# Proliferative Activity and Expression of Cyclin-Dependent Kinase Inhibitor *p21$^{WAF1}$* and *p53* Protein in Endothelial Cells of Human Aorta during Replicative Aging *in Vitro*

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Experiments with bromodeoxyuridine showed that the count of nonproliferating cells in a monolayer culture of aortic endothelial cells from adult humans rapidly increased during long-term subculturing. Cytochemical assay showed that these cells contain neutral β-galactosidase, the marker of aging cells. Immunocytochemical assay demonstrated that most cells express *p53* protein and inhibitor of the cell cycle *p21$^{WAF1}$*.

**Key Words:** *human aortic endothelial cells; proliferation; replicative aging; atherogenesis; p53; p21$^{WAF1}$*

Age-related morphofunctional changes in human vessels determine the course of pathological processes in the vascular wall, including the development of atherosclerotic damages (AD). Our previous studies showed accumulation of giant nonproliferating endothelial cells (EC) in the endothelial layer of human aorta in regions predisposed to AD during aging [10]. This modification of EC observed *in vivo* during aging also occurs in long-term cultures of human and animal EC [6,9,13]. Accumulation of giant EC reflects the intensity of replicative aging in cell populations *in vivo* and *in vitro*. Replicative aging of human vascular EC in regions predisposed to AD was confirmed by the presence of the marker of replicative aging (length of telomeric DNA region [5]) and marker of aging cells (neutral β-galactosidase, senescence-associated β-galactosidase, SA-β-Gal). This was demonstrated *in vivo* and *in vitro* for the endothelium covering AD [13] and long-term EC cultures [6]. Aging cells retain their viability for a long time. However, these cells are characterized by considerable changes in gene expression and impairment of various systems [1]. These changes trigger functional activation of *p53* gene. Biologically, this gene blocks division of genetically abnormal cells and initiates their apoptosis [3]. Similarly to transcription factor, *p53* modulates expression of genes, whose activation is responsible for inhibition of the cell cycle. The major effector gene for *p53* is *p21$^{WAF1}$* protein, which acts as a potent inhibitor of cyclin-dependent kinases [7] and blocks cell division during phase $G_1$ in response to overexpression of *p53*. Overexpression of *p21* in human cells inhibits their growth and determines the phenotype of aging. Previous studies showed that in cells composing AD, including EC, accumulation of *p53* correlates with increased expression of *p21*. These data suggest that during atherogenesis *p53* negatively regulates proliferation through transcriptional activation of *waf1* gene [8].

Here we evaluated the relationship between the expression of *p53* and inhibitor of cyclin-dependent kinases *p21* and accumulation of aged EC in human aorta during experimental replicative aging *in vitro*.

## MATERIALS AND METHODS

The experiments were performed on cultured EC isolated from human aorta as described elsewhere (*n*=9, autopsy specimens, 40-43 years) [4]. Long-term sub-

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culturing of EC was used for modeling replicative aging. Early (0-2), middle (8-10), and late passages (18-21) were compared. The size of EC was measured [10]. Proliferative activity of EC in subconfluent monolayer was estimated by incorporation of bromodeoxyuridine (BrDU). Immunocytochemical assay of *p21*, *p53*, and BrDU was performed routinely using the avidin-biotin method. SA-β-Gal was detected as described elsewhere [6]. For simultaneous studies of proliferating and aging EC in preconfluent monolayer, the cultures were incubated with 100 mM BrDU for 24 h. SA-β-Gal was detected histochemically. Immunocytochemical staining was performed with monoclonal antibodies recognizing BrDU. Colocalization of *p21* and *p53* was determined by double immunocytochemical staining. We calculated the percent of stained EC per 1000 cells in randomly selected fields of view.

The results were analyzed by SigmaStat software.

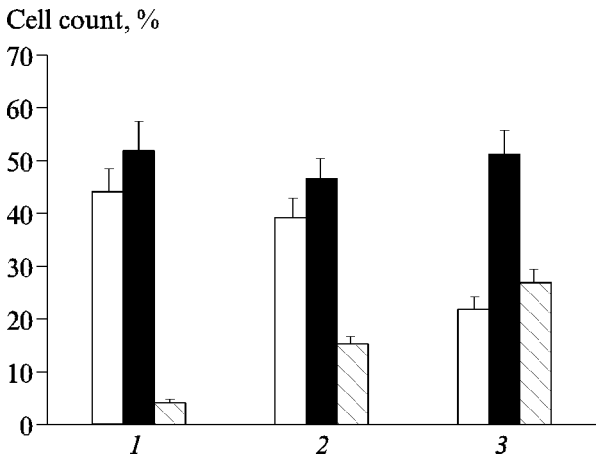


**Fig. 1.** Relative content of human aortic endothelial cells with various sizes in confluent cultures of early (0-2, *1*), middle (8-10, *2*), and late passages (18-21, *3*; *M*±*m*, *n*=3). Light bars: small cells. Dark bars: intermediate cells. Shaded bars: large and giant cells.

## RESULTS

Early passages of EC (0-2) were characterized by insignificant morphological polymorphism and formed regular confluent monolayers within 5-7 days. Long-term subculturing was accompanied by an increase in cell polymorphism and accumulation of giant EC, including EC with a phenotype of aging cells (giant mono- and multinuclear EC). Early passages contained primarily small and intermediate EC, while the number of large and giant cells was low (Fig. 1). After long-term subculturing (passages 8-10 and 18-21) the count of large and giant cells increased. In late passages these EC constituted less than $^1/_3$ of the total cell population. However, these cells occupied more than $^2/_3$ of the monolayer area. The time of attaining confluence progressively increased. After passages 21-22 EC did not proliferate, and confluence was never attained.

The number of EC estimated by the presence of SA-β-Gal marker in late passages 5-fold surpassed that in early passages (Fig. 2). The count of proliferating EC detected with BrDU decreased by 4 times. Co-expression of aging marker SA-β-gal and proliferation marker BrDU was not revealed in these cells. Morphological, cytochemical, and immunocytochemical criteria for the state of cultures proved replicative aging during subculturing of EC. Simiular results were obtained in experiments with multiple passages of EC from human umbilical vein [12] and calf cornea. A correlation was found between the appearance of SA-β-Gal-positive cells in the culture, morphological changes, and shortening of telomeric DNA regions typical of aging [14].

Long-term subculturing was accompanied by accumulation of *p21*-positive EC (Fig. 2). EC expressing *p21* were primarily presented by intermediate and large cells, while most small EC were *p21*-negative (Fig. 3). In passages 8-10 and 18-21 the count of antigen-positive cells increased (Fig. 2). Similar heterogeneity of the expression was observed for *p53* acting as a regulator of proliferation (Fig. 2). Intensive expression of *p21* and *p53* was found in all multinuclear cells. Parallel measurements of SA-β-Gal and *p21* in passages 8-10 and 18-21 showed that 69.2±5.9% cells carrying the aging marker had *p21*-positive nuclei. Moreover, 74.2±7.8% SA-β-Gal-positive cells had *p21*-positive nuclei. Hence, most aging EC express nuclear proteins that negatively regulate the cell cycle. Simultaneous studies of *p21* and *p53* in the same cultures from various passages showed that 88.6±7.5% cells express both proteins in the nucleus; 11.4±1.9% cells had only

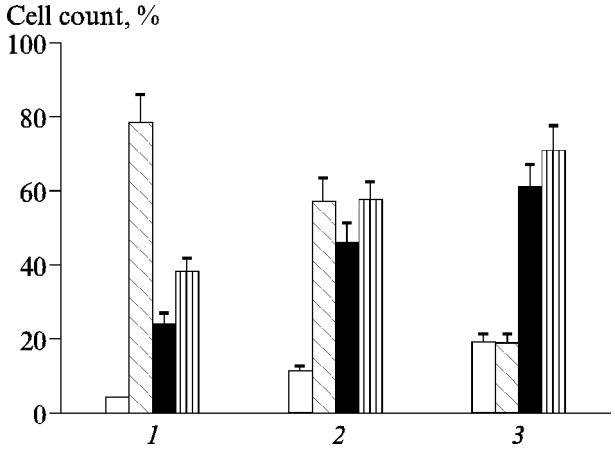


**Fig. 2.** Relative content of endothelial cells containing the marker of aging cells SA-β-Gal (light bars), marker of proliferating cells BrDU (slant shading), inhibitor of cyclin-dependent kinases *p21* (dark bars), and *p53* protein (vertical shading; *M*±*m*, *n*=3). Passages: 0-2 (*1*), 8-10 (*2*), and 18-21 (*3*).

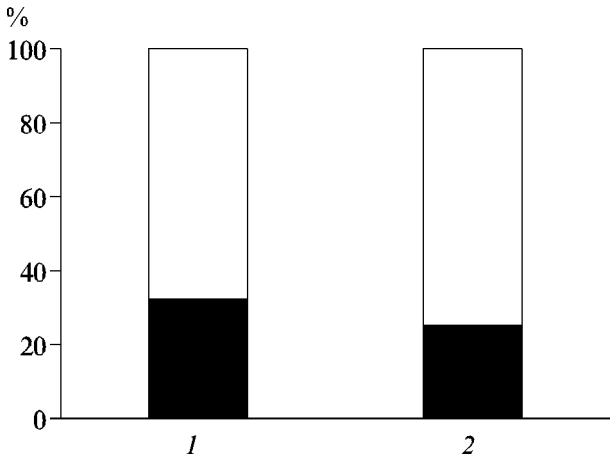


**Fig. 3.** Ratio of small (dark portion), large, and giant *p21* cells (light portion, *1*) and *p53*-positive cells (*2*).

1 antigen in the nucleus (*p53*). Our results indicate that accumulation of cells expressing these proteins during long-term subculturing was accompanied by colocalized gene expression. Coexpression of *p21* and *p53* proteins confirms published data that *p21* acts as a potential mediator for *p53* [7], and that these proteins are involved in physiological aging of cells [11,14].

Experimental modeling of replicative aging in human aortic EC *in vitro* revealed a correlation between expression of *p21* and *p53* proteins regulating transition of the cell into the rest state and the presence of aging marker SA-β-Gal. Previous studies demonstrated accumulation of EC undergoing replicative aging in regions predisposed to AD [5,10,13], coexpression of *p21* and *p53* in the endothelium covering AD [8], and involvement of *p21* in the regulation of genes responsible for the development of age-related diseases [5]. Our results and published data explain the relationship between aging of the endothelium and formation of AD. Aging EC intensively generate reactive oxygen species. This process is associated with disturbed protein repair and increased adhesive, prothrombotic, and proatherogenic activities of leukocytes [1,14]. These data suggest that the appearance of aging EC in the aorta endothelium contributes to the initiation of atherogenesis.

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