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# Modulation of Gene Expression during Aging of Human Vascular Endothelial Cells

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## ABSTRACT

Vascular endothelial cells are thought to play an important role in human aging as their senescence and detachment from a vascular wall may contribute to arteriosclerosis and high blood pressure in the elderly. We investigated the level of fibronectin (FN) expression in aortic endothelial cells aged *in vivo*, because FN is necessary for cell attachment and spreading and its increased expression had been shown in aging fibroblasts. The results showed that the steady state level of expression of FN mRNA increased with advancing donor age, while the labeling index of cultured cells decreased with age. Furthermore, the increased level of FN expression clearly correlated with an increase in cell area. In order to explore whether these changes reflected exhaustion of proliferation potential *in vivo*, we examined FN expression in human umbilical vein endothelial (HUVE) cells aging *in vitro*. Very similar results were obtained, supporting the idea that vascular endothelial cells age *in vivo* by using up division potential. Furthermore, we investigated the level of endothelin (ET) -1 mRNA expression during *in vitro* cellular aging of HUVE cells. The results showed that the expression of ET-1 gene was also up-regulated when the culture became old. It is very interesting that the genes for quite different proteins of FN and ET-1 are both up-regulated during cellular aging.

***Key words***: *Cellular aging, Endothelial cell, Fibronectin, Endothelin*

Vascular endothelial cells have been found to exhibit a finite lifespan[1, 5]. Culture of endothelial cells from elderly persons contains larger cells, characteristic of cells aged *in vitro*, and these become the majority in monolayers of endothelial cells lining the vascular wall of elderly people[10,11]. Furthermore, a negative correlation between *in vitro* lifespan and donor age has been observed[5]. These observations suggest that endothelial cells *in vivo* age in a similar manner to cells aging *in vitro* and that studies of *in vitro* aging of endothelial cells will provide wider knowledge of *in vivo* cellular aging.

Fibronectin (FN) is an approximately 220 kD glycoprotein and usually exists as a heterodimer composed of very similar subunits. Cellular FN, produced by fibroblasts, endothelial cells and other cells, is secreted into their extracellular matrix. FN in this matrix plays an important role in cell adhesion, spreading, cell motility and cell growth. We have reported that fibroblasts which have aged *in vivo* or *in vitro* express a higher level of FN mRNA as compared with their younger counterparts[8,9].

Endothelin (ET) is a 21-amino acid peptide, was originally identified in endothelial cell culture medium, and is a novel and potent vasoconstrictor[12]. It is known that there are three different ETs: ET-1, ET-2 and ET-3, which are encoded by different genes. ET-1 has been detected in many organs, such as the lung, kidney and brain. In a previous report[11], we showed that ET-1 mRNA was expressed in aortic endothelial cells *in vivo*. The level of ET-1 expression was low in fetal aorta, but high in aorta from donors aged 20 year or older. Furthermore, we showed that ET-1 synthesis in cultured human aortic endothelial cells from donors of various ages was elevated in cells from donors over 50 year old. Thus, modulation of ET-1 mRNA expression during aging was suggested.

In this report, we show that the level of FN mRNA expressed in human aortic endothelial cells from donors of different ages increases with the advancing donor age. Human umbilical vein endothelial (HUVE) cells grown *in vitro* also show the up-regulation of FN mRNA expression during cellular aging. These results extend our previous observations that FN expression increases with the aging of fibroblasts[8,9]. It suggest that the up-regulation of FN gene is a common phenomenon during *in vivo* aging of both endothelial cells and fibroblasts and that both endothelial cells and fibroblasts age *in vivo* by exhausting their division potential like in cells aged *in vitro*. Furthermore, we show that the

level of ET-1 mRNA increases when the culture has senesced.

## MATERIALS AND METHODS

### Cell strains and culture conditions

HUE142-2 (a HUVE cell strain) was isolated and established in Mitsui laboratory, Tsukuba, Japan. When the culture was established, population doubling level (PDL) was assigned to 0. The culture was maintained by serial subcultivations. When the cell number doubled, the PDL number of the culture was increased by 1. This culture reached PDL72-74 by senescence. 829AE, 748AE and 791AE were from the aorta of donors aged 5, 50 and 76, respectively, as reported by Tokunaga et al[10]. Endothelial cells were grown in MCDB151 medium (Sigma) supplemented with 15% fetal bovine serum, 5 μg/ml of heparin (Sigma) and 5 ng/ml of recombinant acidic fibroblast growth factor (kindly provided by Dr. T. Imamura) in a plastic flask (Corning) pre-coated with bovine FN solution (1 μg/cm$^2$, Wako pure chemical industries).

### Preparation of cells for *in situ* hybridization

Endothelial cells were grown on glass coverslips in 12 well plates (Linbro). Coverslips were pretreated with bovine FN (1 μg/cm$^2$). Other details are described in a previous paper[9].

### Preparation of probes

The method of preparation of FN probes has been described in a previous paper[9]. For probes of ET-1, a 1.2kb human ET-1 cDNA fragment[6] was cloned into pT7T3 18U vector (Pharmacia) in both sense and antisense orientations, cut with *Bam* HI and transcribed with T7 RNA polymerase. Other details are the same as for FN.

### In situ hybridization

The procedure of *in situ* hybridization used in this study was described previously[9].

### Labeling index

The method to determine the labeling index was described previously[9].

## RESULTS

### Expression of FN during *in vivo* aging of endothelial cells

Endothelial cells, like fibroblasts, have been shown to senesce *in vitro* and have also been used as a model for studies of cellular aging[2,5]. FN is known to be important for cell attachment, spreading and other cellular functions. We and others have shown that the expression of FN is affected significantly during cellular aging of fibroblasts[7,8]. FN is also produced by endothelial cells. We, therefore, decided to study FN expression in aortic endothelial cells from human donors of different ages to elucidate whether the up-regulation of the FN gene during aging is a common characteristic in both endothelial cells and fibroblasts. We analyzed FN expression at the individual cell level by *in situ* hybridization of aortic endothelial cells, 829AE (PDL15), 748AE (PDL13) and 791AE (PDL10) from 5, 50 and 76 year old donors, respectively. The labeling indices of these cells which were 80%, 68% and 27%, respectively, decreased gradually with increasing donor age, showing that cells from the older donor are less active in DNA replication. Mean values of grains per cell and cell area for these endothelial cells were calculated and graphed to show the relationship between the amount of FN mRNA and cell area (Fig. 1A). A very clear correlation between FN expression and cell area is observed. Though the aortic endothelial cells (PDL10) of the 76 year old donor may not have been completely senescent, as suggested by its relatively high level of labeling index, a 2.7 fold increase of the level of FN expression was observed when compared with that of the 5 year old donor.

### Expression of FN during *in vitro* aging of endothelial cells

We investigated FN expression in HUVE cells aged *in vitro*. Labeling indices of the cells used in this experiment were 91% at PDL11, 76% at PDL62 and 13% at PDL74. Thus, the labeling indices decreased with increasing PDL number, showing gradual loss of DNA synthesis activity with aging *in vitro*. The increases in both FN expression and cell area with increased PDL number were clearly observed, especially at the end of their *in vitro* lifespan (Fig. 1B). A 4.9 fold increase of FN mRNA was observed during the *in vitro* cellular aging. The plot is surprisingly similar to that for aortic endothelial cells from donors of different ages (Fig. 1A), supporting the idea that *in vivo* endothelial cells age by dividing and using up their division potential.

### Expression of ET-1

We also analyzed the modulation of expression of ET-1 gene during *in vitro* aging of HUVE cells. Labeling indices of the populations were 91% at PDL21 and 15% at PDL68. The distributions of grain number per cell in young (PDL21) and senescent (PDL68) populations are shown in Fig. 2. In the young population, 90% of cells expressed low levels of ET-1 mRNA (less than 50 grains/cell) and 10% of cells was intermediate levels (51-200 grains/cell). On the contrary, 12% of cells in the senescent population had low levels of ET-1 mRNA and most of cells showed intermediate to high levels (more than 200 grains/cell). The mean grain number per cell of the

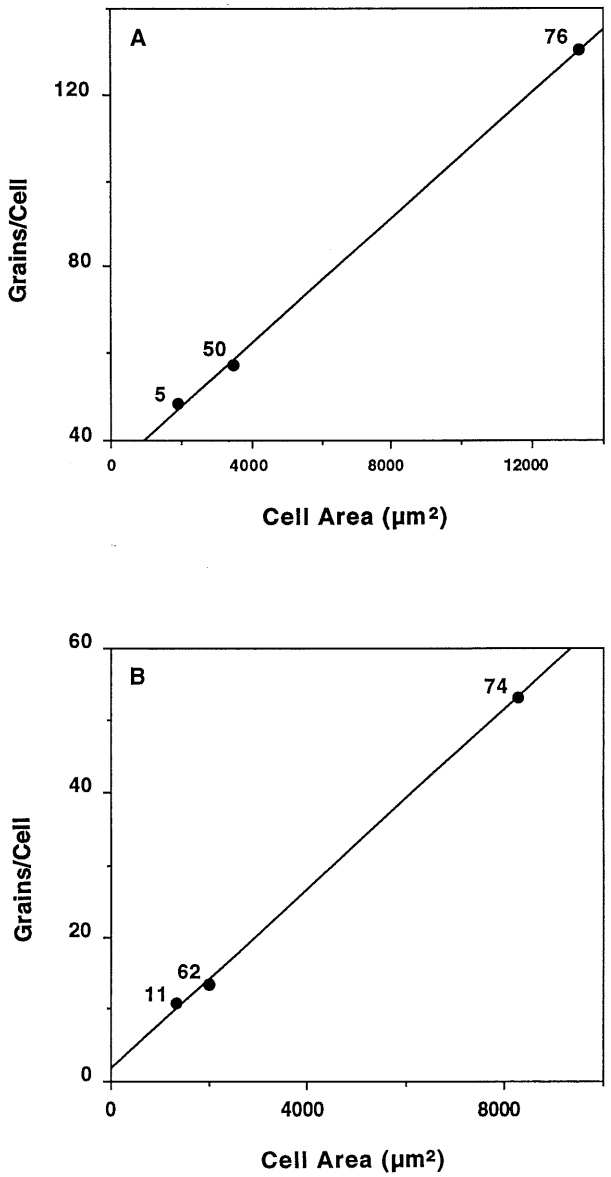


**Fig. 1.** Relationship between the mean of the FN mRNA content and the mean of cell area. Each point was obtained from 100 cells at each age or PDL. A, for aortic endothelial cells aged *in vivo*. Age of the donor is indicated in the figure near the appropriate point. B, for umbilical vein endothelial cells aged *in vitro*. PDL of the cell population is indicated. A correlation coefficient of 0.99 was determined by least squares analysis in both cases.

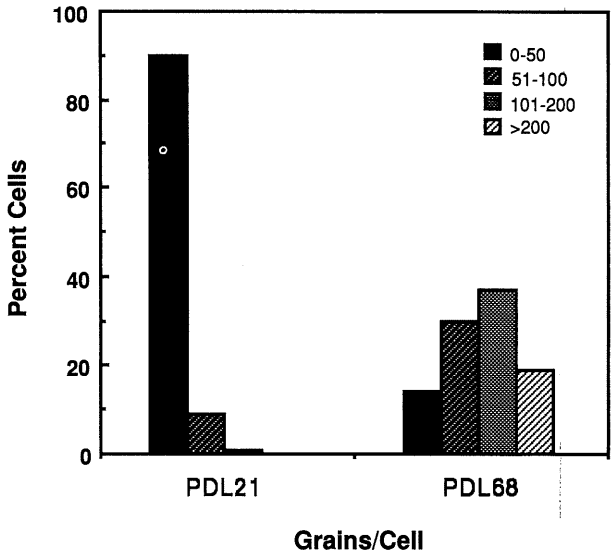


**Fig. 2.** Distribution of level of ET-1 mRNA among cells of culture at PDL21 and 68. Percentage of cells with 0-50 grains, 51-100 grains, 101-200 grains, and greater than 200 grains over the cell is shown.

young population was 27.3 and that of the senescent was 150.6. Thus, a 5.5 fold increase was observed. This result shows that expression of ET-1 is also modulated by cellular aging.

## DISCUSSION

We examined the level of FN expression in aortic endothelial cells from donors aged 5, 50 and 76 year old and showed that the steady state level of FN mRNA increased with advancing donor age. We also showed that the increase in FN mRNA clearly correlated with that of cell area. Furthermore, we investigated the relationship in HUVE cells aged *in vitro* and obtained a similar correlation. These results suggest that endothelial cells are getting old *in vivo* in a manner similar to cells aging *in vitro*; that is, by utilizing division potential. In Fig. 1 A and B, slopes of the lines determined by the least squares analysis for aortic endothelial cells and HUVE cells are almost the same as each other, but the mean grain number, estimated by extrapolation of the lines to 0 of cell area, is larger in aortic endothelial cells than in HUVE cells, suggesting that aortic endothelial cells contain more FN mRNA than HUVE cells. About 4 times more grains were detected in the population from the aortic endothelial cell of a 5 year old donor (PDL15) when compared to that of HUVE cell population (PDL11). Vascular endothelial cells are always exposed to hydrodynamic forces and may be carried away from the vascular wall by the blood stream. Since the aorta has stronger shear forces than the vein, aortic endothelial cells might need more FN to remain attached to the vascular wall than HUVE cells.

After the pioneering works by Hayflick[3,4], extensive works of cellular aging were conducted on fibroblasts. In a previous report[8], we also used a fibroblast strain and showed by Northern analysis that the level of FN mRNA expression was elevated 5 fold during cellular aging, with most of the increase occurring during the last 10% of the *in vitro* lifespan. We also showed that senescent cells produced more FN protein than young cells. Furthermore, the increase of level of FN expression was observed in skin fibroblasts

aged *in vivo*[9]. Thus, the up-regulation of FN gene during cellular aging is a common phenomenon in both endothelial cells and fibroblasts. All these observations indicate the usefulness of cells aging *in vitro* as a model for studies of cellular aging *in vivo*. The observations also suggest the importance of FN in cellular aging.

As previously shown, the synthesis of ET-1 in culture is low in aortic endothelial cells from donors under 50 year old, but is high in those over 50 year old[11]. In order to see whether the modulation of ET-1 synthesis by cellular aging is regulated at the mRNA level, we carried out *in situ* hybridization analysis on HUVE cells aging *in vitro*. The result showed that the level of expression of ET-1 mRNA increased during *in vitro* cellular aging. Thus, it is strongly suggested that the higher level of ET-1 synthesis in elderly people is, at least in part, regulated by the level of transcription and caused by *in vivo* aging of endothelial cells. It is very interesting that expression of both FN and ET-1 are up-regulated during cellular aging, because they are very different as FN is a large glycoprotein of approximately 220 kD and is distributed in extracellular matrix and ET-1 is, on the other hand, an only 21 amino-acid circulating peptide.

As mentioned above, cellular aging has been studied extensively on fibroblasts, but the mechanism of cellular aging has not yet been clarified. Endothelial cells have also been shown to be effective as a model to investigate cellular aging. Studies of cellular aging on cells other than fibroblasts, e.g., endothelial cells, might provide a breakthrough for the clarification of the mechanisms of cellular aging, but fewer studies on such cells have been carried out. Because the up-regulation of FN gene is a common characteristic in both endothelial cells and fibroblasts, as shown in this report, elucidation of the mechanism of up-regulation might be a crucial way to resolve the mechanisms of cellular aging. Besides FN, ET-1 is also up-regulated during aging of endothelial cells. Thus, a common mechanism for the up-regulation of both FN and ET-1 genes might exist. Endothelial cells might be the better material to resolve the mechanism of up-regulation during cellular aging.

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