.** 1984. Structural comparisons of fibronectins isolated from early and late passage cells. Mech. Ageing Dev. **28:** 83–97.